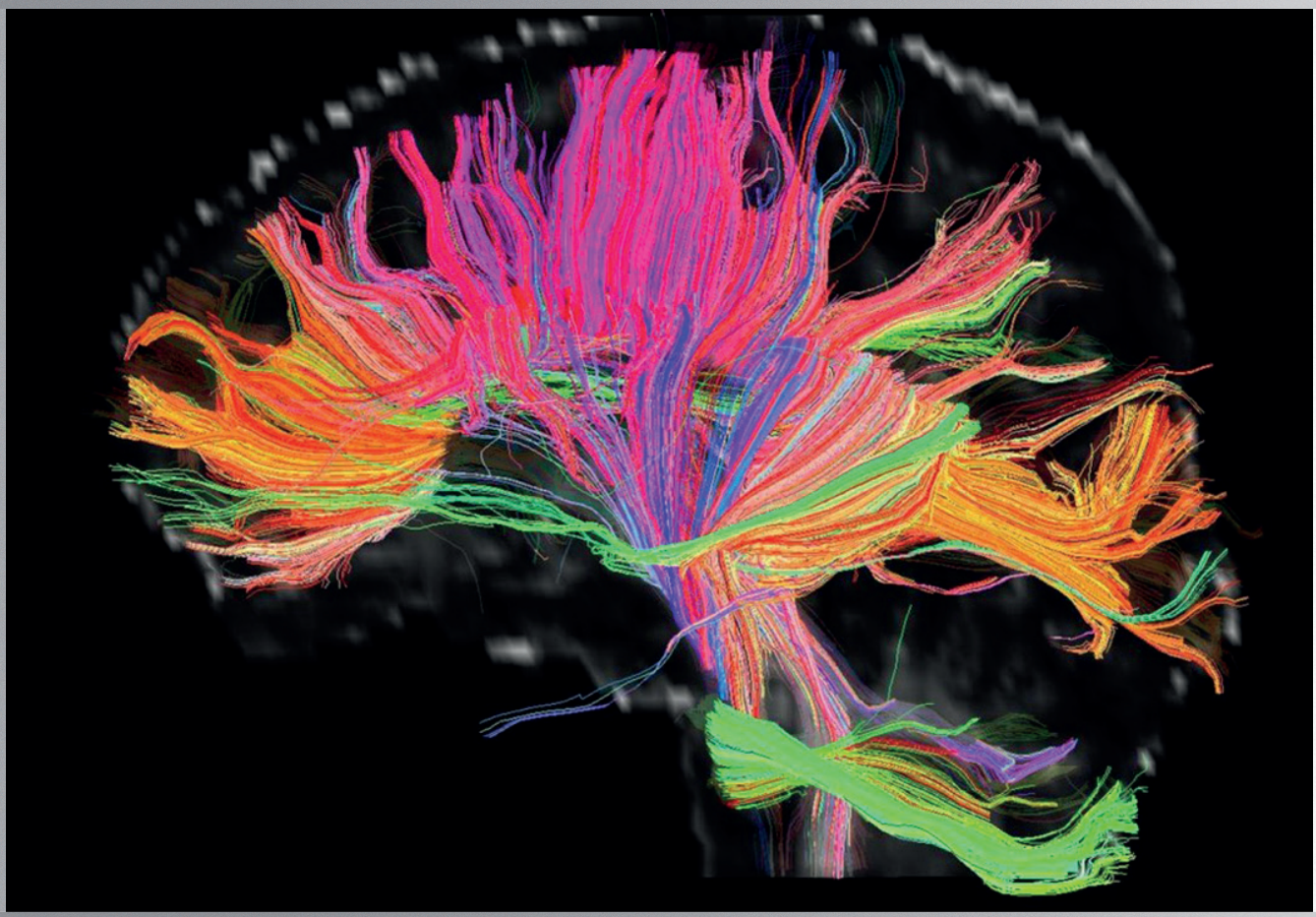
ADVANCES IN ALZHEIMER'S DISEASE 9

Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease



Edited by
Giulio M. Pasinetti

IOS Press

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ADVANCES IN ALZHEIMER'S DISEASE 9

Series editors: George Perry Ph.D and J. Wesson Ashford, MD, PhD

Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease

Despite being confined to the gastrointestinal tract, the gut microbiome has a wide impact on human physiology, supplementing its host's biochemistry in a complex symbiotic relationship. Research in the field has evolved rapidly in the last decade, and we are now developing a better understanding of how our gut microbiome can influence our immune systems, metabolism, neurological signaling, and perhaps most unexpectedly, our brains; a phenomenon described as the gut-brain-axis.

This book, 'Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease', sets out to explore the complex role of the microbiome with regard to Alzheimer's disease (AD). The microbiome is a critical and often overlooked aspect of immunity, which in turn plays a role in cognition. The book presents current research into the gut microbiota and its far-reaching impacts on cognitive function and neurodegeneration. Interventions, including probiotic supplementation, fecal transfer, and supplementation with microbial metabolites, are discussed, as is the use of certain probiotics to study the effects of the gut microbiota on behavior and cognitive function, and as potential therapeutics for AD. Other topics covered include the influence of the gut and oral microbiota on immune inflammatory signals: cytokines, neuroendocrine hormones, bacterial components, neuroactive molecules, and microbial metabolites.

The book is divided into four sections, each covering a research area pertinent to the gut-brain-axis and its relationship with cognitive function and AD. It will be of interest to all those whose work includes the study and understanding of these complex, multi-variable biological mechanisms, particularly in the context of cognitive function and AD.

The cover shows a color edited MRI image of a sagittal section of a neurological control brain of Dr. Giulio Maria Pasinetti.

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HANDBOOK OF MICROBIOME AND GUT-BRAIN-AXIS IN ALZHEIMER'S DISEASE

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Advances in Alzheimer's Disease brings together the latest insights in Alzheimer's disease research in specific areas in which major advances have been made. This book series assembles and builds on work recently published in the *Journal of Alzheimer's Disease* (JAD) and also includes further contributions to ensure comprehensive coverage of the topic. The emphasis is on the development of novel approaches to understanding and treating Alzheimer's and related diseases.

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Dedication

Through your acts
Of respect, kindness, and humility,
Instilling in us a nobility of spirit.
Role models who believed in equality
And human decency
To create a better and more peaceful world.

Written, even as you battled dementia
a tribute to your loving memory…
Witnessing your decline,
My personal Race for a Cure, spurred.

Angelo and Vanna,

For your gift to us of a life of love and caring, we are truly grateful.

Love

Giuseppe, Giulio, Clara, Eleonora

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A special thank you to Joyce Harary (Associate Researcher, Icahn School of Medicine) and Joshua Palmieri (Associate Researcher, Icahn School of Medicine) for their assistance throughout the development of this handbook. This handbook would not have been possible without their diligent efforts!

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Introduction

Current State of Gut Microbiota: Where We Have Been, Where We Are Going

Giulio Maria Pasinetti, MD, PhD

Research in the gut microbiota field has been rapidly evolving over the past decade. Despite being confined to the gastrointestinal tract, the impact of the gut microbiota biology on human physiology is extremely broad. The gut microbiota itself forms a powerful endocrine organ with a unique metabolome that supplements the host's biochemistry beyond what it is designed to provide for itself: a perfect symbiotic relationship! These metabolites, and the breadth of gut microbiota cells themselves, interact with, and/or transverse the protective gut epithelial barrier influencing several physiological systems including the immune system, metabolism, neurological signaling and perhaps the most unexpectedly, the brain, giving rise to the gut-brain-axis [1,2].

While microbiota is referred as all the microorganisms found in an environment, including bacteria or viruses, microbiome is here referred as the collection of genomes of the microorganisms found in a particular environment (i.e., gut or oral microbiota). In addition to the intrinsic heterogeneity in microbiota composition among each person, microbiome diversity also plays a major factor in microbiota heterogeneity. Indeed, microbiota and the microbiome in the gut can be radically different than oral, skin, eye, etc. Based on this, we believe this Handbook collection fully addresses the complex role of the microbiota and its microbiome heterogeneity and will bring to discussion novel approaches in Alzheimer's disease (AD) leveraging, for example, the intervention of prebiotic, probiotic, and synbiotic supplementations. In this regard, we have chosen to discuss the influence of the gut and oral microbiota on the immune inflammatory signals including cytokines, neuroendocrine hormones, bacterial components, neuroactive molecules, or microbial metabolites among others. Given that the microbiome is a critical and often overlooked aspect of innate immunity, and which plays a role in cognition, the overarching goal of this Handbook is to review and summarize recent and current research of microbiota and microbiome and the far-reaching impacts on cognitive functioning and neurodegeneration, possibly through mechanisms involving immune inflammatory mechanisms. Interventions including probiotic supplementation, fecal microbiota transfer, and supplementation with microbial metabolites are also discussed. We want to point out that certain probiotics have been used not only to study the effects that the gut microbiota has on behavior and cognitive function, but also as potential therapeutics for AD.

Traditionally, the gut microbiota and its impact on biology were studied using surveillant and correlative techniques. The composition of the gut microbiota would be determined in the context of a disease state and related to a selection of biological markers such as cytokines, gene expression, or a behavioral output. Causality would be established by using germ-free mice or strong antibiotic treatment where the absence of a gut microbiota would create behavioral or biochemical changes [3,4]. While these seminal studies were absolutely essential to drive our current understanding of the breadth of the gut microbiota's action through the host's physiology, research moving forward must expand and become more sophisticated to address causal mechanisms and direct interactions such that the gut microbiota can be leveraged upon to develop useful therapeutic interventions.

This is especially true with studies of the gut-brain-axis. Current research seems to have reached a standstill with the climate focusing on behavioral correlations and general influences of the broad environmental changes of the gut microbiota. Studies using fecal transplants surmise that pathological neurological changes can be transferred into naive mice with a fecal transplant [5] and could even be used as a therapeutic intervention for Parkinson's disease [6]. But the questions remain regarding how and why this works? One study by Bravo et al. eloquently showed that *Lactobacillus rhamnosus* can innervate the vagus nerve to influence emotional behavioral and GABA expression [7], which demonstrates that causal mechanisms exist—we just need to find them.

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Translational research from mice to humans also forms a barrier to pushing the boundaries of gut microbiota research, which requires the creativity and innovation of the current generation of researchers. Studying artificial systems like heavy antibiotic treatment and germ-free animals does not have translatable potential to human studies as these are artificial environments. Studies understanding the causality of the gut microbiota need to use current tools but must also evolve to develop new tools to gain insight into the causal mechanisms linking the gut microbiota and its metabolome to human disease. Especially for studies of probiotic, prebiotic, or synbiotic interventions, translation to human utility requires pharmacokinetic understanding of metabolite production and the dissection of common molecular pathways.

Collectively, this Handbook contains four broad sections, each covering a pertinent research area pertaining to the overall literature on the gut-brain-axis and its relationship with cognitive functioning and AD. We hope that future research will be able to cohesively build upon the current foundation of rigorous studies by developing innovative and novel approaches that will progress our understanding of these complex, multi-variable biological mechanisms, particularly in the context of cognitive functioning and AD.

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Section 1

Gut Microbiome and Alzheimer’s Disease

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Discovery of Gut Bacteria Specific to Alzheimer's Associated Diseases Is a Clue to Understanding Disease Etiology: Meta-Analysis of Population-Based Data on Human Gut Metagenomics and Metabolomics

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Abstract. Alzheimer's disease (AD)-associated sequence (ADAS) of cultured fecal bacteria was discovered in human gut targeted screening. This study provides important information to expand our current understanding of the structure/activity relationship of ADAS and putative inhibitors/activators that are potentially involved in ADAS appearance/disappearance. The NCBI database analysis revealed that ADAS presents at a large proportion in American Indian Oklahoman (C&A) with a high prevalence of obesity/diabetes and in colorectal cancer (CRC) patients from the US and China. An Oklahoman non-native group (NNI) showed no ADAS. Comparison of two large US populations reveals that ADAS is more frequent in individuals aged $\geq$66 and in females. Prevalence and levels of fecal metabolites are altered in the C&A and CRC groups versus controls. Biogenic amines (histamine, tryptamine, tyramine, phenylethylamine, cadaverine, putrescine, agmatine, spermidine) that present in food and are produced by gut microbiota are significantly higher in C&A (e.g., histamine/histidine 95-fold) versus NNI (histamine/histidine 16-fold). The majority of these bio-amines are cytotoxic at concentrations found in food. Inositol phosphate signaling implicated in AD is altered in C&A and CRC. Tryptamine stimulated accumulation of inositol phosphate. The seizure-eliciting tryptamine induced cytoplasmic vacuolization and vesiculation with cell fragmentation. Present additions of ADAS-carriers at different ages including infants led to an ADAS-comprising human sample size of 2,830 from 27 studies from four continents (North America, Australia, Asia, Europe). Levels of food-derived monoamine oxidase inhibitors and anti-bacterial compounds, the potential modulators of ADAS-bacteria growth and biogenic amine production, were altered in C&A versus NNI. ADAS is attributable to potentially modifiable risk factors of AD associated diseases.

Keywords: Alzheimer's human gut metagenome, angiogenesis, biogenic amines, food, gut metabolomics, protein biosynthesis

INTRODUCTION

In the previous reports, I analyzed the presence of Alzheimer's disease (AD) associated human gut bacterial sequence (ADAS) in health and diseases

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using the National Center for Biotechnology Information (NCBI) sequence databases [1, 2]. More recently (August-October, 2018), the new human gut metagenome Third Party Annotation (TPA) assembly derived from the primary whole genome shotgun (WGS) data set added to the human gut microbiota database [3]. The present study reveals ADAS in the new NCBI additions in human gut metagenomics. The ADAS findings based on the assumption that the biochemical pathway for the tryptamine produced by human gut microbiome and implicated in seizures and neurodegeneration can be targeted in human gut analysis with the usage of newly designed PCR primers. The tryptophan metabolite tryptamine is a dietary biogenic amine (BA) with a dose-dependent cytotoxicity [4, 5]. Tryptamine shows cytotoxicity in cell cultures at the concentrations that can be found in common food, especially fermented food. The human tryptamine content and cytotoxicity are modulated by monoamine oxidases (MAO) and monoamine oxidase inhibitors (MAOI). Elevated concentrations (2-fold) of blood β-carboline alkaloid harmane, a MAO inhibitor, were detected using HPLC in essential tremor cases [6] and in Parkinson's disease (PD) in the study of 113 PD cases and 101 controls [7]. These data correlate with a tryptamine ability to induce tremor potentiated by MAOI pretreatment [8] and with the data on elevated (3.3-fold) urine tryptamine in PD [9]. Moreover, tryptamine is able to form β-carboline in reaction with metabolites such as peruvic acid [10]. Therefore the ADAS discovery in human gut microbiome [1] is originated from the studies of tryptamine-induced neurodegeneration in cell culture and mice [11, 12]. The PCR primers for analysis of DNA from human stool samples of AD patients and controls were designed to target the metabolic pathway that predictably related to tryptamine production in bacteria. The previous and present NCBI database analyses reveal the sequence of the resulted PCR product (ADAS) in associated diseases thus expanding our understanding of human pathophysiology. The frequent presence of ADAS in Oklahoma American Indian Cheyenne and Arapaho (C&A) population with a high prevalence of type 2 diabetes (T2DM, 50%) and overweight/obesity (∼93%); and in the populations of colorectal cancer (CRC) patients of the US and China was recently reported [2]. The seven potentially modifiable risk factors have consistent evidence of an association with the AD: T2DM, midlife hypertension, midlife obesity, physical inactivity, depression, smoking, and low educational attainment [13]. Here, the population-based fecal metabolites levels and the detectability percentage (prevalence % as a fraction above the detection limit) in C&A, in the non-native Oklahoma individuals (NNI) [14, 15], in CRC patients, and controls (Washington, DC) [16] were analyzed. The analyses of dietary MAOI exemplify this approach: MAO-A and MAO-B was found to be potently and reversibly inhibited by common natural isoflavones, genistein (more potent than the standard deprenyl) [17]. Genistein accounts for <2% of the isoflavone content of the soybean, soy proteins, and most Western soy foods including soy infant formulas [18]. Here, the fecal genistein was detected in six (4 adults and 2 children) of 23 NNI (20 adults and 3 children) with prevalence of 26% in the total population or 20% in the adult population and in 13 of 35 C&A adults (prevalence 37%). However, the mean level of detectable genistein was lower in C&A than in NNI. Genistein contents varied among the participants (4053–248115 units) with a highest level detected in the sample with a highest tryptamine level. The drugs (antidepressants) and food-derived MAOI can potentiate the tryptamine and other BA toxicity in human. Tryptamine inhibits tRNA aminoacylation of a vital protein biosynthesis enzyme tryptophanyl-tRNA synthetase (TrpRS) leading to tryptophanyl-tRNA^{trp} deficiency [4]. A subject who presented with early developmental delays, motor weakness, and intellectual disability and who was considered during several years as having a non-progressive encephalopathy, had mitochondrial TrpRS (mtTrpRS) deficiency. The two pathogenic variants were detected in the mtTrpRS gene WARS2 [19]. The cumulative effect of the mtTrpRS deficiency with drug valproate, a MAO A/B inhibitor [20] led to severe hepatopathy and neurological deterioration [19]. It was recently revealed that TrpRS plays an important role in high-affinity tryptophan uptake into human cells [21]. Tryptamine-treatment led to TrpRS deficiency concomitant with manifestations of neurodegeneration [12], while TrpRS hyperphosphorylation [22] and overexpression [23, 24] correlate with malignancy. Inhibition of human TrpRS expression by TrpRS-specific siRNAs decreased while TrpRS overexpression increased tryptophan uptake into the cells. Notably, the TrpRS-mediated uptake system had more than hundred-fold higher affinity for tryptophan than the known L amino acid transporter; had the promoted uptake of low tryptophan concentrations, and also very high tryptophan selectivity [21].

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In order to gain more insight into the role of ADAS, the present population-based study focuses on meta-analysis of fecal metabolites associated with the presence of ADAS as a potentially modifiable risk factor of Alzheimer's disease associated diseases.

MATERIAL AND METHODS

Statistical analyses

Statistical analysis of ADAS detection [1] was performed with Prism 7.02 software (GraphPad, San Diego, CA). Data was expressed as mean ± standard deviation (SD). The groups were compared using unpaired two tailed *t* test. *p*-values <0.05 were considered significant. The initial abundance profiles of metabolites from fecal metabolomics were compared between the NNI (male/female = 14/9) and C&A (male/female = 14/24) sample groups using the non-parametric Kruskal-Wallis tests, in which $p < 0.05$ indicated a significant difference. The significantly different metabolites were visualized in boxplot [14]. Here, the statistical analysis of metabolites was conducted using unpaired two-tailed p value parametric (Gaussian distribution) linear regression "do not assume equal" SD *t* test with Welch's correction (Welch's test) and F test to compare variances using software GraphPad Prism version 8.1.1 (330). The differences between NNI and C&A considered significantly different at $p < 0.05$. The outliers were identified and removed from some evaluations using GraphPad Prism 8.1.1. Taking into account that metabolites are undetectable in some human samples, the prevalence (%) of detectable fecal metabolites of interest in C&A, NNI, CRC casesm and controls is estimated here. "Undetectable" metabolite is defined as present at the lower level than the minimum of detection by fecal metabolomics [14, 16]. The prevalence percentage for identification of the direct or inversed association between C&A and CRC populations based on analysis of ∼150 metabolites. The present metabolomic meta-analyses focus on analysis of both the level and prevalence of metabolites in fecal samples from groups with a frequent ADAS occurrence (C&A and CRC versus corresponding controls). The meaningful data on prevalence of metabolites were not analyzed in the original study of C&A versus NNI [14]. Moreover, the metabolites with a prevalence that essentially lower than 100% were excluded from statistical analysis. The special interest of the present analysis are fecal metabolites with 1) activity toward MAO, 2) food-derived anti-microbial activity, 3) activity linked to tryptamine pathway, 4) bioactive metabolites altered in C&A, obesity, T2DM, and cancer, and 5) metabolites resulted from tRNA aminoacylation activity/protein biosynthesis.

Electron microscopy

Tryptamine treatment and electron microscopy of bovine kidney cells MDBK, human neuronal cells of SH-SY5Y subline, and of mice were described earlier in detail [4, 11].

RESULTS AND DISCUSSION

The ADAS prevalence: Updated NCBI database

ADAS was searched with Basic Local Alignment Search Tool (BLAST) in healthy and diseased populations of different continents [1, 2]. The untargeted human gut metagenomics and metabolomics of AD patients are not available. Here the new data was added (Table 1) to the previously reported ADAS prevalence in the 1,246 participants from 17 human gut metagenomics studies [2].

In the recently updated NCBI database (August-October 2018), the 14 samples with 89% identity to ADAS were revealed in Danish, Spanish, and Swedish individuals in the MetaHIT study of 760 European samples [25]. These samples collected from healthy individuals, participants with obesity, and patients with Crohn's disease (CD) and ulcerative colitis (UC) were combined in the pangenomic study of the European population. The total prevalence of ADAS is 1.84% in the pangenomic 760 MetaHIT metagenomes of the European population. In the Spanish study (study MetaHIT-MGS), ADAS was found in one CD patient and one UC patient detected in the project PRJEB25962, in addition to four CD and three UC patients of the European study PRJEB28245. The CD and UC are types of inflammatory bowel disease (IBD). Irritable bowel syndrome (IBS) is associated with an increased risk of AD and non-Alzheimer's dementia in patients older than 50 years in both male and female in the Chinese nationwide population-based study [26]. IBD and IBS are chronic conditions that affect the gut and are typically diagnosed in young people. IBD and IBS express overlapping symptoms such as belly pain and diarrhea. Moreover, as treatments for inflammation in IBD have become more effective, it is now apparent that ∼1 in 3 patients with IBD in remission from

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Table 1
The nucleotide sequence identity (87–100%) to ADAS as a population-attributable risk of Alzheimer's associated diseases: an analysis of population-based data on age, geographic location, ethnicity, and health status. The NNI control for C&A contained no ADAS, the C&A contained 87%/88% (1 female and 1 male) and 100% (2 females) nucleotide identity to ADAS, Of the C&A population, the 5 individuals are ≥66 years old; the NNI population includes no individuals ≥66. Of the ADAS-comprising human sample size of 2,830 from 27 studies across four continents, the 100% ADAS identity was revealed in only 8 individuals of the entire database. The Boston male study HPFS of aged population of ≥66 and the dbGaP male and female study of 18–40 years old contained 87%/88% ADAS identity. The dbGaP sampling includes an approximately equal number of males and females; the 87%/88% ADAS identity was revealed in 3 females and 1 male in dbGaP. Thus, the 87%/88% ADAS identity is 3.3 times more frequent in females than in males from the healthy population of ages 18–40 of MO/TX, USA

N and reference	Study	Population, amount	Age, y	ADAS prevalence %	Location
1 [14]	C&A	35	20–84	10.8	Oklahoma, USA
2 [15]	NNI control for C&A	23	7–50	0	Oklahoma, USA
3 [3]	HPFS	308	≥66	9.09	Boston, USA
4 PRJNA48479	dbGaP	300	18–40	1.3	MO/TX, USA
5 [25]	MetaHIT	760	NA	1.84	Europe
6 PRJEB26925	DIABIMMUNE*	222	Infants	0.45	North Europe
7 [31]	Healthy	106	32 ± 11	6.6	Japan
8 [278]	CRC	52	mean 61†	27	Washington, USA
9 [278]	Control for CRC*	52	mean 61†	1.9	Washington, USA
10 [279]	CRC	74	median 67	8.1	China
11 [279]	Control for CRC*	54	median 62	1.85	China
12 [36]	Traveler diarrhea*	23	NA	4.3	USA
13 [45]	Healthy*	24	22; 38	4.16	Nebraska, USA
14 [46]	Infant twins*	20	Infants	5	China
15 [37]	Athletes-cyclists*	33**	19–49	3	USA

*ADAS revealed in one individual of the population examined. †age: control 36–82 (SD = 11), CRC 31–89 (SD = 13.6), no statistically significant difference in age between control and CRC. **182 samples from 33 individuals, ADAS was in one of seven samples from one individual at different time points.

inflammation still have persistent abnormalities of sensation, motility, and gut microbiota, which might cause IBS-like symptoms [27]. Damage of the mucus layer is suggested as the possible shared critical common cause for both IBD and IBS [28]. Intestinal barrier dysfunction characterizes both IBS [29] and IBD [30].

In the Japanese study, the 106 participants [age: 32 ± 11, BMI: 22 ± 2.7 (mean ± S.D.), 19–60 years old, male:female = 64:42] were recruited by Azabu University (Japan) from 2010 to 2013 [31]. The ADAS at 88% nucleotide identity was detected in seven of 106 individuals (total 6.6%): in 4 males (6.25% of males) and 3 females (7.14% of females) of ages 19–50. Among them, fecal samples were longitudinally collected multiple times at different time points. A total of 168 fecal samples were collected from the 106 individuals. In the ADAS + seven participants, this sequence was detectable in one of two; one of two; two of four; two of six; one of six; one of one; and one of one samples. The recent study described the increasing trends in AD prevalence in Japan [32] as 1.5% in 1985, 1.4% in 1992, 2.4% in 1998, 3.9% in 2005, and 7.2% in 2012 (p for trend <0.01). Japanese mortality due to CRC is also on the rise [33].

In a longitudinal analysis (DIABIMMUNE study) of the developing gut microbiome in infants from Finland, Estonia, and Russian Karelia (http://www.diabimmune.org/), ADAS was detected in one infant from Finland. The number of children included in this study (BioProject: PRJEB26925) was 74 from each country or 222 total. Inclusion criteria are positive cord blood testing for HLA DR-DQ alleles conferring increased risk for autoimmunity.

In the separate case-control study (California, USA) of 49 pregnant women (age 18 years or older), 15 women delivered preterm [34]. 89% identity to ADAS was revealed in the preterm group that included combined samples of 11 individuals.

A comprehensive metagenomic study of the fecal microbiomes from the 23 traveler's diarrhea (TD) patients and seven healthy travelers was performed, all of which tested negative for the known etiologic agents of TD in standard tests [35, 36]. Samples were collected from adults participating in studies who had traveled from the US to Mexico, India, or Guatemala. The 88% identity to ADAS was determined in one patient with TD of unknown cause and none in the healthy travelers. The ADAS-comprising bacteria was isolated from the fecal sample of the traveler. The TD can be caused by enterotoxigenic

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Escherichia coli, other pathogenic gram-negative pathogens, norovirus, and some parasites. Nevertheless, standard diagnostic methods fail to identify pathogens in more than 30% of TD patients.

The new additions (WGS data set: PRJNA305507) are from the gut metagenomics study of athletes-cyclists aged 19–49 (median age 33) conducted by Jackson Laboratory (CT, USA) [37]. All participants spent a minimum of 6 h exercising per week. The 88% nucleotide sequence identity to ADAS was detected in one athlete of 33 participants (182 samples collected at different time points). Moreover, ADAS was detected in one sample out of seven fecal samples self-collected at different time points by this athlete. It seems that this ADAS-sample was not the last collected sample. The lack of ADAS in six of seven samples can be explained by regular physical activity of the athlete. The highest estimated population-attributable risk (PAR) of AD was for physical inactivity in the USA (21.0%), Europe (20.3%), and the UK (21.8%) [13]. In the human gut microbiota study, the athletes had relative increases in pathways, such as amino acid and antibiotic biosynthesis and carbohydrate metabolism [38].

In the China study, an ankylosing spondylitis (AS) patient (BioProject: PRJEB26158) contained ADAS at 100% nucleotide identity. The one sample of 32 AS patients led to two AS patients with 100% identity to ADAS with one from the BioProject: PRJEB24527. Patients with AS are at increased risk of developing IBD [39].

In the Boston follow-up study (USA) of male health professionals [3, 40, 41] over age 65 (aged 40–75 years at enrollment, 1986), the ADAS containing sequences (87–88% ADAS nucleotide identity) were detected in 28 individuals (32 different samples) of 308 participants (9.09%) in human gut metagenome/whole genome shotgun sequence analysis (stool collection of 2012). Each sample was divided and the handling conducted using different techniques in this study (https://sites.sph.harvard.edu/hpfs/). The Health Professionals Follow-Up Study (HPFS) began in 1986. The purpose of the study is to evaluate a series of hypotheses about men's health relating nutritional factors link to the incidence of serious illnesses, such as cancer, heart disease, and other vascular diseases. Patients diagnosed with the non-metastatic colorectal cancer (1986–2012) [42] and persons with self-reported subjective cognitive function [43, 44] are among the HPFS. Note, the age of participants at enrolment (1986) was 40–75 and became 66–101 at the time of stool sample collection (2012); the NCBI gut microbiome sequences was uploaded on 22–24 October 2018. One in 10 people age 65 and older (10%) has AD. Almost two-thirds of Americans with AD are women. Therefore, the ADAS detectability in the Boston study correlates well with AD estimations (https://www.alz.org/alzheimers-dementia/facts-figures). In the previous study [2], the four stool samples with 87% nucleotide identity (equal to 97% amino acid identity) to ADAS were found in the NCBI dataset of 300 males and females of 18–40 years old (1.3%) from the control healthy population of Houston, TX and St. Louis, MO (Table 1). This population-based control set (dbGaP study) of the Human Microbiome Project (HMP) addresses whether individuals share a core microbiome. Therefore, the Boston group of 66 and older showed higher ADAS prevalence (9.09%) than the younger USA mixed male and female group of 18–40 years old (1.3%), the younger Japanese group of age 32 ± 11 (6.6%), and the European 760 MetaHIT pangenomic group (1.84%). The dbGaP sampling includes an approximately equal number of males and females. The 87/88% ADAS identity was revealed in the 3 females and 1 male in dbGaP. Thus, the 87/88% ADAS identity is 3.3 times more frequent in females (2%) than in males (0.6%) in the healthy population of age 18–40 in MO/TX, USA. In the Boston male group of $\geq$66, the frequency of 87/88% ADAS is 15.15-fold higher than in male group of 18–40 from TX/MO, USA.

Human gut metagenomics of 24 healthy human subjects (22 and 38 years of age) was conducted by University of Nebraska (Bioproject PRJNA324129) with the primary objective to determine acceptability and safety of Bifidobacterium longum subsp. longum AH1206 at a daily dose of 10^{10} cells [45]. ADAS was determined in samples collected at pretreatment (baseline), after probiotic consumption, and around 200 days after consumption in one individual. Therefore, the B. longum AN1206 bacteria showed no impact on the ADAS presence in one of 24 individuals.

In the Chinese study of 10 pairs of infant twins, the gut DNA sequence of one twin showed 88% nucleotide/96% amino acid identity to ADAS [46].

Summarizing, the present additions of ADAS with cases of CD and UC (760 individuals, Europe); preterm birth (49 pregnant women, CA, USA); traveler's diarrhea of unknown cause (30 individuals - patients and controls); health professionals of Boston

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metropolitan area (308 participants, USA); three-countries study (222 infants, North Europe); healthy individuals (106 adults, Japan); athlete-cyclist (33 participants, USA); healthy human subjects consumed Bifidobacterium (24 individuals, Nebraska, USA); Chinese case/control study of ankylosing spondylitis (32 AS patients); and Chinese infant twins study (20 participants) led to ADAS-comprising human sample size of 1,246 in the previous report [2] with 1,584 in the present report = 2,830 from 27 studies of four continents including North America (US and Canada), Australia, Asia (China and Japan), and Europe. The Table 1 shows a prevalence of sequence identity (87–100%) to ADAS as a population-attributable risk (PAR) of Alzheimer's associated diseases.

In oral microbiome of saliva and dental samples, a 55% of amino acid identity to ADAS was detected in four (no correlation with disease) out of 266 individuals of Chinese study (controls and rheumatoid arthritis patients aged 23–74 years) [47] and no significant identity to ADAS was detected in oral cavity in the US study dbGaP in the NCBI dataset of 300 males and females of 18–40 years old from the control healthy population of Houston, TX and St. Louis, MO (PRJNA48479). Therefore, no ADAS was revealed in human oral microbiome of at least 566 individuals from two continents.

Population-based analysis of metabolomics attributable to ADAS presence: Binding and kinetics for tryptamine, MAO, MAOI, and TrpRS

Prevalence of tryptamine is higher in C&A and CRC versus controls (Supplementary Table 1). MAO-A and MAO-B contribute to the catabolism of BA including tryptamine. MAO, a mitochondrial enzyme, oxidizes BA to generate hydrogen peroxide, a major source of oxidative stress. Both MAOs are composed of an flavin adenine dinucleotide (FAD)-binding domain [48]. The influence of inhibitors such as pirlindole (pyrazinocarbazole) on the redox cofactor FAD is a key factor in the MAO inhibition. The data suggest that FAD is a preferred substrate and an inhibitor, suppressing the activities of bacterial NAD(P)H:flavin oxidoreductase at low NADH concentrations [49]. Prevalence of FAD is directly associated with CRC and inversely associated with C&A (Supplementary Table 1). Nevertheless, the FAD level is significantly higher in C&A than in NNI [14]. Here, the FAD is 1.49-fold higher (Fig. 1) in C&A than in NNI (*t* test *p* value 0.0032**, F test *p* value 0.0108*). Therefore, the FAD level would not limit the MAO activity in a majority of C&A individuals. However, levels of many BA are higher in C&A versus NNI (Fig. 1). The magnitude of TrpRS inhibition by tryptamine *in vivo* depends on kinetic parameters of TrpRS inhibition and MAO oxidizing activity toward tryptamine. Chronic administration (s.c. via Alzet osmotic minipumps) of MAOI antidepressant drugs (daily doses: tranylcypromine.HCl, 0.5 and 1.0 mg kg–1; phenelzine sulfate, 5 and 10 mg kg–1, each for 28 days; clorgyline.HCl, 1.0 mg kg–1; (–)-deprenyl.HCl, 1.0 mg kg–1, each for 14 days) to rats resulted in decreases in [3H]tryptamine binding site density in brain cortical membranes [50]. The tryptamine MAO Km [51] and TrpRS Ki values support that tryptamine inhibition of TrpRS can be more efficient than tryptamine oxidation by MAO especially in the presence of MAOI. Taking an initial symmetrical dimeric TrpRS protein the two dissociation constants (KD) for tryptophan at pH 8; 25 C are respectively $K1 = 2.0 \pm 0.5\ \mu M$ and $K2 = 10 \pm 4\ \mu M$. They are respectively $K1 = 1 \pm 0.25\ \mu M$ and $K2 = 20 \pm 8\ \mu M$ if one considers a sequenced binding of the two tryptophan molecules [52]. The bovine pancreatic TrpRS Ki for tryptamine is 6×10^{-7} M or 0.6 μM [4]. In medial frontal cortex of human brain the [^{14}C]tryptamine binding was at the KD value of 5 μM [53]. In bovine liver MAO B, tryptamine was 'partially immobilized' with a KD of 39 μM and 2.2 moles bound per enzyme dimer [54]. These estimations may indicate that tryptamine has higher binding affinity to TrpRS than to MAO assuming that the smaller KD values correspond to the greater binding affinity of the ligand for its target. To determine the stability of tryptamine for potential TrpRS inhibition *in vivo* it is important to estimate the rates for tryptamine as TrpRS inhibitor and as MAO substrate. *In vivo*, the *E. coli* codon GAA was found to be translated with a rate of 21.6 codons/s whereas codon GAG was translated 3.4-fold slower (6.4 codons/s) [55]. Kinetic measurements indicated that the half-life for tryptamine ranges from 0.28 min (16 s in rat brain) to 1.6 min (96 s in rat spinal cord) [53]. In rabbits, radiolabeled iodinated [^{131}I]tryptamine rapidly entered the brain (olfactory bulb and olfactory peduncle) and was completely excreted in the urine 20 min after injection in the marginal ear vein [56]. Thus, tryptamine can lead to Trp codon translation blockage even without MOAI. Unexpectedly, radiolabeled N,N-dimethyltryptamine (DMT) remained in the rabbit brain for at least 7 d after injection. DMT

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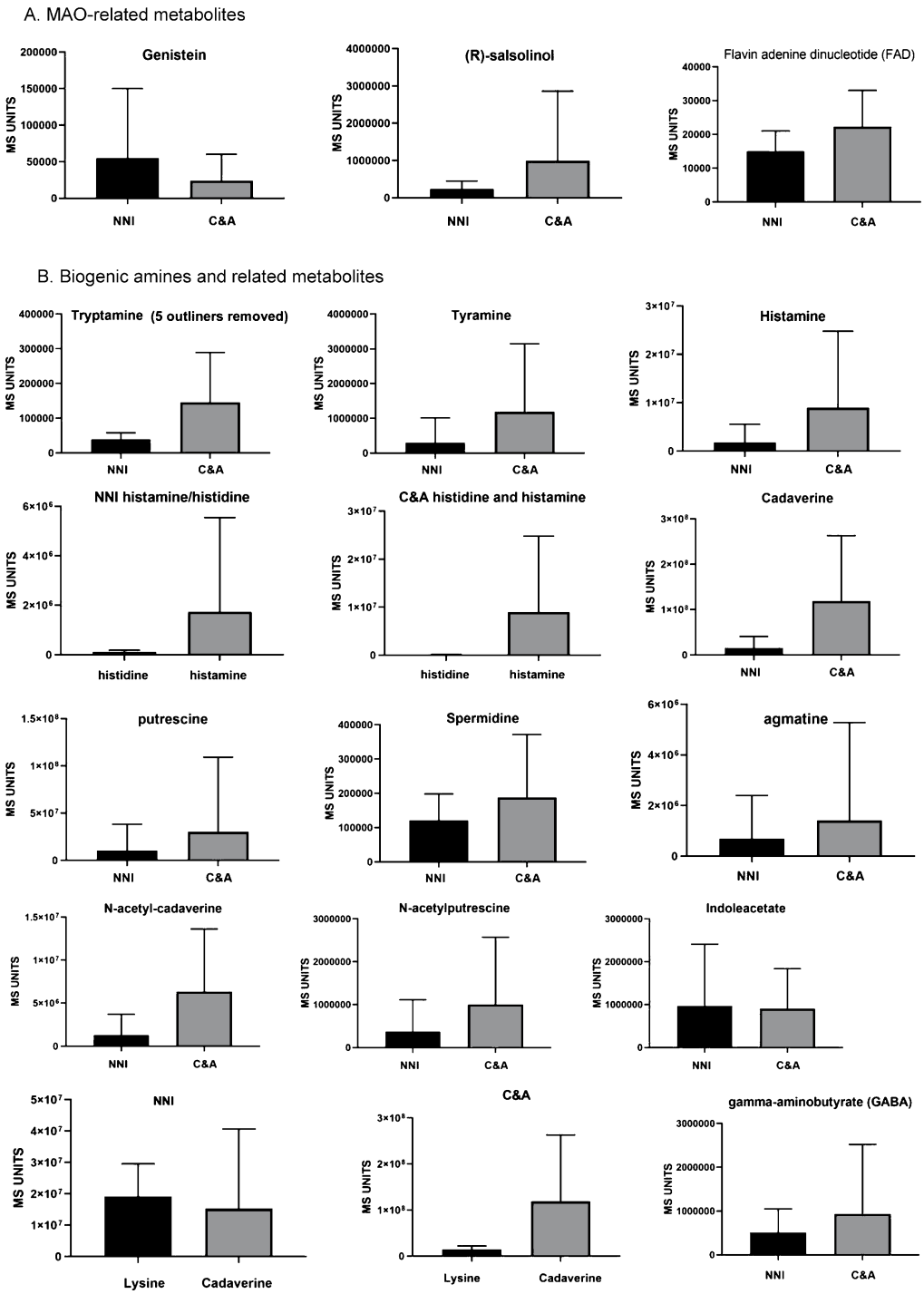


Fig. 1. The population-based statistical analysis of the fecal metabolites for NNI and C&A: correlation of gut metabolites with ADAS occurrence. The metabolomics crude data for NNI and C&A are derived from the Oklahoma study [14]. The 23 NNI (20 adults and 3 children) and 35 C&A adults are included in Oklahoma metabolomic study and all available data for all subjects from this study are analyzed here. The comparison of fecal metabolites from C&A containing 87% and 100% nucleotide identity to ADAS was with the NNI group of no ADAS. Here, the mean data with SD is presented in the graphs built with software GraphPad Prism 8. The outliners identified with this software usage are shown in parenthesis. Panel H shows comparison of the total 81 peptides with the tryptamine (T) level in NNI and C&A. The tryptamine high level correlates with a low level of the total peptides; T high samples (NO1 and NO13) versus T-low (NO2) and non-detectable (ND) tryptamine (NO7 and NO15) of the NNI group.

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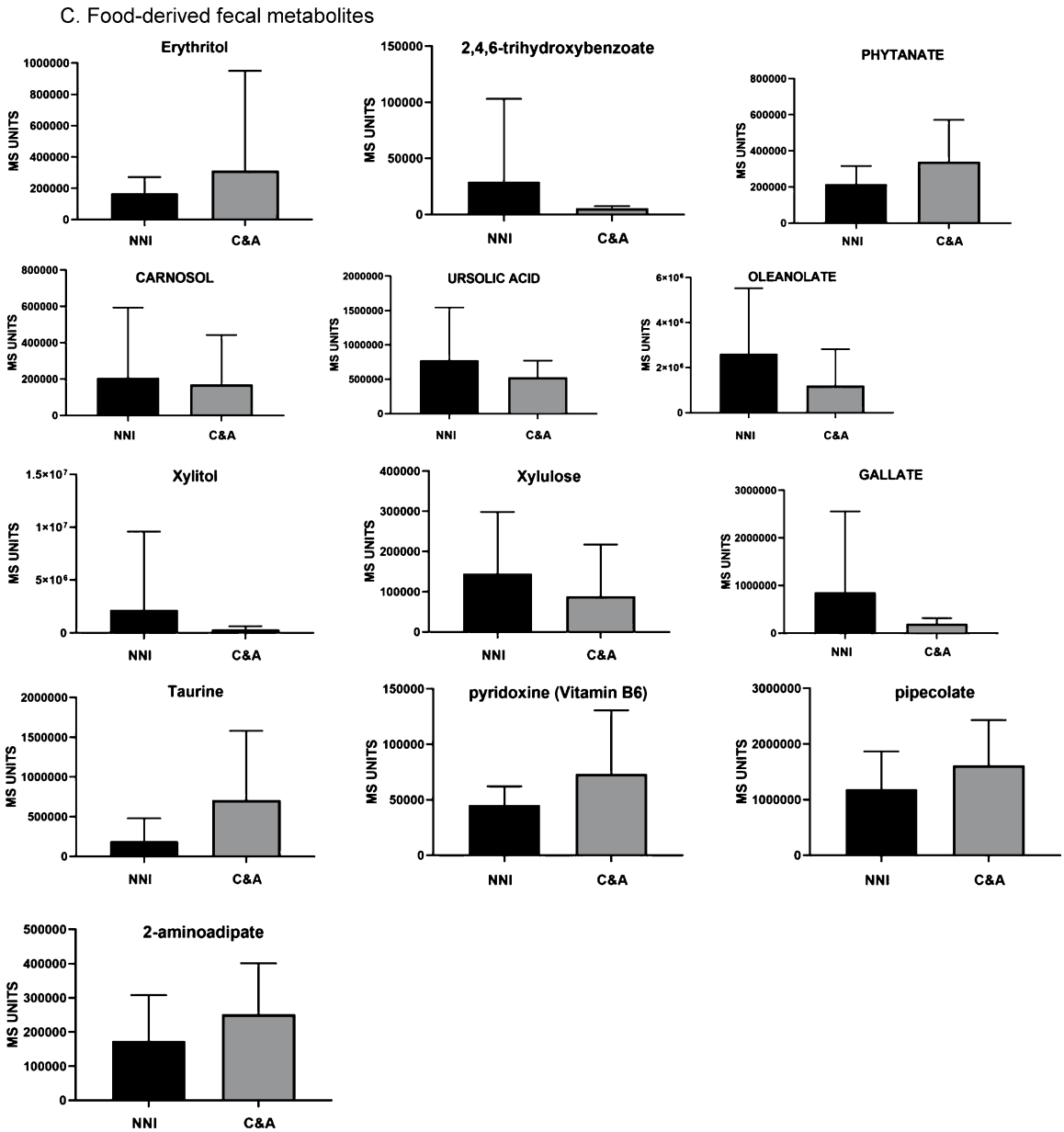


Fig. 1. (*Continued*)

is a potent hallucinogen and an endogenous agonist for the sigma-1 receptor [57]. DMT is a direct metabolite of tryptamine. The DMT degradation by MAO is inhibited by β-carbolines, the reversible MAOI [58]. A significant acceleration in the slope of cognitive decline occurs following an episode of delirium in AD patients [59]. The rate of polypeptide chain elongation in prokaryotes is 4–10 times faster than in eukaryotic cells [60]. On that account, the tryptamine-induced TrpRS-dependent blockage of Trp codon translation may be more pronounced in prokaryotes than in eukaryotes. Tryptamine can lead to dysbiosis in the gut by killing tryptamine-sensitive bacteria.

The MAO enzymes are proteins localized mainly on the outer membranes of mitochondria [61, 62]. Thus, MAO may not present in the cytoplasm in large amounts sufficient to prevent inhibition of the cytoplasmic protein biosynthesis by BA. Nevertheless, the BA can be degraded by MAO on

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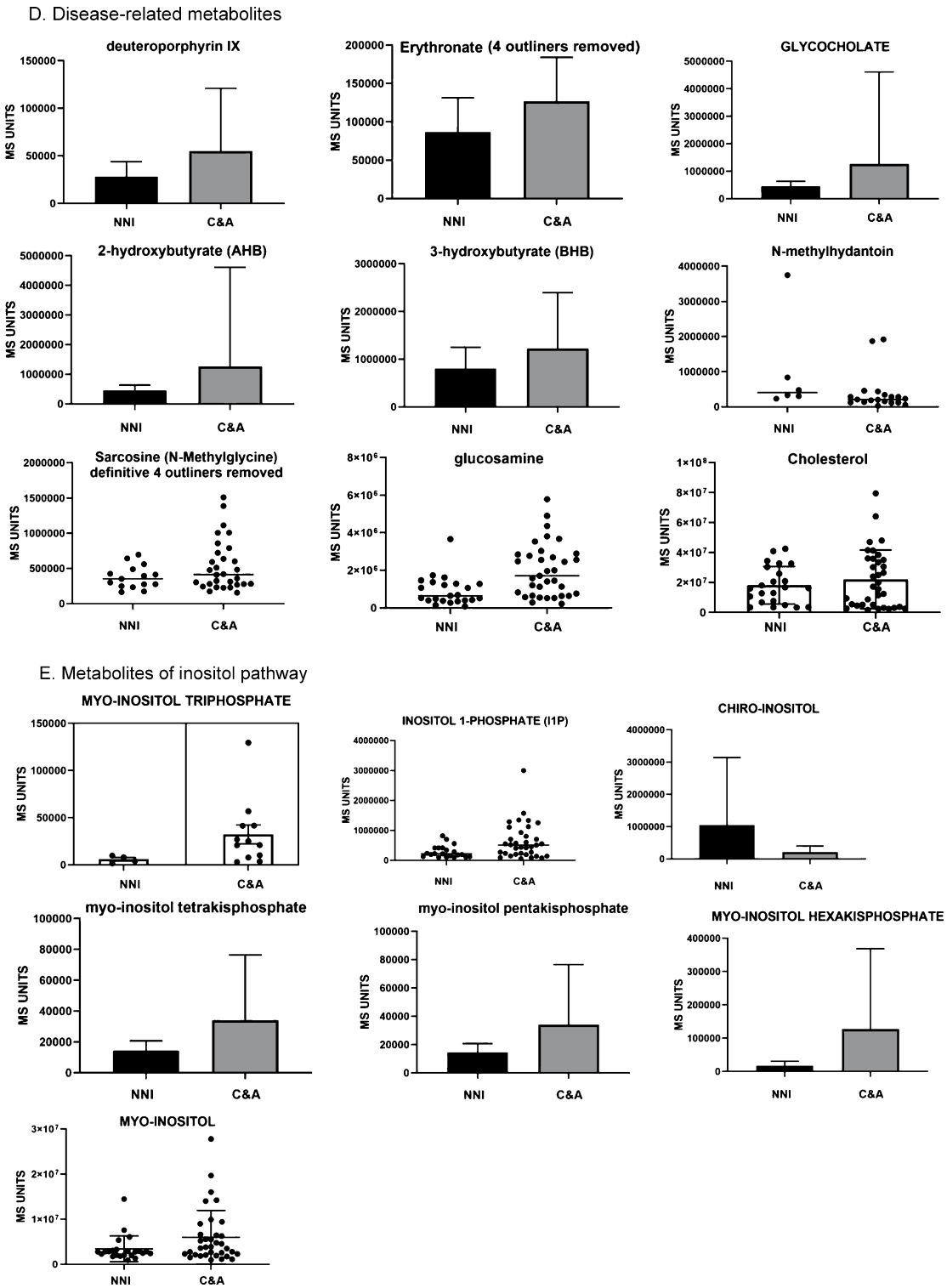


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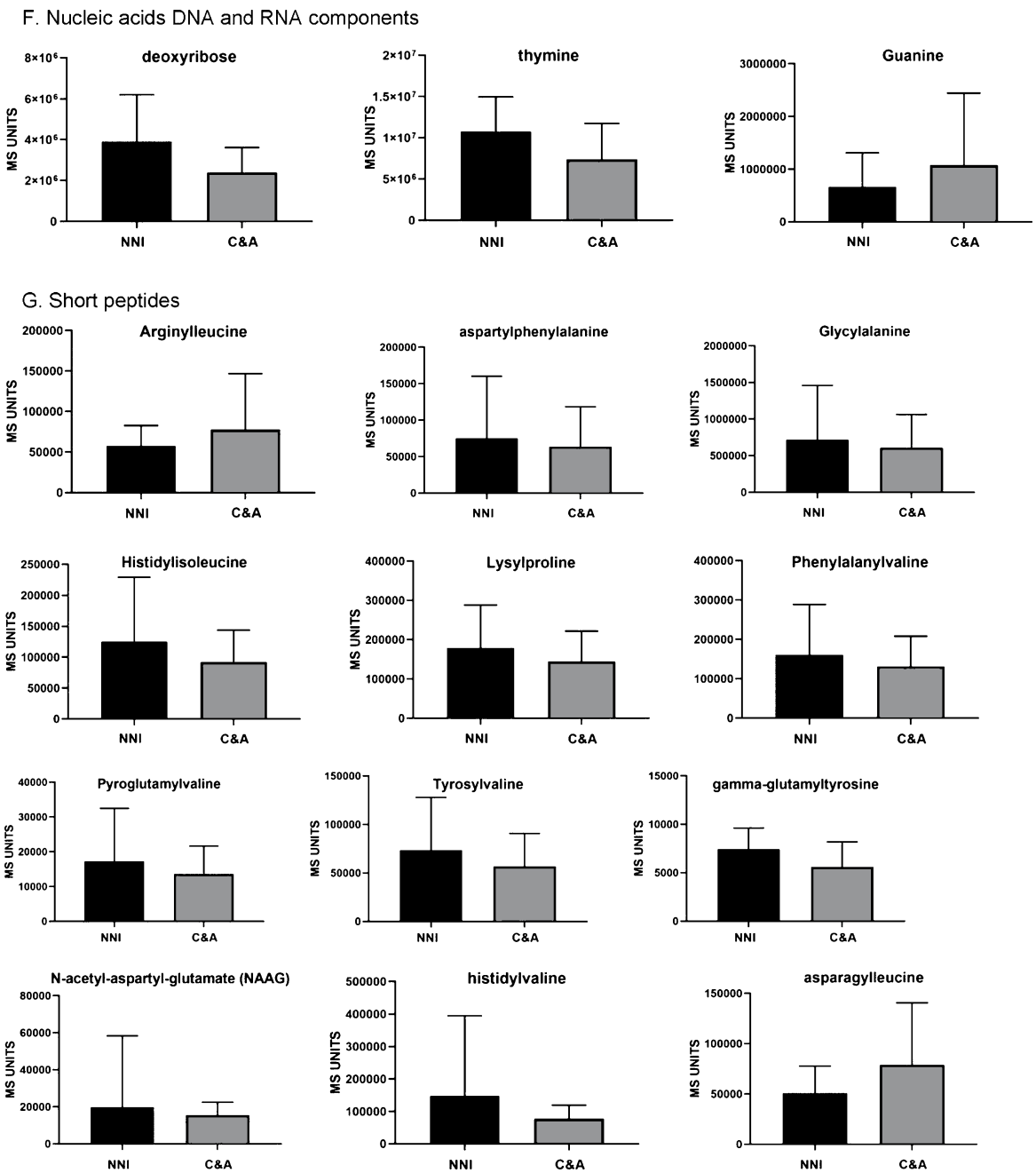


Fig. 1. (*Continued*)

mitochondria and after MAO biosynthesis on the cytoplasmic ribosomes since MAO is encoded by nuclear genes. The genes controlling the expression of MAO A and MAO B are located on the human X chromosome [63]. Semicarbazide-sensitive amine oxidase (SSAO), widely distributed in highly vascularized mammalian tissues, metabolizes endogenous and xenobiotic aromatic and aliphatic monoamines. Widespread SSAO distribution was observed in all tissues except the thyroid gland and endocrine pancreas, which were devoid of specific SSAO immunoreactivity. In most tissues, SSAO immunoreactivity appeared to be bound to the cytoplasmic membrane [64].

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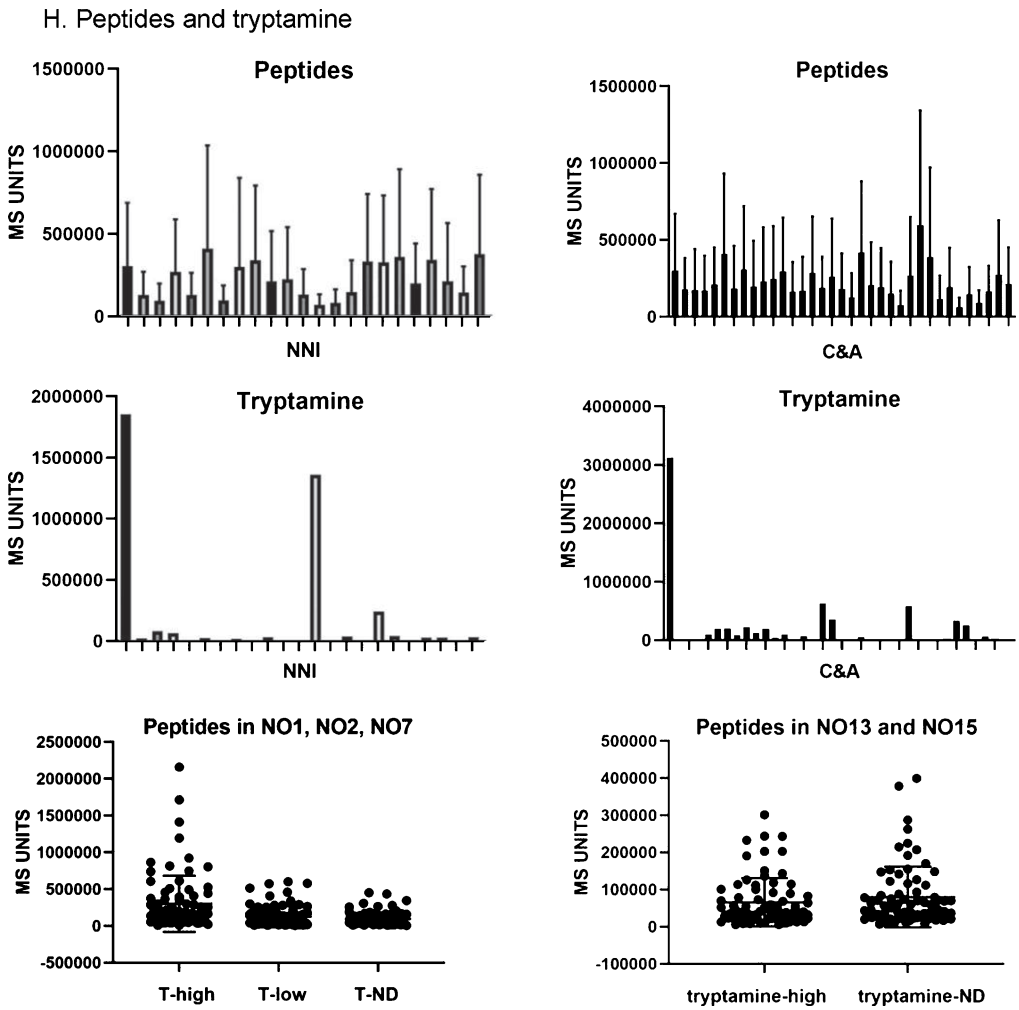


Fig. 1. (*Continued*)

Portal hypertension, circulatory anastomosis, liver bypass, diversion of portal flow of intestinal blood to the systemic circulation, collateral vessels (varices), and fistula

In the previous report, we discussed the possible anatomical routes for tryptamine from the gut microbiome to systemic circulation avoiding degradation by MAO [2]. Here, more detailed analysis of anatomical predispositions suggested for the gut microbiome-produced BA distribution in human organs. Biogenic amines can escape from the MAO-catalyzed degradation in liver by the liver bypass due to the circulatory anastomosis such as portacaval anastomosis. Portacaval anastomosis is a veno-venous anastomosis between a vein of the portal circulation and a vein of the systemic circulation, which allows blood to bypass the liver in patients with portal hypertension, often resulting in hemorrhoids, esophageal varices, or caput medusae. Portosystemic collateral pathways (also called varices) develop spontaneously via dilation of pre-existing anastomoses between the portal and systemic venous systems. Hypertension is produced by biogenic amines tryptamine, tyramine, and phenylethylamine [65–68]. Tryptamine produced vasoconstrictor and vasodilator responses in rat-isolated perfused mesentery vasculature [69]. Histamine produces the hepatic portal vasoconstriction at 0.5–1 μg (causes rise in vascular resistance) and the hepatic arterial vasodilation at 50–100 ng [70]. Histamine injection (10 ml in a dilution of 1 μg/ml) via the cannulated portal vein significantly raised the portal pressure (mean 2.25-fold, $p < 0.001$) in ten normal volunteers [71].

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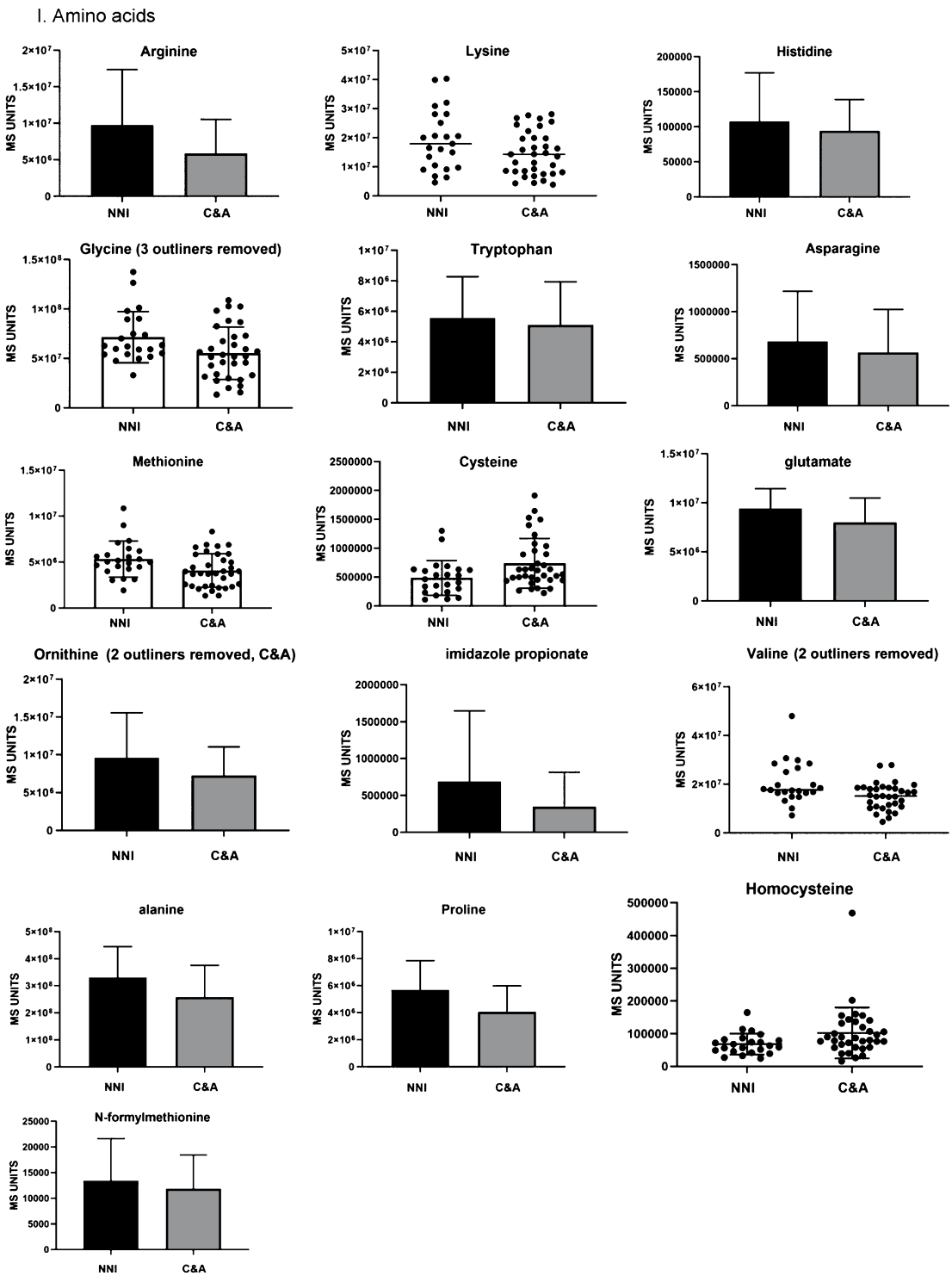


Fig. 1. (*Continued*)

Angiographic study showed that histamine induced intrahepatic portal vasoconstriction in the normal controls. A similar change was obtained in the schistosomal group (parasitic flatworms hepatic fibrosis), particularly on the periportal neovascular formation [71]. In patients with hepatosplenic schistosomiasis

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(8 patients) and controls (7 volunteers), standing heart rate was 60–110 versus 68–74 beats min–1; systolic 105–150 versus 100–120 mm Hg and diastolic blood pressure - 60–90 versus 60–80 mm Hg, respectively [72]. Study of cardiac dysfunction in cirrhotic portal hypertension revealed that left ventricular diastolic dysfunction is commonly associated with advancement of hepatic dysfunction while systolic function is maintained till advanced hepatic failure [73]. Cirrhosis with portal hypertension is associated with increased heart rate.

Tryptamine, infused intravenously in man (4 patients of the National Institute of Mental Health Addiction Research Center, Lexington, KY, USA), facilitates the patellar reflex, dilates pupils and elevates blood pressure (maximal difference between infusion and pre-infusion data for diastolic blood pressure was ~45 mm of Hg). It also causes changes in vision and hearing, as well as nausea, vomiting, dizziness, sweating, and heaviness of body [74]. The tryptamine infusion rates varied from 0.025 to 0.364 mg/kg/min, and periods of time varied from 2 to 7.5 min. The total amounts of tryptamine administered during an experimental day ranged from 23 to 277 mg. Maximal rates and times of infusion were determined by the patient's ability to tolerate the infusion. The control tryptamine excretion in the urine was found to be 0.1 mg/24 h. For the Austrian population, the fresh/cooked fish, preserved fish, cheese, raw sausage, condiments, sauerkraut and fermented tofu maximum tolerable levels were 1,650, 3,200, 2,840, 4,800, 14,120, 1,740, and 2,400 mg kg–1, respectively. For beer, the maximum tolerable limit of 65 mg kg–1 included an uncertainty factor of 10 [75]. A tryptamine dose without effect of 8 mg kg–1 body weight day–1 was derived from literature data. In rats, the i.v. administration of tryptamine (20 mg/kg) induced clonic seizure [76]. Tryptamine-induced (40 mg/kg, administered via the tail vein) clonus of the forepaws was observed in 100% of the animals (20 rats) [77]. Tryptamine-induced clonic seizures are studied at the dose 40 mg/kg in rats (bilateral clonic seizures of the forepaws were observed for a period of 5 min after tryptamine intravenous injection [78]. In our study, convulsions were induced in Balb/c mice by intravenous injections of tryptamine at dose 40 mg/kg of weight [11]. Increase of urine tryptamine from $\leq$120 μg/24 h (normal) to 900 μg/24 h in the patient was demonstrated during MAO inhibition [79].

According to the Mayo Clinic, hemorrhoids are very common. Nearly three out of four adults will have hemorrhoids from time to time. Rectal varices and their relationship to hemorrhoids presented in patients with portal hypertension [80]. Elevated venous portal pressure is an important factor in patients having severe hemorrhoidal bleeding [81]. Portal systemic encephalopathy is a neuropsychiatric disorder that occurs secondary to chronic liver disease. The vein most affected is the portal vein, which contributes 75% of the blood to the liver. To eliminate the high pressure in the portal vein caused by the reduced blood flow, the portal vein develops collateral vessels that bypass the liver and drain directly into the inferior vena cava. Almost any vein in the abdomen may serve as a potential collateral channel to the systemic circulation [82]. Although varices of the gastroesophageal axis are most often the cause of clinical concern in patients with portal hypertension, varices in other locations have been frequently reported [83]. Swollen portasystemic collateral vessels have been found in the duodenum, small bowel, and colon, at intestinal anastomoses and stomas, on the abdominal wall, and in the rectum at the so-called hemorrhoidal plexus [80]. When blood is not cleared, or when hepatic functioning fails, toxins accumulate in the circulation and affect the central nervous system [84]. The normal hepatic venous pressure gradient (HVPG) value is between 1 to 5 mmHg. Pressure higher than this defines the presence of portal hypertension, regardless of clinical evidence [85]. The portal hypertension (defined as HVPG $\geq$10 mmHg) marks the progression to a stage of clinical complications. Diagnosed cirrhosis and portal hypertension might regress after removing the etiological factor causing disease [86]. In the patient with senile dementia and no evidences of portal hypertension, a meso-caval shunt was found in the right lower abdomen. Superior mesenteric arteriography revealed a shunt flowing into the inferior vena cava via the right gonadal vein. The shunt was closed by balloon, and dementia-like symptoms improved [87]. The brain lesions of patients with chronic portal-systemic venous shunts were much the same regardless of the presence or absence of primary liver disease. Alzheimer-type abnormality of astrocytes and areas of spongy degeneration (multiple vacuoles) were found [88]. An autopsy case of portal systemic encephalopathy and senile dementia of the Alzheimer-type coexisting was described [89]. A heterogeneous distribution for the tryptamine binding site was observed in hippocampus $\gg$ frontal cortex = caudate nucleus > temporal cortex = cerebellum. When

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comparing [3H]tryptamine binding site densities in control brain tissue with that in brain tissue from patients with hepatic encephalopathy (HE), significant decreases in densities were observed in the frontal cortex (by 56%, $p < 0.001$), hippocampus (by 43%, $p < 0.001$), and caudate nucleus (by 41%, $p < 0.01$) of the HE group. Binding site affinities were within normal limits. The findings of decreased densities of [3H]tryptamine binding sites taken in conjunction with reports of increased cerebrospinal fluid and brain tryptamine concentrations in HE suggest a pathogenic role for this neuroactive amine in HE resulting from chronic liver failure [90]. Congenital portosystemic venous shunts are rare vascular anomalies resulting in diversion of portal flow to the systemic circulation. They allow intestinal blood to reach the systemic circulation bypassing the liver, resulting in a variety of symptoms and complications in the longer term, although asymptomatic cases, discovered incidentally on imaging, are not uncommon [91]. The BA produced by gut microbes can avoid degradation in liver by spreading through the gastrointestinal fistula, which can be formed due to paraproctitis [92]; inflammatory bowel diseases, such as Crohn's disease [93]; diverticulitis [94]; abscess [95]; or spontaneously [96]. In three-dimensional (3D) cell culture, the vascular anastomosis occurs between sprouts formed by angiogenesis and tubes formed by vasculogenesis [97]. Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system whereas angiogenesis is the process by which new blood vessels take shape from existing blood vessels by "sprouting" of endothelial cells.

In our studies, the cytotoxic concentrations of tryptamine stimulated the angiogenesis in hippocampus of mouse brain [98] and outgrowth of neuronal processes with neurodifferentiation in human cell culture [12]. The endothelial cell tube formation and sprouting are dependent on two essential processes in angiogenesis, endothelial cell migration and differentiation, while the synergistic inhibition of endothelial cell proliferation, tube formation, and sprouting can be induced by cytotoxins [99]. In the 3D collagen gel culture, we demonstrated that tryptamine (5 μg/ml or 30 μM; 25 and 100 μg/ml, bell-shaped concentration-dependence with optimum at 25 μg/ml or 0.15 mM) potentiates the capillary-like tube formation by human umbilical vein endothelial cells (HUVEC) when cultured in the presence of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF)-165 at 100 ng/ml each (data not shown). Tryptamine itself had no direct effects on the processes of angiogenesis in this procedure. The VEGF isoform VEGF-A(165) and HGF stimulate mitogen-activated protein kinases and focal adhesion kinase. This study demonstrated that under angiogenic conditions, combining VEGF-A with HGF can promote neovascularization by enhancing intracellular signaling [100]. Similar to tryptamine [12], histamine induces neural stem cell proliferation and neuronal differentiation [101]. Data showed that another BA histamine (0.1, 1, 10 μM) itself was absent of direct effects on the processes of angiogenesis, including the proliferation, migration, and tube formation of endothelial cells. Nevertheless, it could concentration-dependently enhance basic fibroblast growth factor (bFGF)-induced angiogenesis as well as production of VEGF from endothelial cells [102].

Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver disease worldwide. Global prevalence of NAFLD is 25.24% with highest prevalence in the Middle East and South America and lowest in Africa. Metabolic comorbidities associated with NAFLD included obesity, T2DM, hyperlipidemia, hypertension, and metabolic syndrome [103]. There is evidence that portal venous pressure may begin to rise in the earliest stages of NAFLD when fibrosis is far less advanced or absent [104]. The presence of ADAS was earlier reported in fecal samples of NAFLD patients with obesity [2]. Idiopathic portal hypertension is characterized by a long-standing presinusoidal portal hypertension of unknown etiology in adults. Some unidentified agent(s) affect(s) the intrahepatic small portal veins or portal tracts [105]. A France 2008-13 nationwide retrospective cohort study concluded that alcohol use disorders were a major risk factor for onset of all types of dementia, and especially early-onset dementia [106], while alcohol is a neurotoxin inducing liver and brain damage [107].

Prevalence of fecal metabolites in C&A and CRC

ADAS was found in 4 of 35 C&A individuals [2], while in the gut metagenomics of 23 control rural Oklahoma non-native individuals [15], no ADAS was detected by BLAST analysis of the BioProject Sequence Read Archive (SRA) PRJEB13051.

Approximately one third of CRC patients had at least one comorbidity, with cardiovascular disease and T2DM being most common [108]. CRC is the second most common cancer among American Indi-

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ans. Comparing the epidemiological data on CRC and AD, it was found that CRC and AD have similar epidemiologic feature; both diseases correlate with high prevalence of constipation [109]. This correlation supports a role of gastrointestinal conditions in the pathogenesis of AD.

The detectability as a prevalence of fecal metabolites are varied among participants. In many if not most metabolomics, statistical analyses are conducted for the metabolites with a high prevalence close to 100%, while the metabolites of lower prevalence are frequently unnoticed. However, the prevalence (%) as a fraction above the detection limit can be instrumental in the analysis of comparatively rare samples. Here the prevalence of 132 fecal metabolites are compared between C&A and CRC with a special focus on the food-derived metabolites with MAOI activities, anti-microbial activities, tryptamine pathway, metabolites reflecting protein biosynthesis/proteolysis/tRNA aminoacylation, known signaling molecules, cytotoxic metabolites, and disease-related compounds. In this study, prevalence (%) as a fraction above the detection limit correlates directly or inversely C&A conditions and CRC in a number of fecal metabolites including tryptamine and short peptides. The data on metabolites prevalence presented in Supplementary Table 1, while Fig. 2 demonstrates the possible interactions between fecal metabolites in C&A and CRC. The fecal peptide profiles indicate that both increased protein degradation/proteolysis (higher peptide prevalence) and impaired tRNA aminoacylation (lower peptide prevalence) characterize gut microbiome of C&A and CRC populations in comparison with NNI and controls, similar to findings on short peptides in the blood of AD patients [98].

Fecal tryptamine increase correlates with tryptamine-induced morphological features in cells and mouse brain

Increase in tryptamine prevalence correlates with elevation of the soy product MAOI genistein in human gut of both C&A and CRC compared to controls. Tryptamine treatment induce formation of tangles of twisted filaments in the bovine kidney cells MDBK (15–20 nm thick), in human neuronal cells SH-SY5Y subline and in mouse brain (Fig. 3 A–C) resembling neurofibrillary tangles (NFTs) of paired helical filaments that characterize AD patients and

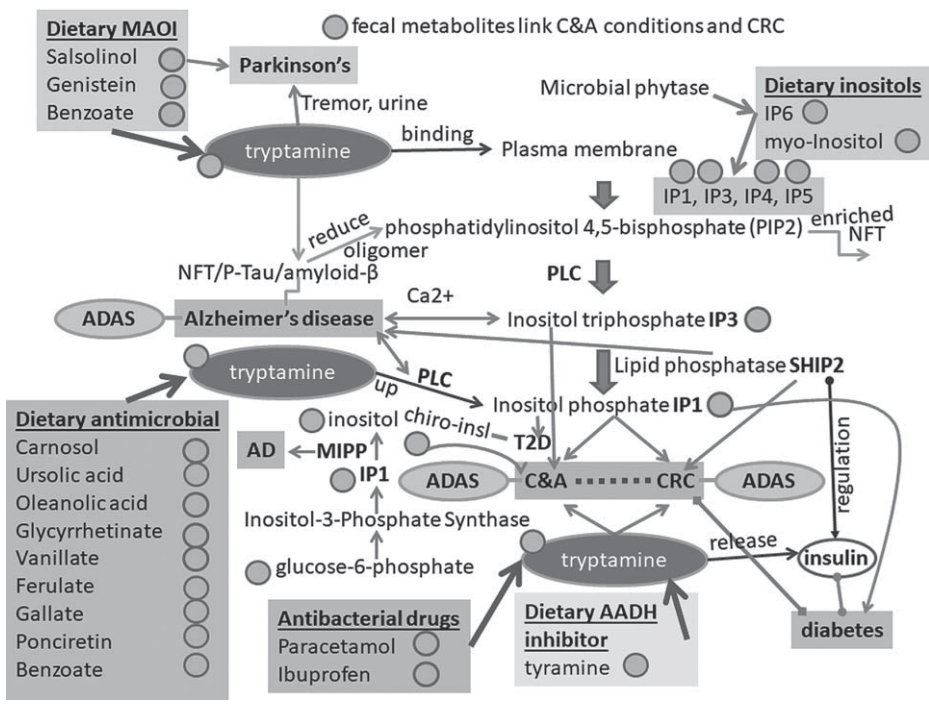


Fig. 2. A metabolic link between CRC, medical conditions prevailing in C&A, and diet. The scheme shows the interactions between the fecal metabolites in the (ADAS+) populations. The gut metabolites are altered in ADAS-containing C&A [14] and CRC [16]. The associated medical conditions (diabetes, Parkinson's disease, and AD) and dietary metabolites present in the scheme are discussed in the Results and Discussion. Abbreviations are in the text.

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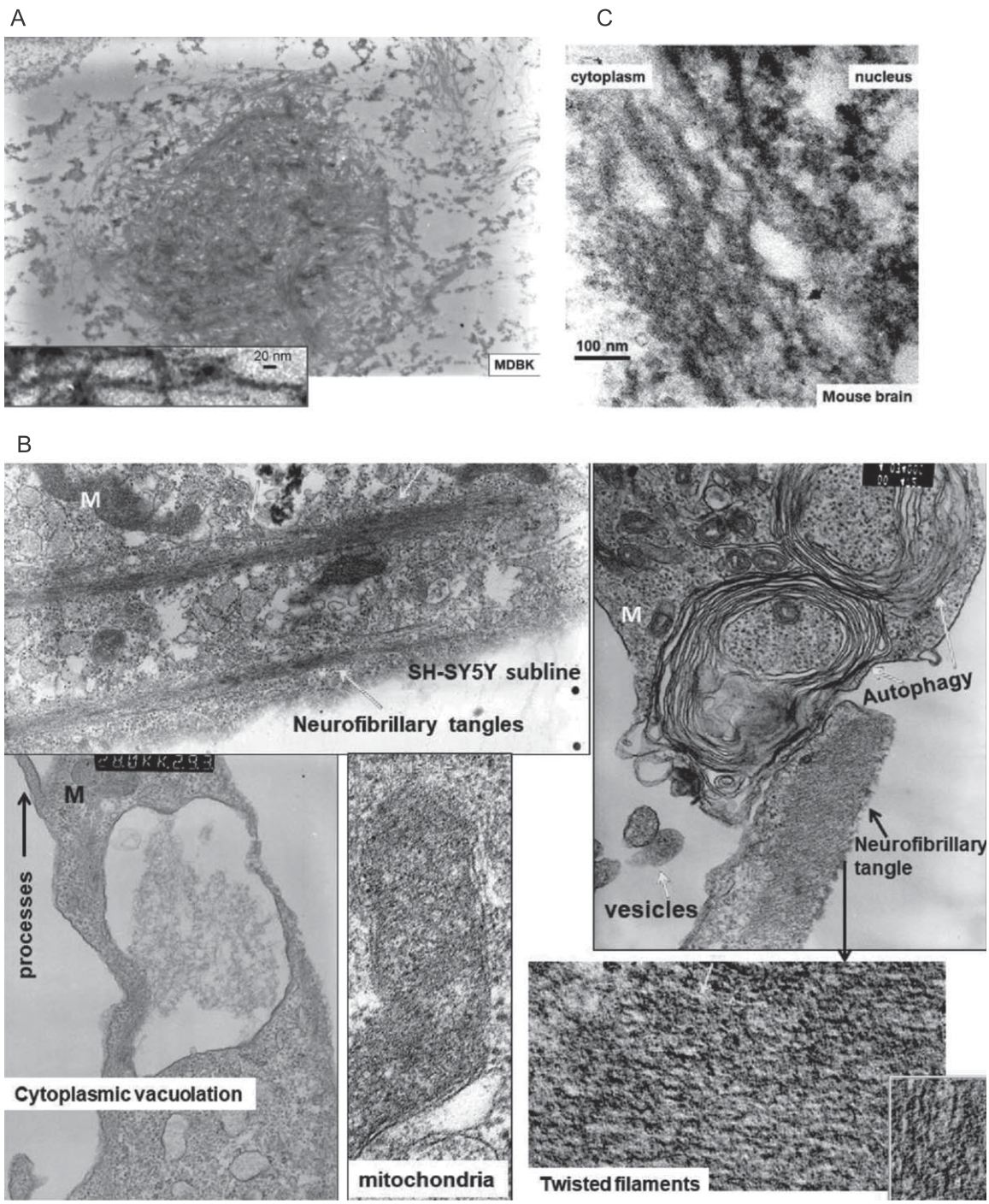


Fig. 3. Electron microscopy of tryptamine-treated bovine kidney (A), human neuronal (B) cells, and mouse brain (C). A) Tangle of twisted filaments (15–20 nm thick) in detergent-pretreated MDBK cells. B) The twisted filaments are visible in mitochondria lacking mitochondrial cristae. M, mitochondria. Blue arrow shows TrpRS-immunopositive twisted filaments revealed with immune-gold particles of 10 nm. The monoclonal antibodies AT8 occasionally show rare *P*-tau positive twisted (helical) filaments. The visible here dramatic cytoplasmic vacuolation in the tryptamine-treated cells probably occur due to overproduction of hydrogen peroxide (H_2O_2), the reactive oxygen species (ROS). Tryptamine degradation produces H_2O_2. Morphological characteristics of cell death induced by H_2O_2 include DNA fragmentation, increased vacuolization, and nuclear condensation. The control cells and mice showed no manifestations of degeneration similar to those visible in tryptamine-treated cells and animals. The treatment of cells and animals and anti-TrpRS antibodies described earlier in details [4, 11, 277].

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other neurodegenerative diseases. The tryptamine cytotoxic effect visualized as cytoplasmic vacuolization or vacuolation of "spongy degeneration" type (Fig. 3B). The vacuolation seems to be a distinct form of cell death, which is different from the conventional lytic and apoptotic modes. Another way of cell death related to tryptamine effect is vesicularization or vesiculation—the formation of vesicles accompanied with neuronal cell fragmentation (Fig. 3B). The abundant vesicularization and neuronal fragmentation was also observed in AD brain [12]. The twisted filaments are visible in cytoplasm and in mitochondria lacking mitochondrial cristae in tryptamine-treated neuronal cells (Fig. 3B). Importantly, the decreased levels of platelet distribution width, red blood cell, hematocrit, hemoglobin, lymphocyte, and basophil revealed in the peripheral blood cells of AD patients compared with normal controls [110]. The blood cells deficiency can be caused by cytotoxic compounds such as tryptamine circulated in the peripheral blood and/or present in the blood cell forming tissues such as bone marrow, the lymph nodes, and spleen of AD patients.

Food-derived fecal metabolites

Here the population-based data on prevalence (Table 2) and levels (Fig. 1) analyzed for food-derived fecal metabolites that include toxins, MAOI, antimicrobial compounds, biogenic amines, and other health-related metabolites. The C&A participants had significantly higher fecal erythritol level (1.87-fold, F test p value <0.0001****) than the NNI population. Participants with adiposity gain had significantly higher blood erythritol, a pentose-phosphate pathway metabolite [111]. It is widely used as sweetener in calorie-reduced food [112]. Erythritol may contribute to association between food and obesity. Food additive erythritol is significantly associated with incident T2DM [113]. Prevalence and level of fecal 2,4,6-trihydroxybenzoate are higher in NNI compared to C&A. In the clinical trial [114], the 2,4,6-trihydroxybenzoate significantly increased by 1.75-fold in the volunteers group consumed cranberry juice versus a placebo group suggesting that this may be a potential biomarker of consumption for cranberry juice. The 2,4,6-trihydroxybenzoate was not found in the cranberry beverage or blood plasma, but was detected in urine (https://www.fasebj.org/doi/abs/10.1096/fasebj.29.1_supplement.249.2).

Prevalence of food-derived MAOI genistein is higher in C&A (1.42-fold) and lower in CRC (1.2-fold) than in controls. The detectible genistein levels varied in both groups: in NNI (8,335–248,115 units) and C&A (4,053–141,578 units). In the NNI fecal samples, the highest tryptamine level corresponds to the highest genistein level in agreement with the genistein MAOI activity [115]. The difference in the genistein detectable levels was NNI/C&A = 2.3-fold (F test p value 0.0061).

Prevalence of food-derived [116] fecal metabolite neurotoxin (R)-salsolinol implicated in PD [117] is higher in C&A compared to NNI (difference in the level C&A/NNI = 4.25-fold, F test p value 0.0044) and lower in CRC than in controls. The dopamine-derived salsolinol derivatives determined as endogenous MAOI [118]. Salsolinol presents in Port wine and dried banana [119].

Prevalence of phytanate is higher in C&A and CRC than in controls. The level of food-derived phytanate is 1.58-fold higher in C&A than in NNI (t test p value 0.02*, F test p value 0.001**). Elevated amounts of phytanate was revealed in cases of multi-neuronal degeneration, hepatosplenomegaly, and adrenocortical insufficiency [120]. Phytanic acid induced neurotoxicity in an animal study [121]. Studies elucidated that the toxic activity of phytanic acid is mediated by multiple mitochondrial dysfunctions, generation of ROS, and Ca2+ deregulation via the Inositol P3–Ca2+ signaling pathway in glial cells [122]. Phytanic acid is a conjugate acid of a phytanate. It derives from a hydride of a phytane. Phytanic acid and phytanate are used as synonyms. Prevalence of phytol, a metabolic precursor of phytanic acid [123] is 100% in both C&A and NNI and is lower in CRC than in control group. The level of phytol is 1.8-fold higher (statistically significant p value <0.0001) in NNI than in C&A.

Prevalence of antimicrobial dietary component Carnosol is higher in NNI than in C&A. Prevalence of antimicrobial dietary components ursolic acid (163,729–3,076,120 units; levels: NNI/C&A = 1.46-fold, p value 0.0004) and oleanolic acid [124] (detected level NNI/C&A = 2.18-fold, p value 0.0395) is considerably lower in C&A and CRC compared to controls. Prevalence of glycyrrhetinate, the antibacterial compound of the plant Licorice [125] used in food, is lower in C&A than in NNI (NNI/C&A level 1.87-fold, p value 0.0002). Prevalence of a rare fecal ponciretin (occurred in the trifoliate orange) with anti-Helicobacter pylori activity [126] is lower in C&A and CRC compared to controls. The xyli-

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Table 2
The estimations of food-derived metabolites: percentage of population with detectable fecal metabolites derived from the studies of C&A, NNI [14], CRC and controls [16]. Prevalence (%) was estimated as a fraction above the detection limit

Metabolites	C&A study, %*		CRC** study, %	
	NNI	C&A	Control	Case
2,4,6-trihydroxybenzoate¤ (food, cranberry juice), cytotoxic[280]	56	26	n/d	n/d
genistein (food, soy, MAOI)	26	37	52	62
phytanate (food, neurotoxic)	73.9	77.1	89	90
phytol (food)	100	100	100	96
carnosol (food rosemary, antimicrobial)	86	71	n/d	n/d
ursolic acid (food, antimicrobial)	74	34	78	60
oleanolate (food, antimicrobial)	74	40	87	83
vanillate (food, antimicrobial)	70	83	77	79
gallate (food, antibacterial)	56	51	47	31
ferulate (food, antibacterial)	100	97	100	96
glycyrrhetinate (plant, antibacterial)	87	57	n/d	n/d
solanidine (food, potato, poisonous)	83	94	91	92
ponciretin (food, antibacterial)	17	14	39	35
(R)-salsolinol (food, neurotoxin, PD, MAOI)	17.4	31.4	78	56
pyridoxine (food, vitamin B6)	57	66	86	100
gamma-CEHC (food desserts, vitamin E metabolism, smocking, breast cancer [281])	30	49	76	52
N-acetylglycine (food, glycine)	100	94	99	98
trans-4-hydroxyproline (food processed meat, prostate cancer [282])	61	86	29	35
taurine(energy drinks, anti-depressant)	48	57	93	90
erythritol (food) sweetener	97	94	93	94
xylulose (carbohydrate) antibiotic [283]	78	91	70	65
xylitol, diabetic food, antibiotic [283]	74	71	61	56
mannitol (diabetic food) sweetener [283]	78	46	51	50
caffeine	91	83	99	96
diaminopimelate (bacteria, plants, lysine synthesis)	70	86	54	56
beta-hydroxypyruvate, food, T2D	26	29	51	54
agmatine	43	60	65	69
cadaverine	100	100	92	94
spermidine	100	91	32	25
N-acetylputrescine	83	86	100	98
sucralose (Splenda)	47.8	57.14	n/d	n/d
4-hydroxycyclohexylcarboxylic acid	31	51	n/d	n/d
anacardic acid (anti-bacterial)	26	25.7	10	17

2,4,6-trihydroxybenzoate is a product of oxidative degradation of quercetin[284]. In this table, the filled pink cells (color shading) represent positive associations and filled green cells represent inverse association between C&A and CRC.

tol level is higher in NNI (NNI/C&A = 7.35-fold, F test < 0.0001). The sugar alcohol xylitol inhibits the growth of some bacterial species including Streptococcus mutans. It is used as a food additive to prevent caries [127]. Doses of xylitol as low as 0.15 g/kg-BW in dogs can result in life-threatening hypoglycemia and acute liver failure [128]. Xylulose is also higher in NNI (NNI/C&A = 1.6-fold, *t* test *p* value 0.02; 2 outliners in each group determined and removed using GraphPad Prism 8 software). The biotransformation from xylitol to L-xylulose by xylitol 4-dehydrogenase was studied intensively [129]. The lower prevalence of the plant-derived metabolites correlates with the C&A characteristics of high rates of processed food consumption and low fresh fruit and vegetable consumption. Concentrations of the antimicrobial dietary compounds varied among participants. These compounds can modify a gut microbial tryptophan metabolism in a dose-dependent manner.

Okra plants (used as food) colonization with *Alcaligenes faecalis* bacterial endophytes induced the plant defense response that can contribute to the biocontrol activity toward pathogenic fungus [130]. Assessment of chemotactic and biofilm formation potential toward individual phenolic acids revealed that different strains of *Alcaligenes faecalis* are maximally attracted toward 1 μM shikimic acid or 1 mM vanillic acid (4-hydroxy-3-methoxybenzoic acid; flavoring agent) or 1 mM ferulic acid, thereby the phenolic acid components are responsible for attracting the endophytic A. faecalis isolates [131].

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Human infections due to *Alcaligenes faecalis* are very rare [132, 133]. Food components vanillic acid [134], ferulic acid and gallic acid [135] exerted antibacterial activity. Prevalence of vanillate (level NNI/C&A = 1.14-fold, p value 0.006), ferulate (no significant difference in level NNI/C&A), and gallate (level NNI/C&A = 4.4-fold, p value <0.0001) are altered in C&A and CRC compared to controls (Table 2, Fig. 1). Gallic acid occurs in a great number of fruits and vegetables. The food compound plant sterol sitostanol is higher in C&A (C&A/NNI = 1.257-fold, F test p value 0.0155, 1 outliner removed) and lower in CRC compared to control. The food compound daidzein prevalence is higher while level is lower in C&A compared to NNI. Prevalence and level of β-hydroxypyruvate (OH-pyr) identified as a potential mediator of T2DM [136] is higher in C&A and CRC. OH-pyr can be biosynthesized from pyruvic acid and L-serine through the action of the enzyme serine—pyruvate aminotransferase. OH-per can be found in a number of foodstuffs such as root vegetables, cascade huckleberry, chervil, and common salsify. In C&A, prevalence of trans-4-hydroxyproline, which present in processed meat is higher (1.4-fold), but the level is lower than in NNI (NNI/C&A = 1.3-fold, F test p value 0.0002). Lower plasma trans-4-hydroxyproline and methionine sulfoxide levels are associated with insulin dysregulation in horses [137]. Methionine sulfoxide level is lower in C&A than in NNI (NNI/C&A = 1.2-fold, F test p value 0.0311). Thermal treatment of milk and milk products leads to protein oxidation, mainly the formation of methionine sulfoxide [138]. Prevalence and level of diaminopimelate are higher in C&A and CRC compared to controls (C&A/NNI = 1.72-fold F test p value 0.0160. Diaminopimelic acid (DAP) is an amino acid, representing an epsilon-carboxy derivative of lysine. DAP is a characteristic of cell walls of some bacteria. Prevalence of pyridoxine (vitamin B6) is higher in C&A and CRC versus controls. The level of vitamin B6 is higher in C&A than NNI (1.62-fold, p values t test 0.03*, F test <0.0001****). Tryptamine is produced by the aid of the vitamin B6-dependent enzymatic reaction. The enzyme employs a pyridoxal 5' phosphate (PLP) cofactor similar to many amino acid decarboxylases. Pyridoxine induced cell death in a concentration-dependent way [139]. The food-derived pipecolate (metabolite of essential amino acid lysine) [140] is elevated in pyridoxine-dependent epilepsy and in C&A compared to NNI (C&A/NNI = 1.37-fold t test p value 0.0303). Another lysine metabolite, the food-produced 2-aminoadipate [141], is elevated in C&A (C&A/NNI = 1.46 t test p value 0.0413). Aminoadipic acid inhibits the production of kynurenic acid, a tryptophan metabolite in brain tissue slices [142].

Biogenic amines (BA), GABA, and amino acids from food and gut microbiome: Decarboxylase activity

Although no significant difference in fecal tryptophan detected between NNI and C&A (NNI/C&A = 1.088-fold, t test p value 0.5445), the decarboxylated tryptophan metabolite tryptamine is higher in C&A (C&A/NNI = 3.73-fold, cleaned data minus definitive 5 outliners, rout $Q = 0.1\%$, identified and removed by GraphPad Prism 8 software; p values t test 0.0031**, F test <0.0001****). Tryptamine metabolite indoleacetate formed via MAO oxidation activity is slightly higher in NNI (NNI/C&A = 1.06-fold, F test p value 0.0239*). Altogether, the data indicate that the tryptamine conversion to IAA in gut can be inhibited in C&A group by MOAI and also by inhibitors of diamine oxidase (DAO), such as copper amine oxidase, which is active toward BA including tryptamine as a substrate [143]. Tryptamine also inhibits irreversibly this enzyme under anaerobic conditions in a manner of substrate inhibition.

In total, 74 BA-producing or BA-degrading strains were isolated from the human gut [144]. The human fecal bacteria *Alcaligenes faecalis* that presents in environment is able to utilize phenylethylamine, tyramine, and tryptamine as both carbon and nitrogen sources via activity of aromatic amine dehydrogenase (AADH). The quinone prosthetic group of AADH is tryptophan tryptophylquinone [145]. While MAO degrading BA present in both eukaryotes and prokaryotes, the AADH have been found only in bacteria. The inhibitors of AADH enzymatic activity can increase BA levels. Tyramine exerts the substrate inhibition activity toward AADH. Notably, the dietary/microbial tyramine is significantly higher in C&A than in NNI. Thus, tyramine can potentially protect tryptamine from the AADH-catalyzed utilization. AADH was inhibited irreversibly by hydrazine, phenylhydrazine, hydroxylamine, semicarbazide, and aminoguanidine while reversibly by isonicotinic acid hydrazide (isoniazid) and isonicotinic acid 2-isopropyl hydrazide (iproniazid) [146].

An increase in polyamines cadaverine (C&A/NNI = 7.8-fold t test p value 0.0002) and N-acetyl-cadaverine (C&A/NNI = 5-fold t test

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p value 0.0006) is significant in C&A compared to NNI. Lysine decarboxylase is an enzyme that converts lysine to cadaverine. N-acetylcadaverine has been found to be a metabolite of several bacteria species. The polyamine putrescine is also elevated in C&A (C&A/NNI = 2.9-fold F test *p* value <0.0001). N-acetylputrescine prevalence and level are elevated in C&A (C&A/NNI = 2.7-fold F test *p* value 0.0017). The biogenic amines putrescine and cadaverine show *in vitro* cytotoxicity at concentrations that can be found in foods [147]. A level of polyamine spermidine is elevated in C&A (C&A/NNI = 1.55-fold, f test *p* value <0.0001), while its prevalence is lower in C&A and CRC compared to controls. Spermidine exhibits anti-inflammatory properties [148]. The three low pH inducible amino acid decarboxylases specific for arginine, lysine, and ornithine were annotated in the bacterial genome [149]. It has been extensively demonstrated that the transcription of genes of many decarboxylase clusters are induced by low pH [150]. Tyramine biosynthesis is transcriptionally induced at low pH in human gut bacteria Enterococcus faecalis [151]. The histidine decarboxylase gene cluster of Morganella morganii DSM30146(T) was sequenced, and four open reading frames, named hdcT1, hdc, hdcT2, and hisRS, were identified. Two putative histidine/histamine antiporters (hdcT1 and hdcT2) were located upstream and downstream the hdc gene, codifying a pyridoxal-P dependent histidine decarboxylase, and followed by hisRS gene encoding a histidyl-tRNA synthetase [152]. The hdc and hisRS genes were highly induced under acidic and histidine-rich conditions. The food-borne biogenic amine histamine, a decarboxylated histidine is significantly higher in C&A than in NNI (C&A/NNI - 5.2-fold t test p value 0.0167) while the level of essential amino acid histidine is 1.14-fold lower in C&A than NNI (F test *p* value 0.02). In NNI, ratio histamine/histidine = 16-fold (F test *p* value <0.0001) whereas in C&A, the ratio histamine/histidine = 95-fold (*t* test *p* value 0.0031; F test *p* value <0.0001). Histamine is one of the few central nervous system neurotransmitters found to cause consistent blood-brain barrier opening [153]. Histidine is transported across the blood-brain barrier by its transporter, but histamine, a singly charged cation, can hardly penetrate the blood-brain barrier [154]. Pretreatment of mice, rats, and guinea pigs with inhibitors of histamine methylation, followed by systemic injection of 14C-histamine, results in a significant increase in brain 14C-histamine relative to controls [155]. Prevalence of N-acetyl-1-methylhistidine is 1.6-fold higher in C&A versus NNI. The two genes within the his operon implicated in histidine biosynthesis, trpR and hisB, were required for rice pathogen Xanthomonas virulence and bacterial growth in planta [156]. In Lactobacillus, the histamine-producing pathway encoded on an unstable plasmid [157]. Histamine at the concentration 16 μM significantly inhibited protein biosynthesis in the rat liver microsomal fraction with aminoacyl-tRNA synthetases [158]. Tyramine level is 4-fold higher in C&A than in NNI [2] (*t* test *p* value 0.0194*; F test *p* value <0.0001****). Tyramine and histamine were toxic for intestinal cell cultures at concentrations commonly found in biogenic amines-rich food [159]. Moreover, the dietary biogenic amines tyramine and histamine show synergistic toxicity toward intestinal cells in cultures [160]. The cytotoxic histidinol, a histidine precursor, inhibits histidyl-tRNA synthetase. In addition to inhibiting protein synthesis, L-histidinol acts as an intracellular histamine antagonist [161, 162]. Human gut bacteria strains convert dietary histidine into histamine [163]. Bacterium that produces histamine utilizes histidine as its sole energy source, and it could play a role in bovine and equine laminitis [164]. This histamine-producing bacterium was isolated from the feces of cattle fed grain and the cecum of a horse. Histamine-secreting microbes are increased in the gut of adult asthma patients [165]. Histamine level in cattle rumen fluid increased from 0.013 mM (control) to 3.23 mM following soybean meal (at 48 h during *in vitro* rumen fermentation) [166]. Imidazole propionate as a microbially produced histidine-derived metabolite present at higher concentrations in subjects with versus without T2DM [167]. The imidazole propionate is lower (1.967-fold, F test *p* value 0.0002***) in C&A versus NNI. The prevalence (1.45-fold) and the level (1.85-fold, *t* test *p* value 0.0074) of histidine catabolite formiminoglutamate is lower in C&A versus NNI. A dietary biogenic amine agmatine is a decarboxylated arginine elevated in C&A and CRC (prevalence C&A/NNI = 1.4-fold or 60%/43%, level C&A/NNI = 2.06-fold F test *p* value 0.016). In targeted plasma metabolomics, the amino acids histidine, arginine, and glutamine are lower in schizophrenic patients than in controls [168]. The fecal arginine is lower 1.677-fold in C&A compared to NNI (*t* test *p* value 0.0334*, F test *p* value 0.012*). The mean lysine is 1.32-fold lower in C&A compared NNI (unpaired *t* test *p* value

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0.0559). Methionine is 1.3-fold lower in C&A than in NNI (Welch's *t* test *p* value 0.0184*). Cysteine is higher 1.52-fold in C&A than in NNI (*t* test *p* value 0.0113*). Taurine is derived from cysteine. Taurine prevalence and level are higher in C&A than in NNI (C&A/NNI 3.76-fold in *t* test, *p* value 0.0213*, F test *p* value 0.0011**). In this pathway, cysteine sulfinic acid is decarboxylated to taurine precursor. Although the aged or diseased brain might benefit from taurine food supplementation, it appears that adolescents are not likely to benefit from supplementation and may, in fact, suffer ill effects from chronic ingestion of high doses [169]. A previous study [2] showed the increase of biogenic amine phenylethylamine (PEA) in C&A versus NNI. The PEA abundant in fermented foods [170] is cytotoxic for some cells [171]. PEA is an inhibitor of bacterial phenylalanyl-tRNA synthetase [172]. PEA elevates mood and favors aggression [173]. The ratio cadaverine/Lys for NNI is 0.79 (*t* test *p* value <0.0001****), while the ratio cadaverine/Lys is 8.15 for C&A (*t* test *p* value 0.0001***, F test *p* value <0.0001****). The cadaverine/Lys ratio is 10.3-fold higher for C&A than for NNI. Cadaverine is a decarboxylation product of lysine and a competitive inhibitor of lysine binding to its lysyl-tRNA synthetase (LysRS) [174, 175]. The Km for lysine is 23.6 ± 2 μM and Ki for cadaverine is 77 ± 8 μM in ATP-PP, exchange reaction of LysRS from Bacillus stearothermophilus. Thus, the 8-fold molar excess of cadaverine over lysine in C&A can result in LysRS inhibition. Increased metabolites such as methionine sulfoxide, 3-methoxy-anthranilate, cadaverine, guanine, and histamine were observed by widely targeted metabolomics of postmortem cerebrospinal fluid from the AD subjects [176]. Fluor 555-cadaverine exudes into most tissues, but normally is excluded from the central nervous system, the vasculature of which is protected with the blood-brain barrier [177]. The metabolic link of histidine to tryptophan was demonstrated in Neurospora crassa, in which the starvation of tryptophan mutants for tryptophan resulted in the de-repression of tryptophan, histidine, and arginine biosynthetic enzymes [178]. Ornithine level is 1.32-fold lower in C&A compared to NNI (F test *p* value 0.0199*). Ornithine is a non-proteinogenic amino acid. Ornithine via the action of ornithine decarboxylase is the starting point for the synthesis of polyamines such as putrescine. The mean level value of gamma-aminobutyrate (GABA) synthesized from glutamate via the enzyme glutamate decarboxylase with pyridoxal phosphate (the active form of vitamin B6) as a cofactor is 1.8-fold higher in C&A compared to NNI (*t* test *p* value 0.1605; F test *p* value <0.0001****) while glutamate is 1.18-fold lower in C&A than in NNI (*t* test *p* value 0.0203*, F test 0.2835). Data suggest that GABA and glutamate play an important role in the etiology of pain and depression [179]. GABA and cadaverine inhibit LysRS and GABA inhibits *in vivo* growth of Bacillus subtilis strains [180]. Level of essential amino acid valine is 1.36-fold lower in C&A versus NNI (*t* test *p* value 0.0114*, F test *p* value 0.0178*). The level of norvaline is also lower in C&A versus NNI (1.28-fold, F test *p* value 0.0015**). The misincorporation of a non-proteinogenic amino acid norvaline found in *E. coli* strain on 10% of all measured leucine residues under microaerobic conditions [181]. The level of essential amino acid isoleucine is 1.25-fold lower in C&A versus NNI (*t* test *p* value 0.0294*). The level of essential amino acid threonine is 1.39-fold lower in C&A versus NNI (*t* test *p* value 0.0002***). The level of allo-threonine is 1.43-fold lower in C&A versus NNI (*p* values *t* test 0.0342*, F test 0.0109*). Allo-threonine found in immunogenic tetra-peptide of the Mycobacterium avium [182]. The level of essential amino acid phenylalanine is 1.25-fold lower in C&A versus NNI (*t* test *p* value 0.0341*, one outliner removed). The level of essential amino acid leucine is not significantly different in C&A versus NNI (NNI/C&A = 1.2-fold, *t* test *p* value 0.0838). Thus, the essential amino acids (Met, Ileu, His, Val, Thr, Phe, Lys) excluding leucine and tryptophan are statistically lower in C&A versus NNI. The semi-essential amino acid tyrosine is 1.2-fold lower in C&A versus NNI (statistically non-significant, *t* test *p* value 0.0808). The nonessential amino acids are significantly lower in C&A versus NNI: proline is 1.4-fold (*t* test *p* value 0.0059**), glycine - 1.295-fold (*t* test *p* value 0.0288*), and alanine - 1.278-fold (*t* test *p* value 0.0253*). The nonessential amino acids glutamine (1.07-fold, *t* test *p* value 0.67), asparagine (1.21-fold, *t* test *p* value 0.3860) and serine (1.13-fold, *t* test *p* value 0.3358) are non-significantly lower in C&A versus NNI. The non-essential amino acid aspartate showed non-significantly higher level in C&A versus NNI (NNI/C&A = 1.009, *t* test *p* value 0.9490). Significant decrease was observed with AD progression for asparagine, methionine, histidine in the serum samples [183]. Therefore, the only one proteinogenic amino acid cysteine (Cys) was significantly higher in C&A versus NNI (Fig. 1,

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Table 3
Estimation of NNI/C&A ratio for proteinogenic amino acids derived from the Oklahoma metabolomics study of fecal samples from C&A and NNI populations [14]. Here, the Welch's *t* test used for statistical analysis of crude data derived from the Oklahoma study [14]

N	Amino acids	NNI/C&A fold	*t* test *p* value	F test *p* value
1	tryptophan	1.088	0.5445	
2	histidine	1.14	0.02*	
3	lysine	1.32	0.0559	
4	methionine	1.3	0.0184*	
5	valine	1.36	0.0114*	0.0178*
6	isoleucine	1.25	0.0294*	
7	leucine	1.2	0.0838	
8	phenylalanine	1.25	0.0341*	
9	threonine	1.39	0.0002***	
10	arginine	1.677	0.0334*	0.012*
11	tyrosine	1.2	0.0808	
12	proline	1.4	0.0059**	
13	glutamate	1.18	0.0203*	0.2835
14	glutamine	1.07	0.67	
15	serine	1.13	0.3358	
16	cysteine	–1.52	0.0113*	
17	glycine	1.295	0.0288*	
18	asparagine	1.21	0.3860	
19	aspartate	–1.009	0.9490	
20	alanine	1.278	0.0253*	
21	homocysteine	–1.5	0.0268*	<0.0001****
22	N-formylmethionine	1.135	0.4512	0.2825
23	allo-threonine	1.43	0.0342*	0.0109*

Table 3). Notably, plasma total concentrations of the sulphur amino acid Cys are linearly associated with fat mass in large population studies. Animal and cellular experiments support the concept that Cys may be obesogenic [184]. The PLP-dependent cysteine sulfinic acid decarboxylase catalyzes the decarboxylation of L-aspartate, 3-sulfino-L-alanine (cysteine sulfinic acid), and L-cysteate to beta-alanine, hypotaurine, and taurine, respectively. The expression of cysteine sulfinate decarboxylase of seawater Pacific oyster was upregulated hundreds of times in response to low salinity in salinity stress experiments [185]. Aromatic L-amino acid decarboxylase (AADC) activity in the intestinal mucosa of old rats was higher than in adult rats. High salt diet increased the AADC activity in adult rats, but not in old rats [186]. The biogenic amines tyramine, histamine, putrescine, cadaverine, and agmatine increased during the salt-ripening process of anchovies [187]. The highest BA values were found in the batch where initial microbial load was highest, especially for enterobacteria and enterococci. The presence of NaCl led to an upregulation of histidine decarboxylase gene in the bacterial strain grown on skim milk [150]. The BA were reduced during salted fish fermentation by starter bacterial culture of Bacillus polymyxa possessing amine degrading activity [188]. The disease-linked homocysteine, a protein amino acid in humans [189] is higher in C&A versus NNI (1.5-fold, *p* values *t* test 0.0268*, F test <0.0001****). Homocysteine is suggested for the risk prediction of cognitive impairment among ischemic stroke patients with elevated blood pressure [190]. Prevalence of N-formylmethionine (fMet), a derivative of the amino acid methionine in which a formyl group has been added to the amino group for initiation of protein synthesis in bacteria, mitochondria and chloroplasts is lower in C&A (80%) versus NNI (100%) and no difference is in CRC versus NNI (98%). The fMet level is non-significantly lower in C&A versus NNI (1.135-fold, *t* test *p* value 0.4512). A lowest content for proteinogenic amino acids is revealed here for histidine in C&A. In the Chinese study, dietary histidine was significantly lower in overweight ($p < 0.05$) and obese ($p < 0.01$) participants of both sexes [191]. As many as one-third of mutations in a gene result in the corresponding enzyme having an increased Michaelis constant, or K(m), (decreased binding affinity) for a coenzyme, resulting in a lower rate of reaction. The defective enzymes can be remedied or ameliorated by the administration of high doses of the vitamin component of the corresponding coenzyme, which at least partially restores enzymatic activity [192]. The amino acid decarboxylases are enzymes that use a PLP cofactor, the active form of vitamin B6. Plausibly the 1.62-fold increase in vitamin B6 in C&A versus NNI is linked to coordinated increase in decarboxylase enzymatic activities toward the PLP-dependent production of biogenic amines from corresponding amino acids. Over 80% of the histidine and 95% of the tyrosine had been converted into histamine and tyramine in cheese by day 60. Substrate (amino acids) depletion played an important role in the rate of biogenic amine buildup, becoming a limiting factor in the case of some amino acids. Tryptamine reached 0.4 mg/g of cheese during 240 days of ripening and storage [193].

The secreted protein diamine oxidase (DAO) has a slow turnover. This, together with the efficacy of the homeostatic regulation of the polyamines via the interconversion reactions and by transport pathways, renders a role of DAO in the regulation of polyamine concentrations unlikely [194]. Histamine intolerance is a disorder attributed to the deficiency in the enzyme DAO, which degrades histamine. In patients with

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episodic migraine treated with DAO supplement, the mean headache durations of 6.14 (±3.06) and 4.76 (±2.68) h were registered before and after treatment, respectively [195].

Administration of BA (putrescine, cadaverine, tryptamine, β-phenylethylamine, spermine, and histamine) to 15 weaned Saanen (goat) kids (2 months old; 11.6 ± 1.4 kg body weight) for 14 days (1.4 g per day of amines by oral administration or 122 mg/kg per day) had a negative effect on the histological characteristics of rumen, abomasum, liver, pancreas, duodenum, jejunum, and distal ileum [196]. If intestinal concentrations of these amines exceed the capacity of gut wall amine oxidizing enzymes, they will be absorbed causing stress to intermediary metabolism with detrimental effects on health and growth performance. Mitotic figures in intestinal glands appeared more common than normal following oral administration of BA. The entire alimentary tract of the treated animals (goats) showed evident signs of inflammation and epithelial shedding. Pathological epithelial cell shedding occurs in many intestinal diseases. The cell loss from the villus exceeds the regenerative capacity of the crypts, due to epithelial injury to one or both components [197]. Shedding of intestinal epithelial cells (IEC) from the epithelial monolayer may cause transient gaps or microerosions in the epithelial barrier, resulting in increased intestinal permeability. The pathological IEC shedding has been observed in mouse models of inflammation and human intestinal conditions such as IBD. Inflammatory infiltrates were present in numerous areas of the rumen musculature following direct oral administration of BA. In the liver inflammatory foci containing eosinophilic granulocytes were often observed in the parenchyma. In the pancreas, the islets of Langerhans endocrine cells appear swollen. The islets contained sometimes cells of abnormally small size. The exocrine pancreas also appeared disorganized [196]. Cell swelling is an early change in most types of acute cell damage. The increased intestinal permeability or "leaky gut" was revealed in AD [198]. Increased concentrations of fecal calprotectin indicate a disturbed intestinal barrier function in AD patients. Fecal calprotectin may be a marker for subclinical intestinal inflammation [199].

Proliferation of mammary epithelial cells was stimulated at low concentrations, while at high concentrations it was inhibited by BA cadaverine, putrescine, spermine, spermidine, and β-phenylethylamine [200]. In other words, the BA tested had both proliferative and cytotoxic effects similar to our findings for tryptamine [12].

Effect of biogenic amines on activity of aminoacyl-tRNA synthetases (ARS) and protein biosynthesis

Tryptamine inhibits TrpRS [98]. Histamine at the concentration 16 μM significantly inhibited protein biosynthesis in the rat liver microsomal fraction with the presence of aminoacyl-tRNA synthetases [158]. Cadaverine is a decarboxylation product of lysine and a competitive inhibitor of lysine binding to its lysyl-tRNA synthetase (LysRS) [174, 175]. Phenylethylamine (PEA) is an inhibitor of bacterial phenylalanyl-tRNA synthetase [172].

Biogenic polyamine spermine stimulates activities of microbial argynyl-tRNA synthetase [201] as well as bacterial [202] and rat-liver isoleucyl-tRNA synthetase [203] but inhibits tyrosyl-tRNA synthetase from Drosophila [204]. In the study of Holler (Lawrence Berkeley Laboratory, University of California, Berkeley), isoleucyl-tRNA synthetase of Escherichia coli is inhibited by spermine [205]. Spermine produced from putrescine, which is produced in the cytoplasm of cells by decarboxylation of a non-protein amino acid ornithine.

Disease-related bioactive metabolites

The level and prevalence of deuteroporphyrin IX described as a fecal porphyrin in patients with endemic chronic arsenic poisoning (http://www.hmdb.ca/metabolites/HMDB0000579) are higher in C&A compared to NNI (C&A/NNI level = 1.96-fold, F test p value 0.0009).

Glycolithocholate sulfate, an urinary marker of liver diseases [206], showed a lower prevalence in C&A (no significant difference in NNI/C&A level) and CRC compared to controls. The urinary glycolithocholate 3-sulfate was markedly increased in hepatitis B-induced cirrhotic patients compared with healthy controls [207].

Prevalence of 1-methylimidazoleacetate is higher in C&A than in NNI with no statistically significant difference in levels. Increase of this histidine metabolite in blood is associated with mortality related to cerebral malaria encephalopathy due to infection with Plasmodium falciparum [208].

Prevalence of 3-ureidopropionate (pyrimidine, uracil-containing metabolite) is increased in both C&A (no significant difference in the levels of C&A versus NNI) and CRC. This metabolite predicted mortality in cirrhosis [209].

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The 103 subjects with cirrhosis had measured glomerular filtration rate, which was strongly associated with erythronate. The erythronate level (100% prevalence in C&A and NNI) is significantly higher in C&A than in NNI (1.46-fold, *t* test *p* value 0.006**). High fecal erythronate is associated with Enterobacteriaceae predominated in CRC [210].

The prevalence of bile acid metabolite glycocholate is lower in CRC and C&A and significantly higher in fecal samples of individuals with Crohn's disease (CD) in the twin study [211]. ADAS presents in this CD group. The glycocholate level is 1.9-fold higher (F test *p* value <0.0001) in C&A compared to NNI. Glycocholate was suggested as a biomarker of cholangiocarcinoma, a bile duct cancer [212] and of obesity [213]. Bile acids are important endocrine signaling molecules, modulating glucose homeostasis through activation of cell surface and nuclear receptors. Bile acid metabolism including glycocholate is altered in T2DM [214]. Median fecal glycocholate excretion is 111,000 for NNI and 213,000 for C&A with the highest values in individuals with T2DM/obesity. A number of bile metabolites are altered in C&A and CRC in comparison with NNI and controls (Supplementary Table 1).

For the 2-hydroxybutyrate (AHB) prevalence is NNI/73.9% versus C&A/62.85%; the levels C&A/NN = 2.78-fold (F test *p* value <0.0001). The 2-hydroxybutyrate (alpha-hydroxybutyrate) and 3-hydroxybutyrate (beta-hydroxybutirate) suggested as prognostic biomarkers to predict the onset of diabetic complications in women with gestational diabetes after delivery [215]. Alpha-hydroxybutyrate is an early plasma biomarker of insulin resistance and glucose intolerance in a nondiabetic population [216]. The four highest fecal AHB values of C&A belongs to individuals with obesity/T2DM. AHB is produced by the intestinal bacterium *Escherichia coli* K12 strain [217].

3-hydroxybutyrate (BHB) shows 100% prevalence in NNI and C&A, while the level ratio C&A/NNI = 1.51-fold (F test *p* value <0.0001). This ketone body is produced in alcoholic ketoacidosis and fasting [218].

Prevalence of N-methylhydantoin is higher in C&A (54.28%) versus NNI (26%) and in CRC (77%) versus controls (73%). The mean level of detectable N-methylhydantoin is lower in C&A versus NNI (2.52-fold, F test *p* value 0.0028**). 1-Methylhydantoin is produced by bacterial creatinine deaminase in the intestinal tract of uremic patients and retaken up into the body. The 1-methylhydantoin (N-methylhydantoin) induced apoptosis as well as cell changes consistent with necrosis [219].

Prevalence of sarcosine (N-Methylglycine) is higher in both C&A and CRC versus controls. The sarcosine level is higher in C&A versus NNI (1.43-fold, Welch's *t* test *p* value 0.0434*, F test 0.0030**, the four definitive outliners removed). Sarcosine, an N-methyl derivative of the amino acid glycine and a metabolic product of choline, plays an important role for prostate cancer aggressiveness and progression [220].

The amino sugar glucosamine is 2.18-fold higher in C&A versus NNI (*p* values *t* test 0.0004***, F test 0.0047**). The anti-inflammatory actions of glucosamine on arthritic disorders are suggested. Glucosamine infusion in rats mimics the beta-cells dysfunction of T2DM [221]. Cholesterol level is 1.22-fold higher in C&A versus NNI (F test *p* value 0.0298*).

In exhaled breath condensate samples of human patients, the two metabolic discriminant features, 4-hydroxycyclohexylcarboxylic acid (higher prevalence in C&A than NNI) and pyroglutamic acid, differentiated the acute pulmonary exacerbations from the stable cystic fibrosis with 84.6% accuracy [222]. 4-hydroxycyclohexanecarboxylic acid is derived from Spanish-style green table olives [223].

Inositol pathway: Inositol/inositol phosphates

Prevalence of inositol-1,4,5-triphosphate (IP3), which is implicated in Ca2+ signaling, is higher 2-fold in C&A than in NNI (Supplementary Table 1). The difference in the level of IP3 between C&A and NNI is statistically significant (C&A/NNI = 5.48-fold; *p* value 0.0037). A variety of alterations in [Ca2+]-regulation may contribute to the neuronal damage in stroke and AD [224]. In AD, Ca(2+) dyshomeostasis represents a global phenomenon affecting virtually all cells in the brain [225]. The nanomolar concentrations of amyloid β-peptide (component of AD plaques) increase the IP3 receptor and mitochondrial calcium concentrations [226]. Prevalence of inositol 1-phosphate (IP1) is higher in C&A and CRC than in controls. Heatmap depicting metabolites of statistically significant differences between C&A and NNI populations [14] includes IP1 with a higher C&A level. Here, in the Welch's test, the difference in the IP1 levels between C&A and NNI is statistically significant (C&A/NNI = 2,218-fold; *p* value <0.0001). Figure 2 illustrates the inositol phosphate interactions in the general scheme linking

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ADAS to AD, T2DM, and CRC. The IP3 produces by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), a phospholipid (phosphoinositide) that is located in the plasma membrane, by phospholipase C (PLC). Oligomeric amyloid-β decreases the level of PIP2 that regulates key aspects of neuronal function [227]. PIP2 was selectively enriched in granulovacuolar degeneration bodies and NFTs [228]. It is widely accepted that IP1 is a major product of the dephosphorylation of inositol polyphosphates. Inositol polyphosphate 5-phosphatase subfamilies include SHIP2, OCRL, and INPP5B involved in insulin regulation and Lowes syndrome [229]. SHIP2 expression is increased in CRC tissue in comparison to adjacent normal tissue, and this is correlated with decreased patient survival. Moreover, SHIP2 is more active in CRC tissue, suggesting that SHIP2 can induce oncogenesis in colonic epithelial cells [230]. SHIP2 inhibition decreases tau protein (component of AD NFT) hyperphosphorylation (P-tau) induced by amyloid-β thus supporting usage of SHIP2 inhibitors for AD treatment [231]. Tryptamine stimulates inositol phosphate accumulation in a dose-dependent way in rat cortical slices with dominated form inositol monophosphate [232]. Tryptamine functions as a full agonist at the PLC signaling pathway [69, 233]. The results suggest that the PLC isozymes are differentially involved in AD [234]. Tryptamine binds to phospholipid liposome [235] and to lipid fraction containing phosphatidylserine and phosphatidylinositol [236]. [3H]tryptamine binds also to whole rat brain synaptic plasma membranes. These binding was decreased by treatment with Azure A (Giemsa solution) and phospholipase A2, while phospholipase D had no effect [236]. Moreover tryptamine upregulates (4.1-fold) the gene encoding amyloid-β precursor-like protein 2 [237]. In urinary metabolomics of newborns infected by human cytomegalovirus, the most important metabolites characterizing the clustering of the samples were: myo-inositol, glycine, 3-hydroxybutyrate, 3-aminoisobutyrate (BAIBA), creatine, taurine and betaine [238]. A stereoisomer of inositol, myo-inositol, presents in a variety of cell types and is obtained from the following three major sources: de novo synthesis from glucose-6-phosphate (G-6-P), sequential dephosphorylation of phosphoinositides, and membrane transport from extracellular fluid. The biosynthesis of inositol follows a common pathway involving first the conversion of G-6-P to myo-inositol-1-phosphate (MIP) catalyzed by MIP synthase, followed by dephosphorylation catalyzed by MIP phosphatase (MIPP). In AD brains, the MIPP activity and its protein level were significantly higher than in control brains [239]. Disruption of inositol homeostasis has been associated with a number of illnesses, including bipolar disorder, AD, bulimia, metabolic syndrome, T2DM, and epilepsy [240]. Fecal chiro-inositol is lower in C&A than in NNI: prevalence (1.76-fold) and level (5.1-fold, F test p value <0.0001****). The urinary excretion of chiro-inositol was much lower in T2DM patients than in control subjects [241]. The enhanced inositol phosphate accumulation was demonstrated in platelets from the diabetic patients [242]. In plant foodstuffs, inositol is preferentially present as phytic acid (IP6). The main sources of phytate are cereals and legumes, including oil, seeds and nuts [243]. Phytases are phosphohydrolytic enzymes that initiate stepwise removal of phosphate from phytate [244]. Phytase, a phosphatase that catalyzes the hydrolysis of indigestible phytic acid (myo-inositol hexakisphosphate), present in Lactobacillus strains isolated from farmhouse goat's milk cheeses—in the 10 strains of L. plantarum and in 2 strains of L. paracasei of 80 strains [245] (15% of isolated strains). Lactobacillus can be found in plants or material of plant origin, silage, fermented food (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, gastrointestinal tracts, and vaginas of humans and animals [246]. Prevalence of inositol phosphates (IP) IP3, IP4, IP5, and IP6 is higher in C&A than in NNI and unidentified in CRC and the controls while the tryptamine-inducible IP1 is higher in both C&A and CRC compared to controls. The difference in the level of IP6 (myo-inositol hexakisphosphate) between C&A and NNI is statistically significant (C&A/NNI = 7.69-fold; F test p value <0.0001). Myo-inositol pentakisphosphate (IP5) level is 2.37-fold and myo-inositol tetrakisphosphate (IP4) is 3.3-fold higher in C&A than NNI. When applied extracellularly, myo-inositol hexakisphosphate (IP6) and myo-inositol pentakisphosphate (IP5) can inhibit the growth and proliferation of tumor cells [247–249]. C&A participants originate from five different towns in western Oklahoma: Clinton, Concho, Geary, Hammon, and Kingfisher, and NNI from Norman, Oklahoma. Subsistence strategy for both populations C&A and NNI is Industrial Agriculture. Phytate-degrading recombinant Lactobacillus cultures expressing Bacillus subtilis phytase used as probiotics in broiler chickens [250]. I hypothesize that a part of Western Oklahoma participants may possess enzyme phytase in gut microbiota with a higher proportion of microbial

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phytase carriers in C&A than in NNI population or/and C&A consume more processed food containing IP. Increase in specific kinase activity can also cause increase in IP, particularly, inositol trisphosphate 3-kinase B (ITPKB) phosphorylates inositol 1,4,5-trisphosphate into inositol 1,3,4,5-tetrakisphosphate. The ITPKB protein level was increased 3-fold in the cerebral cortex of most patients with AD compared with control subjects, and accumulated in dystrophic neurites associated to amyloid plaques [251].

The environmental bacterium Legionella pneumophila, a natural parasite of environmental amoebae and the causative agent of a severe pneumonia termed Legionnaires' disease, metabolizes myo-inositol through the iol gene products, thus promoting the growth and virulence of the pathogen [252]. Myo-inositol detected in all the samples of both NNI and C&A is 1.75-fold higher in C&A than in NNI (statistically significant difference: *t* test *p* value 0.03*, F test *p* value 0.0006***). Increased cerebral myo-inositol revealed with proton MR spectroscopy was demonstrated in AD [253], in the dementia of Down syndrome [254,] and in patients with glioblastoma multiforme [255]. Myo-inositol is clustered in human cytomegalovirus infection with 3-aminoisobutyrate, which prevalence is higher in CRC while 3-aminoisobutyric acid (or β-aminoisobutyric acid, BAIBA) prevalence is lower in C&A compared to controls. The 3-aminoisobutyrate is a conjugate base formed by deprotonation of BAIBA carboxylic acid group. BAIBA level is 1.9-fold higher (*p* value <0.0001 in F test) in C&A compared to NNI. The 3-aminoisobutyrate was good discriminator for CRC patients [256]. In humans, plasma BAIBA concentrations increase with exercise [257]. Prevalence of chiro-inositol and three other serum metabolites theobromine, 7-methylurate and 3-methylxanthine of the 10 most influential metabolites for discriminating between the Dietary Approaches to Stop Hypertension (DASH) and control dietary patterns altered in C&A and CRC compared to controls [258]. The DASH dietary pattern is recommended for cardiovascular disease risk reduction.

Nucleic acids DNA and RNA components

The fecal deoxyribose level was 1.6-fold lower in C&A than in NNI (*t* test *p* value 0.0074, F test *p* value 0.0009). This can be due to the presence of gut bacteria that utilize deoxyribose. The bacterium that can grow with deoxyribose as its sole source of carbon was characterized [259]. Deoxyribose is one of the building blocks of DNA and is released when cells die and their DNA degrades. Another DNA component thymine is 1.46-fold lower (*t* test *p* value 0.0046) in C&A compared to NNI. Lethality under thymine starvation or thymineless death in bacteria is known for decades. Given that thymineless death is similar in mammalian cells [260], this phenomenon can be related to the C&A health status. The plasma metabolite thymine was altered in AD (2.05-fold higher level) compared to normal controls [261]. Prevalence of both fecal metabolites deoxyribose and thymine is lower in CRC than in control. Guanine, one of the four main nucleobases found in the nucleic acids DNA and RNA, is 1.62-fold higher in C&A compared to NNI (*t* test *p* value 0.0005***), increased in AD [176].

Short peptides in human fecal samples/gut microbiota

The prevalence and levels of short peptides are demonstrated in Supplementary Table 1 and Fig. 1. The level of histidylvaline is 1.92-fold higher in NNI versus C&A (F test *p* value <0.0001****). The level of aspartylphenylalanine is 1.18-fold higher in NNI than in C&A (F test *p* value 0.0382*); serylleucine - C&A/NNI = 1.4-fold (*t* test *p* value 0.042*, F test *p* value 0.0001****); glycylalanine – NNI/C&A = 1.18-fold (F test *p* value 0.0351*); phenylalanylvaline - NNI/C&A = 1.22-fold (F test *p* value 0.0084**); arginylleucine C&A/NNI 1.35-fold (F test *p* value <0.0001****); tyrosylvaline level NNI/C&A = 1.29-fold (F test *p* value 0.0146*); lysylproline NNI/C&A = 1.23-fold (*t* test *p* value 0.2, F test, *p* value 0.0631); pyroglutamylvaline NNI/C&A 1.27-fold (F test *p* value 0.0019**); histidylisoleucine NNI/C&A = 1.357 (*t* test *p* value 0.2, F test *p* value 0.0004***); gamma-glutamyltyrosine level NNI/C&A = 1.3 (*t* Test *p* value 0.0233*, 3 outliners removed by GraphPad Prism), the prevalence ratio NNI/C&A = 1.52. The level of asparagylleucine is higher (1.55-fold) in C&A versus NNI (*p* values *t* test 0.0256*, F test 0.0002***). N-acetyl-aspartyl-glutamate (NAAG), a peptide neurotransmitter, is lower in both C&A and CRC versus controls. The NAAG level is lower in C&A versus NNI (1.28-fold, *t* test *p* value <0.0001****). The Welch's *t* test indicates that along with an increase in some short peptides in C&A that can reflect higher proteolysis, the decrease is also revealed in C&A. The decrease in short peptides detected in C&A group can be

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caused by deficiency in tRNA aminoacylation/protein biosynthesis in microbes found predominantly in human stool. The resulting average values for some peptides are with no significant differences between NNI and C&A because the data reflect both decrease and increase. Based on the peptide prevalence (Supplementary Table 1), the peptide decrease is expected to be more profound than could be estimated by Welch's t test in the C&A gut microbiota. Because at least two processes are coincided, the increased proteolysis and tRNA aminoacylation/protein biosynthesis impairment, the data show the combined processes. In such a situation, the analysis of prevalence is informative. Both the deficiency in amino acids and abundance of biogenic amines (Table 3, Fig. 1) lead to the protein biosynthesis decline. The decline in NAAG exemplifies the reduction in bioactive peptides. The levels of the total 81 peptides is compared with the tryptamine levels in NNI and C&A (Fig. 1H). The one of the three highest tryptamine samples correspond to the lowest total peptide's level (sample NO13). Figure 1H shows the tryptamine (T)-high samples (NO1 and NO13) versus T-low (NO2) and non-detectable (ND) tryptamine (NO7 and NO15) samples of NNI group. The distribution of individual values demonstrates both high and low peptide levels in the samples with detectable tryptamine. In the microbial cheese ripening, the greatest range of proteolysis correlates with the BA content [262, 263]. Amino acid starvation-induced macroautophagy leads to selective degradation of proteins important for protein translation [264]. In C&A, the amino acid decrease (starvation) correlates with BA abundance and rise of some peptides that characterize the protein degradation. It was shown that the tryptamine effects are dose-, time-, and diet-dependent and include tryptamine-induced seizures and death in animals [2]. It is noteworthy that the traditional diet of American Indian including C&A people contains maize, or corn, an important crop to tribes, and beans. In rumen fluid of the ruminant animal goat, metabolomics revealed that a high grain feeding (50% maize) increased tryptamine levels (284.08 μM) compared to control with no maize feeding (145 μM) [265]. Tryptamine was the main BA detected in beans, and its concentration considerably increased during the germination [266]. In Saskatchewan, Canada between 2005 and 2010, the overall age-standardized incidence of epilepsy was 62 per 100,000 person-years, while for self-declared registered Indian aboriginal population of the same province, the overall age-standardized incidence of epilepsy was 122 per 100,000 person-years [267]. Epilepsy was a well-recognized disease in pre-Columbian cultures of Native American tribes [268]. The disproportionately high rates of T2DM-related mortality and complications among Indigenous populations of Australia, Canada, New Zealand, and the United States were reported [269, 270]. The causal link between seizure and constipation is a common belief among patients and physicians while constipated mice are more prone to seizure [271]. This correlation supports a role of gastrointestinal conditions in the pathogenesis of epilepsy affecting about 60 million people worldwide. Constipation is also associated with cardiovascular events [272].

Public health

The control of tryptamine content is utmost important for human life because tryptamine readily crosses the blood-brain barrier [53] and induces seizures [8, 11, 76, 273] at concentrations than can be detected in food. In the US, seizure-related deaths take more than 5,000 lives each year [274]. High seizure frequency is strongly associated with increased mortality. Seizures and epileptic activity is frequently associated with AD [275], particularly in the early stages of AD [276]. Epilepsy is associated with a high rate of premature mortality from direct and indirect effects of seizures. Sudden unexpected death in epilepsy (SUDEP) is the second leading neurologic cause of total lost potential life-years after stroke. The present and earlier reported [2] analysis of tryptamine produced by human gut microbiome is a response to a call for action against seizure-related deaths [274]. Controlling seizures will save lives. Death records show that American Indian and Alaska Native death rates for both men and women combined were nearly 50% greater than rates among non-Hispanic whites during 1999–2009 (https://www.cdc.gov/media/releases/2014/p0422-natamerican-deathrate.html).

Conclusions

The present study reveals: 1) the common signaling pathways in AD and other diseases marked by ADAS; 2) the alterations in metabolic pathways uncovered in this study correlate with dysbiosis in C&A [14]; 3) the ADAS-linked prevalence of fecal metabolites in C&A and CRC shed light on interfacing mechanism of tryptamine-induced inositol phosphate signaling; 4) L-amino acids decarboxylase activity (AAD) of

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human gut is critical for production of cytotoxic BA in ADAS-linked pathology. The AAD modifying factors discussed here are vitamin B6, salt stress, and low pH; 5) impairment of protein biosynthesis machinery in human gut microbiome and human cells can play a causative role in ADAS linked pathologies. The amino acids-to-biogenic amines conversion regulates the protein biosynthesis at the levels of: 1) aminoacyl-tRNA synthetase (ARS) enzymatic activities, and 2) transcription of genes encoding ARS in the decarboxylase gene clusters. Altogether the data support conclusion that neurodegeneration is a part of the generalized protein biosynthesis machinery dysfunction. The targeted metagenomics revealed the gut microbial metabolites that expand the knowledge of human pathophysiology. The data on AD related comorbidity and concomitant diseases along with our data on targeted gut metagenomics sequence analysis support that AD is a systemic widespread disease that start from the human gut dysbiosis. The BA produced from amino acids by gut microbiome spread to the system through the damaged vascular and intestinal cells. This damage results from the BA non-specific cytotoxicity that can target and kill any cells in any organs of the human body including brain, vascular, and intestinal cells while the BA affinity to its degrading MAO enzymes is lower than BA affinity to ARS.

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SUPPLEMENTARY MATERIAL

Discovery of Gut Bacteria Specific to Alzheimer's Associated Diseases Is a Clue to Understanding Disease Etiology: Meta-Analysis of Population-Based Data on Human Gut Metagenomics and Metabolomics

Supplementary Table 1

Population-based detectability of fecal metabolites in C&A, NNI [1, 2], CRC and controls [3]. In this study, the prevalence (%) was estimated as a fraction above the detection limit for the crude data derived from the Oklahoma study [1] and compared with the prevalence data (%) from the CRC fecal metabolomics study of three Washington DC area hospitals [3]

Metabolites	C&A study, %*		CRC** study, %	
	NNI	C&A	Control	Case
tryptamine	61	66	60	65
benzoate	100	100	99	100
2,4,6-trihydroxybenzoate *currency* (consumption of cranberry juice), cytotoxic [4]	56	26	n/d	n/d
benzene-1,2,3-triol (toxic)	91	89	n/d	n/d
1H-quinolin-2-one	26	13	77	79
2,8-quinolinediol	43	37	89	73
ICA	17	34	75	92
Kynurenate	83	71	98	100
IAA	100	97	98	98
2-oxindole-3-acetate (uremic toxin)	100	97	100	98
Genistein (MAOI, soy-food)	26	37	62	52
Skatole	52	66	48	48
IPA	96	97	98	96
quinate	100	100	99	100
tyramine	96	97	98	100
4-methylcatechol sulfate (benzoate metabolism)	87	80	90	73
catechol sulfate (benzoate metabolism)	63	37	73	54
carnosol (food rosemary, antimicrobial)	86	71	n/d	n/d
ursolic acid (food, antimicrobial)	74	34	78	60
oleanolate (food, antimicrobial)	74	40	87	83
vanillate (food, antimicrobial)	70	83	77	79
ferulate (food, antibacterial)	100	97	100	96
gallate (food, antibacterial)	56	51	47	31
phytanate (food)	74	77	89	90
solanidine (food, potato, poisonous)	83	94	91	92
dihydroferulic acid (food, coffee)	96	89	100	98
Piperine (food, pepper)	78	91	95	90
daidzein (soy-food)	70	80	97	94
erythritol (food) sweetener	97	94	93	94
erythronate (CRC)	100	100	99	100
glycyrrhetinate (plant, antibacterial)	87	57	n/d	n/d
5-ketogluconate	70	71	47	33
4-hydroxycyclohexylcarboxylic acid	39	51	n/d	n/d
ponciretin (food, antibacterial)	17	14	39	35
diaminopimelate (bacteria, plants, lysine synthesis)	70	86	54	56
brilliant blue FCF (blue 1) food	39	54	n/d	n/d
4-acetamidophenol (paracetamol) antibacterial	22	51	98	92
Ibuprofen (antibacterial)	17	23	4	2
carboxyibuprofen (ibuprofen metabolite)	26	37	n/d	n/d
2-hydroxyibuprofen (ibuprofen metaboloite)	17	31	n/d	n/d
Allantoin (purine, cosmetics)	52	46	55	58
Inosine (purine; Parkinson's trial)	39	57	92	88
2'-deoxyadenosine 3'-monophosphate (purine metabolism)	35	74	85	90
2'-deoxycytidine (pyrimidine)	100	91	90	73
3-ureidopropionate (pyrimidine, uracil) cirrhosis mortality [5]	43	57	81	85
3-(4-hydroxyphenyl)lactate [5]	100	100	86	83
3-hydroxy-3-methylglutarate ¶	57	46	48	44

(*Continued*)

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Supplementary Table 1
(*Continued*)

Metabolites	C&A study, %*		CRC** study, %	
	NNI	C&A	Control	Case
glycocholate (bile) cholangiocarcinoma marker [6]	87	69	94	83
glycolithocholate sulfate (bile)	61	34	70	52
12-dehydrocholate (bile)	79	83	79	67
chenodeoxycholate (bile, cholesterol	35	49	n/d	n/d
Squalene (lipid, sterol) adjuvant in vaccines	91	83	98	94
glycochenodeoxycholate (sclerosing cholangitis [7])	22	6	46	40
7-ketolithocholate (bile acid)	35	51	29	31
tauroursodeoxycholate (bile)	43	49	44	40
taurolithocholate 3-sulfate (bile)	35	20	56	52
7,12-diketolithocholate secondary bile); antibiotic-induced shift [8]	39	54	73	58
palmitoyl sphingomyelin (lipid)	61	71	95	98
N-acetylsphingosine (C2-ceramide) calcium Influx, cytotoxic	57	71	74	81
pregnen-diol disulfate (steroid)	61	69	75	83
(R)-salsolinol (food, neurotoxin, PD)	17.4	31.4	78	56
gentisate (3- and 4-HBA catabolism)	83	80	88	71
pyridoxine (food, vitamin B6)	57	66	86	100
gamma-CEHC (food desserts, vitamin E metabolism, smocking, breast cancer [9]	30	49	76	52
flavin adenine dinucleotide (FAD)	91	86	54	58
palmitoylcarnitine fatty acid oxidation	43	57	48	60
sarcosine (N-methylglycine); cancer	78	91	89	92
N-acetylglycine (food, glycine)	100	94	99	98
alpha-hydroxybutyrate (T2D marker)	74	63	99	100
3-hydroxybutyrate (BHBA) T2D	100	100	100	98
beta-hydroxypyruvate, food, T2D	26	29	51	54
formiminoglutamate (histidine)	87	60	n/d	n/d
1-methylimidazoleacetate (histidine)	57	77	n/d	n/d
trans-4-hydroxyproline (food processed meat, prostate cancer [10])	61	86	29	35
N-acetylproline	96	83	100	98
Taurine (energy drinks, anti-depressant)	48	57	93	90
nervonate (24:1n9) long fatty acid	74	97	90	94
10-nonadecenoate (19:1n9) long fatty	74	60	n/d	n/d
1-stearoylglycerophosphoethanolami	82	83	95	98
1-stearoylglycerophosphoinositol	74	77	67	79
1-oleoylglycerol (1-monoolein)	87	89	82	71
diglycerol (probably food)	74	94	n/d	n/d
Phosphoethanolamine (cytotoxic)	61	83	84	90
bilirubin (Z,Z)	78	86	86	73
I-urobilinogen	83	86	60	54
myo-inositol triphosphate (1,4,5 or 1,3,4) ¥interface in Alzheimer's	17	34	n/d	n/d
myo-inositol pentakisphosphate (1,2,4,5,6 or 1,3,4,5,6)	22	54	n/d	n/d
inositol 1-phosphate (I1P)	91	100	97	100
myo-inositol hexakisphosphate (food)	48	54	n/d	n/d
myo-inositol	100	100	100	98
myo-inositol tetrakisphosphate (1,3,4,6 or 3,4,5,6 or 1,3,4,5)	13	26	n/d	n/d
chiro-inositol (T2D; **DASH [11]**)	30	17	49	50
3-aminoisobutyrate (cytomegalovirus	85	54	41	54
Xylitol (pentose) diabetic food, antibiotic [8]	74	71	61	56
Mannitol (diabetic food) sweetener [8]	78	46	51	50
Arabitol (carbohydrate) antibiotic [8]	74	80	75	81
Xylulose (carbohydrate) antibiotic [8]	78	91	70	65
fuculose	78	69	n/d	n/d
Glucose-6-phosphate (G-6-P)	n/d	n/d	58	52
caffeine	91	83	99	96
Paraxanthine (caffeine metabolite)	57	86	91	88
1-methylxanthine (from caffeine)	70	74	91	96
3-methylxanthine (**DASH** [11])	35	26	83	85

(*Continued*)

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Supplementary Table 1
(*Continued*)

Metabolites	C&A study, %*		CRC** study, %	
	NNI	C&A	Control	Case
Theophylline (drug, asthma)	48	54	87	81
Theobromine (caffeine metabolite) **DASH** [11]	57	63	90	90
1-methylurate (theophylline metabolite)	35	51	73	77
7-methylurate (**DASH** [11])	65	69	68	73
xanthosine (endometriosis [12])	52	60	84	92
2-hydroxyadipate	56.5	71.4	80	83
2_DEOXYRIBOSE	100	100	79	73
THYMINE	100	100	100	98
N-methylhydantoin	26	54.3	73	77
N-acetyl-1-methylhistidine	39	63	n/d	n/d
SITOSTANOL	95.6	100	99	90
deuteroporphyrin IX	34.7	48.6	n/d	n/d
N-formylmethionine	100	80	98	98
glucosamine	100	100	89	90
N-acetylaspartate (NAA)	91	100	100	98
PUTRESCINE	95.6	97.1	99	96
N-acetylputrescine	82.6	85.7	100	98
Dipeptides	**C&A reduced prevalence %**		**CRC**	
tryptophylvaline	96	91	97	100
aspartylphenylalanine	87	77	99	100
alpha-glutamylthreonine	96	83	n/d	n/d
phenylalanylleucine	100	91	99	100
pyroglutamylvaline	96	83	98	98
serylleucine	96	89	99	100
alanylphenylalanine	96	83	99	100
glycylalanine	78	66	80	81
cyclo(leu-phe)	30	11	52	50
gamma-glutamylleucine	100	91	99	100
gamma-glutamylisoleucine	100	91	99	100
gamma-glutamylphenylalanine	100	89	99	100
gamma-glutamyltyrosine	91	60	n/d	n/d
phenylalanylisoleucine	96	91	99	100
serylphenyalanine	100	89	99	100
isoleucylleucine	100	94	99	100
asparagylisoleucine	100	94	100	98
N-acetyl-aspartyl-glutamate (NAAG)	95.6	91.4	21	19
Di- tripeptides	**C&A elevated prevalence %**		**CRC**	
leu-leu-leu	56	83	94	92
val-val-val	87	97	100	98
histidylleucine	78	97	99	96
alanylalanine	39	74	99	100
arginylphenylalanine	74	89	97	94
valylserine	87	91	99	100
leucylphenylalanine	91	94	99	100
isoleucylvaline	91	94	99	100
histidylisoleucine	87	100	99	100
glutamine-leucine	74	86	n/d	n/d
tryptophylproline	74	86	91	94
histidylvaline	95.6	97.1	98	98

*Percentage calculations based on 23 (NNI) and 35 (C&A) as 100% in controls and C&A, respectively; background: pink – direct association, green - inversed association between C&A and CRC. **CRC is the third most common cancer diagnosed in both men and women in the United States (https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html) and the second most common cancer among American Indians (following lung cancer) https://www.americanindiancancer.org/colon. *currency* Sporobacterium can utilize and produce 2,4,6-trihydroxybenzoate [13]. n/d, data are not available. ¶3-hydroxy-3-methylglutarate (meglutol) is an antilipemic agent which lowers cholesterol [14]. [yen] inositol triphosphate (IP3) receptor interface in AD [15]. DASH - Dietary Approaches to Stop Hypertension.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Investigation of Potential Brain Microbiome in Alzheimer's Disease: Implications of Study Bias

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Abstract.
Background: Dysbiotic microbiota in the gastrointestinal tract promotes and aggravates neurodegenerative disorders. Alzheimer's disease (AD) has been shown to correlate to dysbiotic bacteria and the immune, metabolic, and endocrine abnormalities associated with abnormal gut-brain-axis signaling. Recent reports also indicate that brain dysbacteriosis may play a role in AD pathogenesis.
Objective: To evaluate the presence and differences of brain-region dependent microbiomes in control and AD subjects and the contribution of study bias.
Methods: Two independent cohorts of postmortem AD brain samples were collected from separate locations, processed with different extraction protocols and investigated for the presence of bacterial DNA indicative of a brain microbiome with V4 16S next generation sequencing.
Results: In both cohorts, few differences between the control and AD groups were observed in terms of alpha and beta diversities, phyla and genera proportions. Independent of study in both AD and control subjects the most abundant phyla were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Variations in beta diversity between hippocampal and cerebellum samples were observed indicating an impact of brain region on the presence of microbial DNA. Importantly, differences in alpha and beta diversities between the two independent cohorts were found indicating a significant cohort- and processing-dependent effect on the microbiome. Finally, there were cohort-specific correlations between the gut microbiome and subject demographics indicate that postmortem interval may have a significant impact on brain microbiome determination.
Conclusion: Regardless of the study bias, this study concludes that bacterial DNA can be isolated from the human brain suggesting that a brain microbiome may exist; however, more studies are required to understand the variation in AD.

Keywords: Alzheimer's disease, gut-brain-axis, microbiome, neurodegeneration, 16S sequencing

INTRODUCTION

Gastrointestinal (GI) dysbiosis in the microbiome has broad-acting effects on the health of both the gut and the brain with important implications in neurodegenerative diseases such as Alzheimer's disease (AD) [1, 2]. One of the mechanisms that drives dysbiosis-induced neurodegeneration is a compromised GI and blood-brain barriers (BBB) facilitating the infiltration of immune cells, neuroactive products, viruses and even bacterial cells from the GI lumen into the brain. This also supports the inflammatory hypothesis of AD where a dysbiotic microbiome creates an aberrant

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innate-immune crosstalk between the GI tract and the brain, promoting the neuroinflammatory milieu: a recognized risk factor of AD and other neurological disorders. One group has even postulated a "cooperative pathogenesis" where less virulent organisms, such as the dysbiotic microbiota, can enable infection of typically non-virulent organisms in the brain by facilitating their incursion into the brain [3]. These mechanisms challenge the previously assumed sterility of the brain. Several studies have demonstrated that there indeed exists bacterial and fungal species in the brain, and that these species could drive neurodegenerative pathogenesis. Indeed, some studies have detected, albeit in low quantities, bacteria in the brain of healthy individuals [4–6] insinuating the possibility of a brain microbiome.

It is highly possible that the opening of the BBB and consequent infiltration of pathogenic bacteria into the brain is a driving force for neurodegenerative pathologies [7]. 16S rRNA sequencing of frozen multiple sclerosis (MS) patients' brain sections revealed that Proteobacteria, associated with increased inflammatory gene expression, may be the dominant phylum with restricted diversity in cerebral white matter compared to non-MS patients. Both clinical groups displayed 1,200–1,400 bacterial genomes/cm^3 [4]. Several groups have also demonstrated the possibility of polymicrobial infections in AD patients. In both formalin fixed and frozen sections, postmortem AD tissue samples were identified to have greater incursion of bacterial species compared to controls including Firmicutes, *Staphylococcus*, and the family *Propionibacteriaceae* [5]. Another group identified immunolabelled prokaryotic structures in the postmortem brain specimens from AD patients [8], which were later confirmed using anti-peptidoglycan, anti-*Clamidophyla*, and anti-*Borrelia* antibodies [6]. This group went further with NGS to identify that the phylum Proteobacteria was the most prominent in both AD patients and controls, followed by Firmicutes, Actinobacteria, and Bacteroidetes whereas at the family level, *Burkholeriaceae* and *Staphylococcaceae* were found in greater abundance in the AD brains compared to control brains. Based on these results, this group also investigated the brain-region specificity of bacterial infiltration in amyotrophic lateral sclerosis (ALS) and AD patients. Using a nested polymerase chain reaction (PCR) approach to specifically amplify bacterial species instead of human, the bacterium *Cutibacterium acnes* (formerly known as *Propionibacterium acnes*) was found in abundance in all regions investigated in the ALS patients. Other species detected included *Corynebacterium sp.*, *Fusobacterium nucleatum*, *Lawsonella clevelandesis*, and *Streptococcus thermophilus*. To get a comprehensive view of the bacterial species in a region-specific manner, NGS was performed in the motor cortex, medulla, and spinal cord of 11 ALS patients. The phyla Actinobacteria and Proteobacteria were found in all three regions of the 11 ALS patients, while the order *Sphyngomonodales*, the family *Methilobacteriaceae*, and the genus *Cupriavidus* seemed to be the most prevalent bacterial groups in ALS patients. The phyla Firmicutes and Bacteroidetes were also prevalent, whereas other phyla were less represented. Multivariate principal coordinates analysis (PCoA) showed no significant clustering among the three central nervous system (CNS) regions. Interestingly, region-dependent bacterial enumeration of ALS patients were significantly different compared to the enthorrinal region from AD patients as previously reported [6]. For instance, the bacterial genera *Acrobacter, Thermomonas, Hemophylus, Propionibacterium*, and *Corynebacterium* were more represented in motor cortex, medulla, and spinal cord regions in ALS patients compared to the AD patients. Many of these variations were also confirmed using immunohistochemical techniques confirming that there is the infiltration of GI bacteria into the brain, with possible enrichment in AD patients [9].

There are several caveats to investigating bacterial populations in human brain sections. One is the possibility of contamination of brain sections with the skin microbiome during the autopsy, unless proper sterilization techniques are observed. Storage, processing, and timing (postmortem interval; PMI) are also significant issues and difficult to control when utilizing postmortem human tissue specimens. As such, the thanatomicrobiome has recently been coined to describe the colonization of microbes in internal organs and orifices after death, a study normally used in forensics to identify time of death [10]. One study concluded that there are time-, organ-, and sex-dependent alterations in the thanatomicrobiome, including samples taken from the brain [11]. Finally, extraction, amplification, and sequencing protocols also induce bias into NGS results compromising comparative and meta-analyses within and between studies [12, 13].

To address these caveats and gather more information on the potential microbial load in the brain

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of AD patients, we conducted NGS sequencing of two independent study groups from samples collected from three independent sites in two separate brain regions: hippocampus and cerebellum. Samples from two independent cohorts (Study 1 and Study 2) were extracted with different methods yet all samples were processed with the same NGS protocol. We also conducted correlative analyses between subject demographics collected from the AD patients with the variations in brain microbiome samples to understand if specific bacteria are associated with AD pathophysiology. Despite there being few differences in the gut microbiome between control and AD cohorts, we did identify significant study biases that need to be addressed as studies move forward in investigating the possibility of a brain microbiome.

METHODS

Tissue specimens

Two tissue cohorts are included in this study. In the first study (Study 1, Supplementary Table 1), frozen postmortem hippocampal formation specimens from 10 neurological control (7 females and 3 males) and 10 AD cases (7 females and 3 males) were provided by The Mount Sinai/JJ Peters VA Medical Center NIH Brain and Tissue Repository. The second cohort (Study 2, Supplementary Tables 2 and 3) was received from the Brain Endowment Bank from the University of Miami Health System containing 22 AD specimens (16 females and 7 males) and 19 neurological controls (13 females and 6 males) from the hippocampus. Included in Study 2 were 12 control (8 females and 4 males) and 20 AD cerebellum samples (15 female and 5 males) that were obtained from The Mount Sinai/JJ Peters VA Medical Center NIH Brain and Tissue Repository. Sample details and demographic information can be found in Supplementary Tables 1–3. Information regarding the sterilization and autopsy technique were not provided.

DNA extraction

In study 1, samples were extracted with the Qiagen Stool Kit (catalogue # 19590) following company instructions. In study 2, specimens were placed into a MoBio PowerMag Soil DNA Isolation Bead Plate. DNA was extracted following MoBio's instructions on a KingFisher robot.

PCR, sequencing, and sequence processing

DNA from extracted samples was amplified using Invitrogen's AccuPrime High Fidelity kit (Cat# 12346094). The primers used for amplification contain adapters for MiSeq sequencing and single-end barcodes allowing pooling and direct sequencing of PCR products. As all samples were amplified using the same method, there should not be any significant hindrance in relation to sequencing due to extraction method. Each PCR reaction is prepared by combining 16 μl of the master mix (13.85 μl water, 2 μl 10X reaction buffer, and 0.15 μl Taq DNA polymerase), 2 μL template DNA, 4 μL forward (515F 5′ AATGATACGGCGACCACCGAGATCTA CACTATGGTAATTGTGTGCCAGCMGCCGCGG TAA- 3′ and reverse (806R 5′-CAAGCAGAAGACG GCATACGAGATTCCCTTGTCTCCAGTCAGTCA GCCGGACTACHVGGGTWTCTAAT-3′) primers. The DNA samples are amplified using the following thermocycler program: 95°C for 2 min followed by 30 amplification cycles of 20 s at 95°C, 45 s at 50°C and, 90 s at 72°C followed by final extension at 72°C for 10 min. The PCR product was quantified using Invitrogen's Quant-iT Picogreen dsDNA assay kit (Cat # P7589). Amplicons are pooled and purified using the QIAquick PCR purification kit (Cat# 28104).

16S Sequencing

The 16S rRNA gene sequencing was conducted by Diversigen (Houston, TX) [14]. The 16S rRNA V4 gene region was amplified from the extracted community DNA by PCR and sequenced in the MiSeq platform (Illumina) using the 2 × 250 bp paired-end protocol. This generates paired-end reads that overlap almost completely. The primers used for amplification contain adapters for MiSeq sequencing and single-end barcodes allowing pooling and direct sequencing of PCR products [15]. Sequences that passed chimera slaying post sequencing were merged and blast against the 16S specific curated Silva database (v132). Of the kept sequences, only 35.56% were successfully mapped due to the exclusion of host 16S mitochondrial contamination. Samples were subsequently rarefied to 356 to effectively eliminate noise but retain as many samples as possible. With this cut-off, 18 samples from Study 2 were excluded from the analysis including 21, 23, 24, 25, 26, 29, 33, 34, 37, 39, 42, 44, 46, 49, 64, 70, 81, and 84.

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Statistical analysis

Alpha diversity was calculated for the Shannon diversity index and observed operational taxonomic unit (OTU) abundances. As microbiome data are usually non-parametric in nature, a Mann-Whitney U test was used for comparisons between two groups. Beta diversity was estimated both qualitatively with unweighted UniFrac and quantitatively with weighted UniFrac analysis distance matrices. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) between groups with visualization of the data using PCoA. Between group statistics for control and AD subjects of the phyla and genera were conducted with the Mann-Whitney U test with false discovery rate (FDR) corrections using the Benjamini-Hochberg method. To preform correlation data with patient metadata, we used the Spearman's Rho test and incorporated FDR for multiple testing corrections. All metrics and statistical analyses were conducted in R (Version 3.5.1 with the exception of the Spearman's Rho which was conducted in GraphPad Prism (version 8.0)).

RESULTS

Microbiome abundances in AD versus control subjects in Study 1

In samples derived from The Mount Sinai/JJ Peters VA Medical Center NIH Brain and Tissue Repository (Study 1) consisting only of hippocampal specimens, there was no difference in the alpha diversity shown with observed OTUs or Shannon index estimation between the AD and control subjects (Fig. 1a). In addition, beta diversity estimated with both unweighted (not shown) and weighted (Fig. 1b) UniFrac representation showed no clustering between the AD and control subjects. The most abundant phyla in both the AD and control subjects were the Proteobacteria followed by Firmicutes, Actinobacteria, and Bacteriodetes with lower abundances of Cyanobacteria, Deinococcus-Thermus, Acidobacteria, Verrucomicrobia, and Gemmatimonadetes. Regardless, there were no differences between AD and control subjects for any of the observed phyla (Fig. 1c, upper) and the variable distribution between individuals in Study 1 can be observed in Fig. 1d (left). The most abundant genera included *Acinetobacter, Pseudomonas, Escherichia-Shigella, Staphylococcus, Chryseobacterium, Stenotrophomonas, Massilia, Bacillus, Corynebacteriaceae*, and *Lawsonella*, with again, no differences between the AD and control subjects (Fig. 1c, lower). Again, the significant variation between individuals in Study 1 for the enumeration of genera can be observed in Fig. 1d (right). Although not shown, the potential gender differences within the Study 1 cohort were also examined, but no differences or trends were found in alpha diversity, beta diversity, phyla or genera quantification.

Microbiome abundances in AD versus control subjects in hippocampal and cerebellum samples in Study 2

Study 2 contains a more substantial data set and compared samples between the hippocampus, typically associated with AD pathology, and the cerebellum, which is not typically associated with AD. Within the hippocampal sections, there were no differences in the alpha diversity indices between control and AD subjects (Fig. 2a). There is a trend toward clustering of AD and control subjects in the weighted UniFrac PCoA analysis indicating that the quantities of the different microbial OTUs might be different (Fig. 2b). The same trend was not observed in the unweighted UniFrac PCoA analysis (not shown). Similar to Study 1, the most four most abundant phyla were Proteobacteria followed by Firmicutes, Actinobacteria, and Bacteriodetes, with no differences between the AD and control groups (Fig. 2c, upper; Supplementary Figure 1a). Interestingly, different genera abundances were observed in Study 2 hippocampi compared to Study 1. The most abundant genera in Study 2 include *Bacillus, Staphylococcus, Pseudomonas, Acinetobacter, Enhydrobacter, Massilia, Escherichia-Shigella, Sphinogomonas, Roseomonas*, and *Stenotrophomonas*. But again, there were no differences between the AD and control samples (Fig. 2c, lower; Supplementary Figure 1a). Comparing in parallel, the cerebellum samples in AD and control subjects showed no differences in alpha diversity (Fig. 2d) or weighted UniFrac PCoA analysis (Fig. 2e). Like the hippocampus, the cerebellum samples had the same distribution of phyla abundances Proteobacteria followed by Firmicutes, Actinobacteria, and Bacteriodetes (Fig. 2e, upper). There was again a unique distribution of genera with a relative abundance order of *Pseudomonas, Acinetobacter, Escherichia-Shigella, Burkholderiaceae* (OTU)*, Stenotrophomonas, Pedobacter, Staphylo-*

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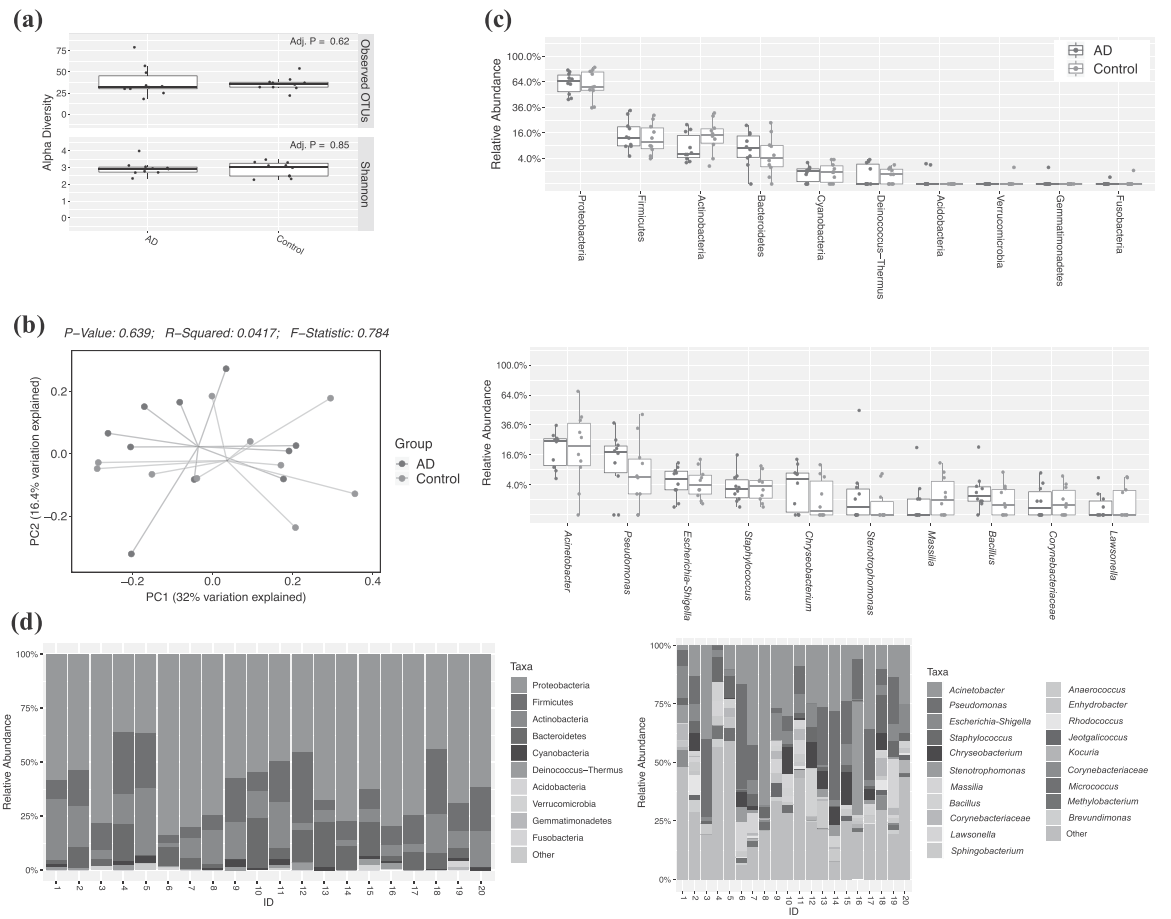


Fig. 1. Microbiome Distribution of in Alzheimer's Disease and Control Subjects in Study 1. a) Alpha diversity was estimated with total OTU counts (upper) and Shannon Index (lower), respectively. b) Beta diversity estimated with a weighted UniFrac analysis is represented as a PCoA plot to observe clustering between control and AD samples. c) Microbiome enumeration in control (blue) versus AD (red) samples are shown for the phyla (upper) and genera (lower) divisions. d) Individual representations of the phyla (left) and genera (right) are shown with the most abundant groups shown.

coccus, Bacillus, Corynebacteriaceae (OTU), and *Burkholderiaceae* (OTU) (Fig. 2f, lower) respectively. No differences in either phyla or genera between AD or control subjects were detected.

When directly comparing the control hippocampal to control cerebellar samples, no differences were observed in the alpha diversity (Fig. 3a), beta diversity (Fig. 3b), phyla, or genera (Fig. 3c). However, when comparing the hippocampal to cerebellar samples in AD subjects, albeit no differences in alpha diversity (Fig. 3d), there was a significant difference in the clustering of the weighted UniFrac analysis of the beta diversity (Fig. 3e; $p < 0.05$) indicating that there are potentially different quantities of the bacterial populations in the hippocampal versus cerebellar samples. Breaking these down into the quantification of the phyla and genera, no differences between specific phyla or genera were observed post FDR corrections.

Study bias in brain microbiome samples

Considering that there, unexpectedly, few differences in the AD subjects compared to the control subjects, we wanted to determine if there was an implicit study biases as each study was sourced from separate locations and importantly, bacterial DNA was extracted in different ways. In control subjects, there were significant variations in the alpha (Fig. 4a; $p < 0.01$) and beta diversity (Fig. 4b; $p < 0.01$) between studies with Study 2 having reduced bacterial richness compared to Study 1. Looking at the individual phyla, there was a significant reduction of

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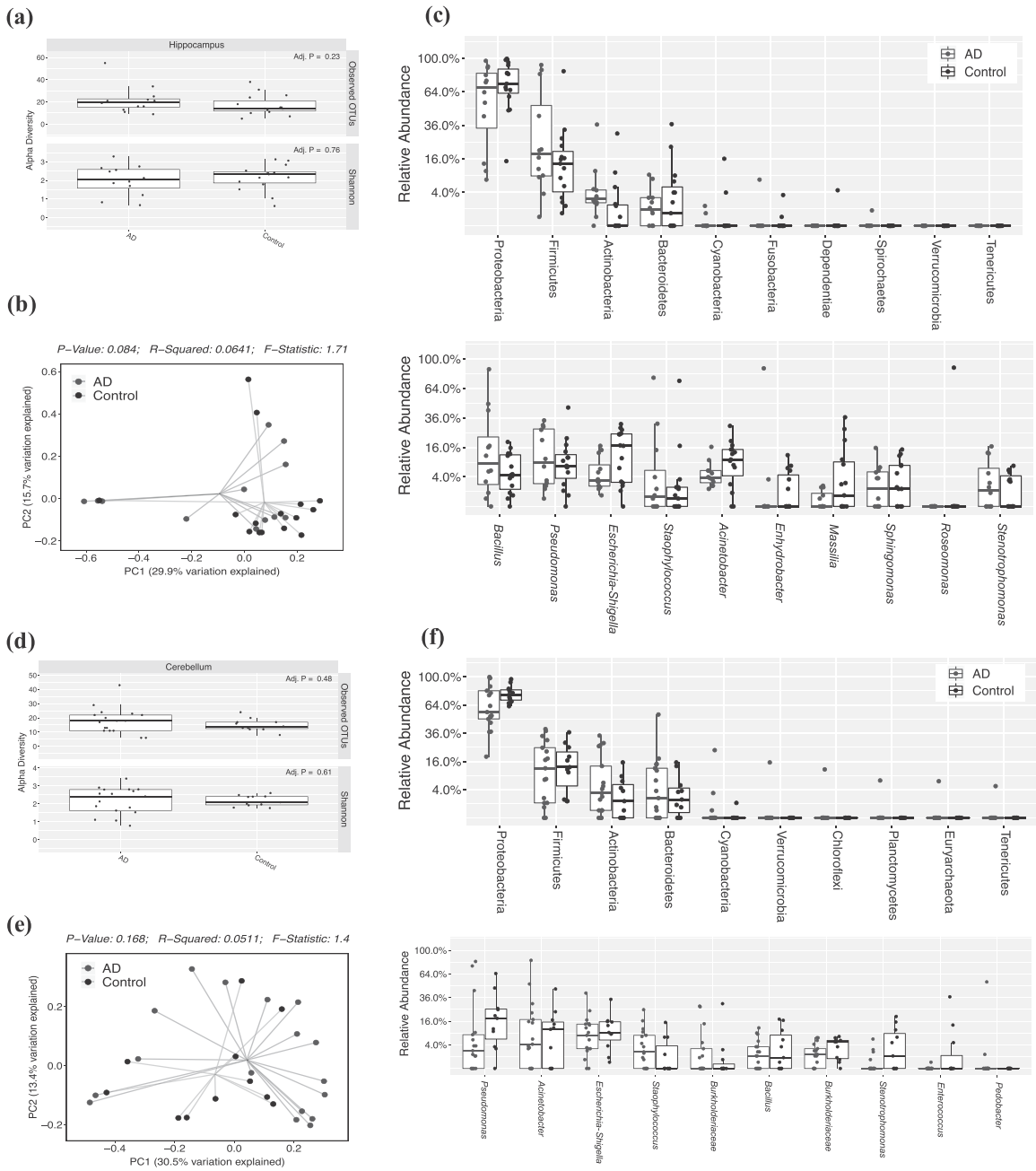


Fig. 2. Microbiome Distribution of in Alzheimer's Disease and Control Subjects in Study 2. In the study 2 subjects, microbiome presence in AD and control subjects in the hippocampus identify (a) alpha diversity, (b) beta diversity, and (c) phyla (upper) and genera (lower) comparative enumeration. Microbiome presence also in the cerebellum of AD and control subjects are shown identifying (d) alpha diversity, (e) beta diversity, and (f) phyla (upper) and genera (lower) comparative enumeration.

the phyla Actinobacteria and Deinococcus-Thermus compared to Study 1 controls (Fig. 4c, upper) with no variations in the genera between studies. Similarly, AD subjects had significant variations in alpha diversity (Fig. 4d; $p < 0.01$) and beta diversities (Fig. 4e; $p < 0.01$), again with Study 2 demonstrating lower bacterial richness compared to Study 1. Comparing the phyla and genera abundances in AD subjects,

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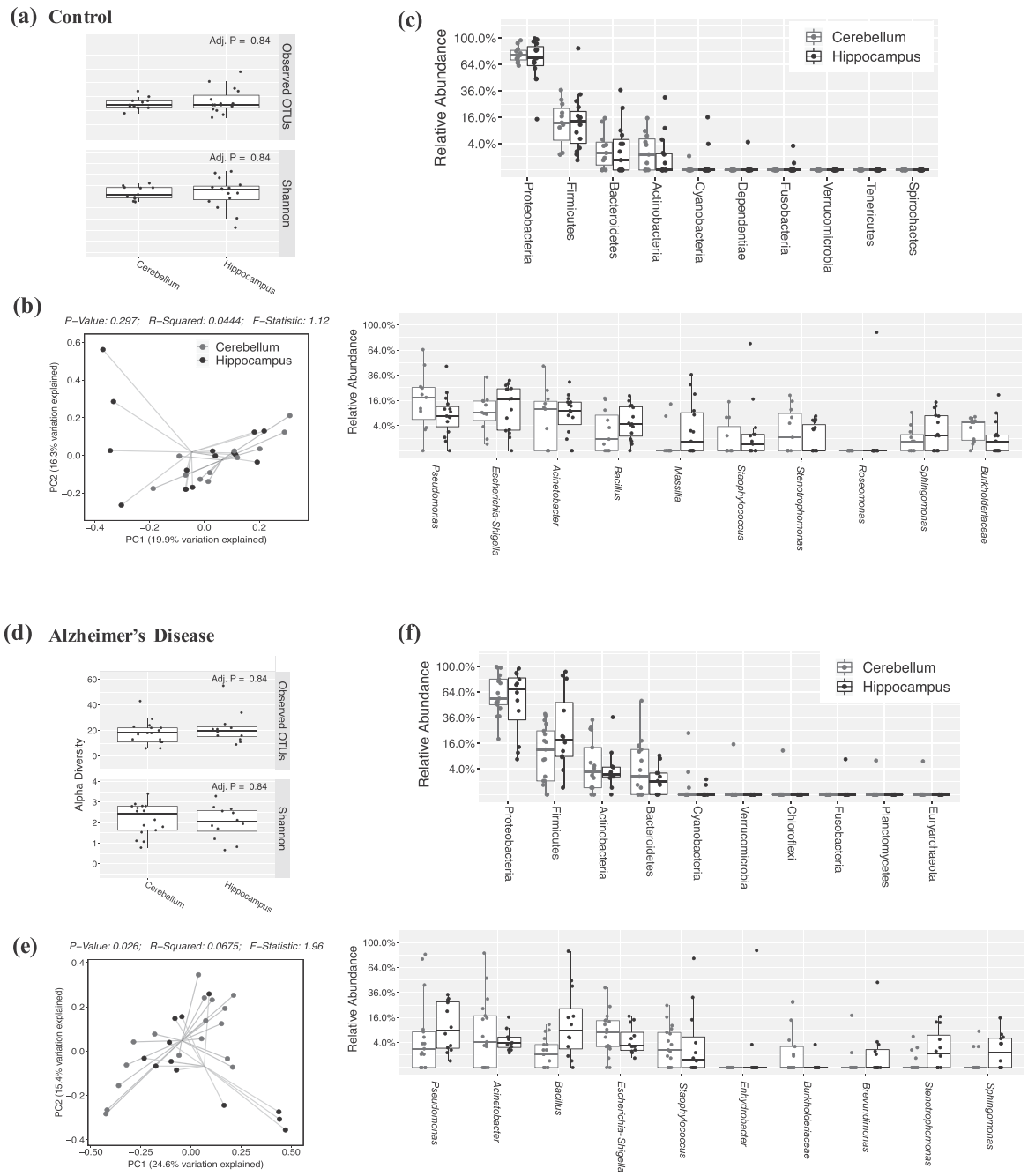


Fig. 3. Hippocampal versus Cerebellar Microbiome Distributions in Study 2. Microbiome enumeration is compared between hippocampus and cerebellum samples in Study 2 among controls (a–c) and AD subjects (d-e). Alpha diversity in controls (a) and AD (d) are estimated with OTU counts and Shannon indices while beta diversity in controls (b) and AD (e) estimated with a weighted UniFrac analysis in PCoA plots are shown. Finally, phyla (upper) and genera (lower) quantifications are shown for control (c) and AD (f) subjects.

no significant variations between phyla or genera were observed (Fig. 4f), likely due to the increased variability in the Study 2 sample cohort and a very conservative FDR. When assessing for biomarker significance, there is an increased risk of accepting false positives as a greater number of tests are conducted

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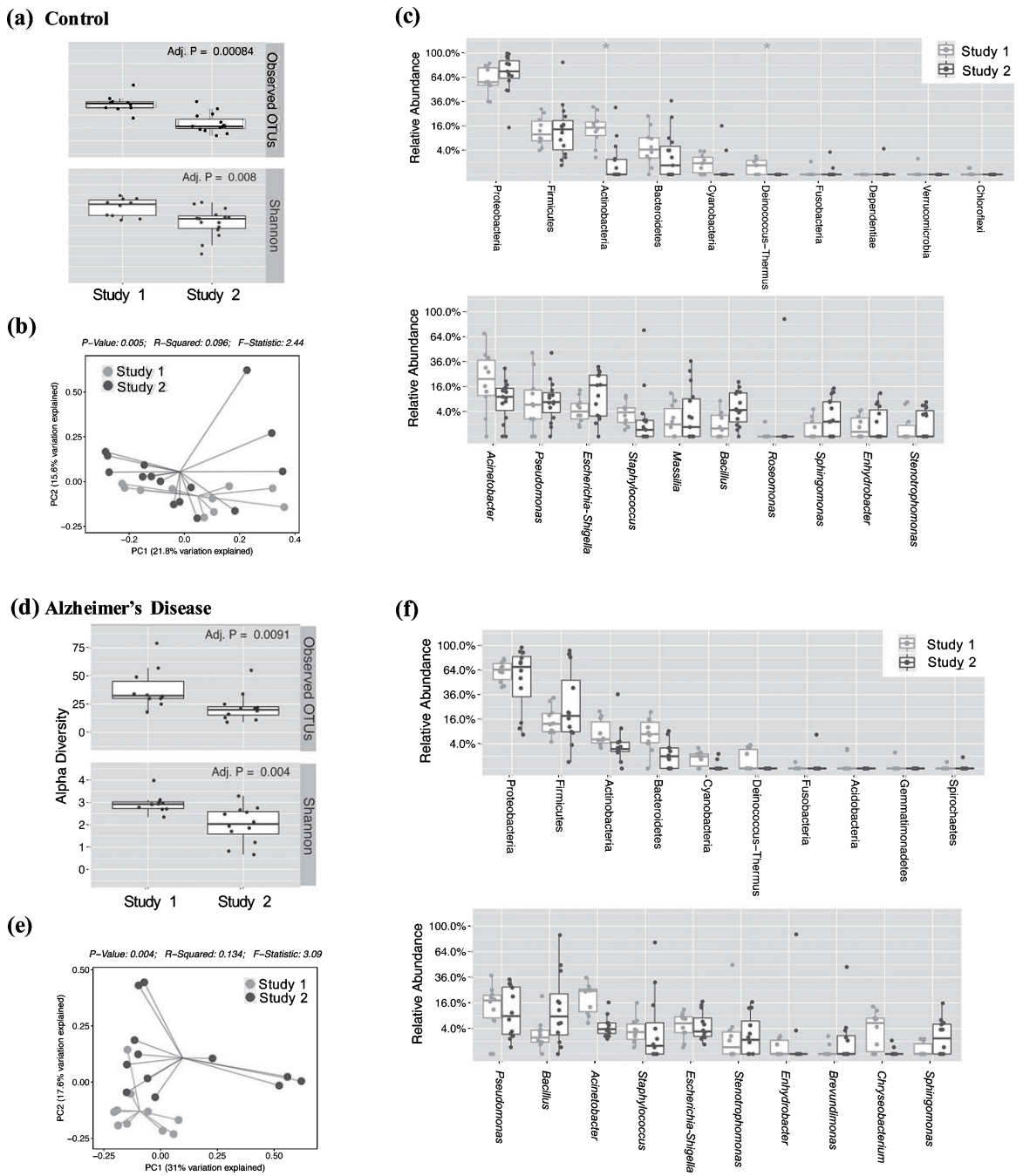


Fig. 4. Study Bias between Microbiome Cohorts of Independent Studies. Measures of microbiome differences are directly compared between Study 1 and 2. Alpha diversity in controls (a) and AD (d) are estimated with OTU counts and Shannon indices while beta diversity in controls (b) and AD (e) estimated with a weighted UniFrac analysis in PCoA plots are shown. Finally, phyla (upper) and genera (lower) quantifications are shown for control (c) and AD (f) subjects. Significant differences are represented (*) is $p < 0.05$.

based on random chance alone. To combat against accepting a high number of false positives, FDR corrections, as described in the statistical methods, were applied to lower the rate of accepting positives. The FDR correction method is considered a moderate method for false positive corrections.

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Correlation of Variations in Gut Microbiota Populations with Alzheimer's disease Metadata

AD is defined along a spectrum of pathologies including the accumulation of amyloid-β plaques, tau tangles, and levels of dementia with the confound of age and PMI. In particular, the PMI has been shown to be a major issue for the integrity and quality of the tissue sections and could influence not only the macromolecule (protein, DNA, RNA, etc.) reads, but also the brain microbiome [16]: factors which can be exasperated by the diseased state in AD. Nevertheless, several interesting correlations were observed in both studies (Table 1). Interestingly, the genera *Corynebacteriaceae* (OTU) was found to be correlated in Study 1 positively to PMI and negatively to age, amyloid-β plaque, and Clinical Dementia Rating (CDR); however, the significance of these differences were lost post FDR correction. In Study 2, *Corynebacteriaceae* (OTU) negatively correlated to PMI, and positively to amyloid-β, Braak, and CDR in the cerebellum, but not the hippocampus, while following FDR correction, only the correlation between *Corynebacteriaceae* (OTU) and PMI remained significant. Considering correlations in difference phyla, Proteobacteria were negatively associated with age in Study 1 (insignificant post FDR correction), while total OTUs and Actinobacteria were negatively correlated to PMI in the hippocampus of Study 2 (significant post FDR correction) and positively correlated to Braak (insignificant post FDR correction). In the cerebellum, the adjusted p value but not post FDR correction, *Enterococcus* was negatively while *Staphyloccocus* was positively associated to Braak. Notably, the positive correlations to Braak in the hippocampi of Study 1 and 2 were different, with the genus *Kocuria* and fungal mitochondria having a positive correlation in Study 1 and *Actinobacteria, Lawsonella and Methylobacterium* in Study 2. The variations in populations correlated either positively or negatively with the aforementioned metrics of AD support the study-specific bias in 16S sequencing of brain microbiome samples.

DISCUSSION

Dysbiotic gut microbiota has become over the last decade a well-known comorbid condition of AD [17, 18]. In clinical studies with AD patients, reported microbiome profiles contain many vari-

Table 1
Correlation of Brain Microbiome Quantification to Patients Metadata for Alzheimer's Disease Markers

Study	Study 1 (Hippocampus)	Study 2 (Hippocampus)	Study 2 (Cerebellum)
Age	Proteobacteria (–) $p < 0.01$ *Corynebacteriaceae* (–) $p < 0.05$	*Kocuria* (–) $p < 0.05$	Cyanobacteria (+) $p < 0.05^{**}$
PMI	*Corynebacteriaceae* (+) $p < 0.05$ *Enteractinococcus* (–) $p < 0.05$ *Enterobacteriaceae* (+) $p < 0.05$ *Massilia* (+) $p < 0.01$ *Rhodococcus* (+) $p < 0.0001$	Observed OTU (–) $p < 0.01^{**}$ Actinobacteria (–) $p < 0.01^{**}$ *Alkanindiges* (–) $p < 0.05$ *Chryseobacterium* (–) $p < 0.01^{**}$ *Lactobacillus* (–) $p < 0.05$ *Peptoniphilus* (–) $p < 0.001$ *Veillonella* (–) $p < 0.05$	*Corynebacteriaceae* (–) $p < 0.05^{**}$ *Moraxella* (–) $p < 0.01$ *Ruminococcaceae* (–) $p < 0.05$ *Tepidimonas* (+) $p < 0.05$
Amyloid-β	*Corynebacteriaceae* (–) $p < 0.05$ *Enterobacteriaceae* (+) $p < 0.05$ *Rheinheimera* (+) $p < 0.05$	Not Available	*Corynebacteriaceae* (+) $p < 0.05$
Braak	*Kocuria* (+) $p < 0.05$ Fungal Mitochondria (+) $p < 0.05$	Actinobacteria (+) $p < 0.05$ *Lawsonella* (+) $p < 0.05$ *Methylobacterium* (+) $p < 0.05$	*Enterococcus* (–) $p < 0.05$ *Staphylococcus* (+) $p < 0.05$ *Corynebacteriaceae* (+) $p < 0.05$ *Brevundimonas* (–) $p < 0.05$ *Burkholderia-Caballeronia-Paraburkholderia* (–) $p < 0.05$ *Tepidimonas* (–) $p < 0.05$
CDR	*Brachybacterium* (–) $p < 0.05$ *Corynebacteriaceae* (–) $p < 0.05$ *Facklamia* (+) $p < 0.05$	None	*Corynebacteriaceae* (+) $p < 0.05$ *Brevundimonas* (–) $p < 0.05$ *Burkholderia-Caballeronia-Paraburkholderia* (–) $p < 0.05$ *Moraxella* (+) $p < 0.05$ *Tepidimonas* (–) $p < 0.05$

Significant correlations between all features (phyla, genera, alpha and beta diversities) are shown for instances where $p < 0.05$. Positive/negative signs in parentheses indicate directionality of the *r*-value from the Spearman rho correlation. PMI, postmortem interval; CDR, clinical dementia rating.

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abilities; however, reports are generally consistent with showing that AD patients have a reduction in microbiome diversity, abundance, and butyrate-producing species. Many postulate that this dysbiotic microbiome is a major driving force to the onset and progression of AD [1, 19, 20] through mechanisms involving gut-brain-axis communication. Notably, the inflammatory destabilization created by a dysbiotic gut microbiome is considered a major factor in the pathogenesis of AD. One of the consequences of the inflammatory response to dysbiotic bacteria in AD is an opening of the epithelial barriers in the gut and the brain allowing increased infiltration of bacterial products and cells from the GI lumen into the brain [21, 22]. Based on this notion, several groups have begun a systematic exploration of the presence of a gut microbiota in the brain, with the potential increased incursion or altered diversity in AD patients.

In parallel with other studies [5, 8, 9], an enrichment of Proteobacteria, Firmicutes, Actinobacteria, and Bacteriodetes phyla were observed in both the control and AD subjects in both Study 1 and 2. However, unlike these studies, there were no significant differences in the composition of phyla between controls and AD in either the hippocampus or cerebellum, or in the higher resolution assessment of the genera. This is in contrast to a previous study which showed a clear increase in bacterial load in the brain of AD subjects compared to controls in both frozen and formalin-fixed samples, but in the prefrontal cortex [5]. One reason could be the sample extraction protocol, which was different than the current study. In the study of Emery et. al., a phenol/chloroform method was used to extract DNA from the frozen tissue segments while the Qiagen Formalin-fixed, Paraffin Embedded (FFPE) kit was used for the formalin-fixed samples.

Although there were no differences in bacterial population or diversity (alpha and beta diversities) observed between the AD and control subjects in Studies 1 and 2, the results remain significant since the taxa reported are not typically exclusively associated with the skin, thus can be assumed to be derived from the GI tract [23]. In general, the human skin microbiome is composed primarily of *Staphylococcus epidermidis*, *Corynebacterium*, and *Propionibacterium acnes* [24], which are distinct from the significant phyla and genera observed in the current study. In addition, there were significant variations in the genera between individuals within a single group (AD or control), allowing the assumption to be made that these reads are not artifacts of contamination but rather representative of the actual variability in the brain sample cohorts. In addition, groups have shown unequivocally that several fungal and bacterial proteins can be found in the corpora amylacea of AD patients [25]. This is an interesting observation as the corpora amylacea, spherical bodies composed of polyglucans and proteins, accumulate gradually in the CNS of aging persons, but to a greater extent in patients with neurodegenerative disorders and AD. This indicates that fungi and bacteria may be present in the body for a significant amount of time, and hence not merely an artifact of postmortem tissues. This group additionally showed using immunohistochemistry with anti-peptidoglycan and Clostridium antibodies and imaging with confocal microscopy that fungal or prokaryotic structures were present in the brains of AD patients confirming that microbial products are present in the brain of AD patients [8].

In addition to assessing the composition of the brain microbiome, the current study correlated variations in the brain microbiome and its complexity to the patient metadata for measures of AD. One potential confound based on the patient metadata is the variation in PMI. Previously, the gut microbiota was predicted to be an indicator of PMI, as determined through a random forest correlation in cases when the time of death was uncertain [26, 27]. Interestingly, between Studies 1 and 2, there was a different trend between positive and negative correlation of bacterial populations with PMI. Study 2 consistently showed a negative correlation between bacterial groups and total OTU counts in the hippocampus and cerebellum while Study 1 showed a mix of positive and negative correlations. This indicates that there is not a consistent bias for PMI with microbiome populations in the brain; however, it must be considered a potential factor based on the results from other studies. In addition, the average and range of PMI is very different between the two studies. In Study 1, the PMI ranges from 125 to 1,437 hours with a median of 265 hours, whereas in Study 2, the range is much smaller from 1.5 to 29.6 hours with a median of 14.2 hours. It has been suggested that studying multiple brain regions in the same subject can determine if PMI is actually a factor in the sample integrity and in this case, bacterial richness. As such, in Study 2, the alpha diversity, observed OTUs, levels in hippocampus was negatively associated with PMI indicating that PMI may be a factor in the richness and/or diversity of the brain microbiome. Interestingly, we

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found a limited number of correlations between different genera and PMI. *Corynebacteriaceae* (OTU) had a negative correlation with PMI in the cerebellum whereas *Chryseobacterium* and *Peptoniphilus* were negatively correlated with PMI in the hippocampus.

An obvious population of interest in the correlative study is the presence of the genera *Corynebacteriaceae* (OTU) and its correlation with multiple AD metadata in both Studies 1 and 2. As noted before, bacterial species in the family *Corynebacteriaceae* are also found on the skin as well as in the GI tract. In general, bacteria in the family *Corynebacteriaceae* are generally pathogenic causing a range of serious infections in humans and animals including diphtheria, endocarditis, and lymphadenitis [28], although some non-pathogenic strains have also been identified [29]. In Study 1, *Corynebacteriaceae* in the hippocampus correlated negatively to age, amyloid-β, Braak, and CDR while retaining a positive correlation to PMI; however, the significance of these results was lost post FDR correction. In contrast, in Study 2, there were no correlations of *Corynebacteriaceae* to AD metrics in the hippocampus while in the cerebellum, *Corynebacteriaceae* was positively associated to amyloid-β, Braak, and CDR and negatively to PMI again outlining the Study-specific bias in studying the brain microbiome.

The most interesting correlation observed was the positive correlation between the phyla Actinobacteria and Braak in the hippocampus samples of Study 2 and the positive association between *Staphylococcus* and Braak in the cerebellum. Despite there being no significant differences in the control versus AD populations of these phyla, the trending changes could be a significant driver of AD pathogenic markers in humans.

Another important fact that should be acknowledged regarding brain infection and the pathogenicity of AD is that there is a commensal fungal population specifically in AD CNS tissue, which is not present in controls [30]. One group determined fungal material in both intra- and extracellular tissue in multiple brain regions including the cerebellar hemisphere and the hippocampus. This group also identified evidence of several fungal species in these regions and in the vascular tissue [31]. Similarly, immunohistochemical assessment of fungal components were observed in the cytoplasm of approximately 10% of AD patient's frontal cortex tissue, but not control subjects confirming the intracellular localization [32]. These results have been confirmed by other groups where the most abundance fungal genera were identified as *Alternaria, Botrytis, Candida*, and *Malassezia*, which were distinctly different from healthy control subjects [6]. The colonization of fungi and bacteria in the brain could contribute to the abnormalities in the innate immune system in both the periphery and the brain: a widely accepted part of AD's pathophysiology [30]. In contrast, the disrupted innate immune response consequent of possibly other underlying factors may facilitate the incursion of fungi particles or bacterial particles into the brain, exasperating the AD phenotype. Regardless, infection of the aging adult brain may be a significant source of the AD pathophysiology opening novel avenues for therapeutic approaches.

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SUPPLEMENTARY MATERIAL

Investigation of Potential Brain Microbiome in Alzheimer's Disease: Implications of Study Bias

Supplementary Table 1
Patient Demographics for Study 1. Patient demographic information collected from Study 1 patients

Group	Sample ID	Source	Age (y)	Race	Gender	PMI (mhr)	AD Amyloid Plaque	Braak	CDR	pH
Control	1	MSSM/	95	ND	M	245	0	2	0.5	ND
	2	BVAC	84	ND	F	1110	0	1	0	ND
	3	(Study 1)	69	ND	M	255	0	1	0.5	ND
	4		89	ND	F	140	1.28	2	0	ND
	5		83	ND	F	370	0	1	0.5	ND
	6		66	ND	F	1355	0.67	0	0	ND
	7		68	ND	F	1437	0	3	0	ND
	8		85	ND	F	260	4.08	2	0	ND
	9		75	ND	M	300	0	1	0	ND
	10		76	ND	F	255	0	2	1	ND
AD	11	MSSM/	84	ND	F	435	15.68	6	5	ND
	12	BVAC	85	ND	F	130	18.88	5	5	ND
	13	(Study 1)	83	ND	F	125	27.04	6	3	ND
	14		93	ND	M	230	17.92	5	3	ND
	15		66	ND	F	260	36.72	2	5	ND
	16		69	ND	M	178	33.6	6	5	ND
	17		68	ND	F	620	31.88571	5	3	ND
	18		85	ND	M	375	28.8	6	5	ND
	19		75	ND	F	310	21.44	6	5	ND
	20		76	ND	F	270	11.12	6	3	ND

PMI, postmortem interval; CDR, Clinical Dementia Rating.

Supplementary Table 2
Patient Demographics Hippocamps Samples Study 2. Patient demographic information collected from Study 2 patients

Group	Sample ID	Source	Age (y)	Race	Gender	PMI (mhr)	AD Amyloid Plaque	Braak	CDR	pH
Control	43	Miami	82	W	F	14.2	NA	ND	ND	5.92
	44	(Study2)	83	W	F	6	NA	ND	ND	6.02
	45		83	W	M	25	NA	ND	ND	6.39
	46		83	W	M	29.55	NA	ND	ND	6.24
	47		84	W	M	15.5	NA	ND	ND	6.09
	48		85	W	F	25.83	NA	ND	ND	6.34
	49		86	W	F	37	NA	ND	ND	6.69
	50		86	AA	M	8.05	NA	ND	ND	5.96
	51		86	W	F	25.11	NA	ND	ND	5.81
	52		87	W	F	5.2	NA	ND	ND	6.06
	54		89	W	F	12	NA	ND	ND	6.26
	55		89	W	M	25.5	NA	ND	ND	6.47
	56		83	W	F	7.1	NA	ND	ND	6.16
	57		87	W	F	13	NA	ND	ND	5.42
	58		88	W	M	19.4	NA	ND	ND	6.18
	59		87	AA	F	20	NA	ND	ND	6.32
	60		88	W	F	3.3	NA	ND	ND	ND
	61		85	W	F	4	NA	ND	ND	5.92
AD	21	Miami	80	W	M	22	NA	6	3	5.92
	22	(Study2)	80	W	F	6	NA	6	ND	6.19
	23		85	W	M	26	NA	6	3	6
	24		81	W	F	11	NA	5	3	5.84
	25		81	W	F	19	NA	5	3	6.61
	26		81	W	F	21	NA	4	0	5.67
	27		81	W	M	24	NA	3-4	1	6.46

(*Continued*)

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Supplementary Table 2
(*Continued*)

Group	Sample ID	Source	Age (y)	Race	Gender	PMI (mhr)	AD Amyloid Plaque	Braak	CDR	pH
	28		81	W	F	4.5	NA	6	3	6.34
	29		82	W	M	11	NA	6	3	6.25
	30		82	W	F	14.5	NA	5-6	3	5.91
	31		83	W	F	3.9	NA	6	3	5.76
	32		85	W	F	7.92	NA	6	3	6.45
	33		85	W	M	17.6	NA	3	ND	5.96
	34		85	W	F	12.3	NA	6	3	6.3
	35		80	W	F	3.5	NA	6	3	NF
	36		85	W	F	9.1	NA	6	3	NF
	37		88	W	M	3.66	NA	6	3	6.35
	38		88	W	F	18	NA	6	3	6.29
	39		88	W	F	16.5	NA	6	3	6.32
	40		81	W	M	9.5	NA	5	3	nf
	41		88	W	F	20.3	NA	6	ND	6.44
	42		88	W	F	24	NA	6	3	5.83
	53		88	W	F	10.1	NA	6	3	6.4

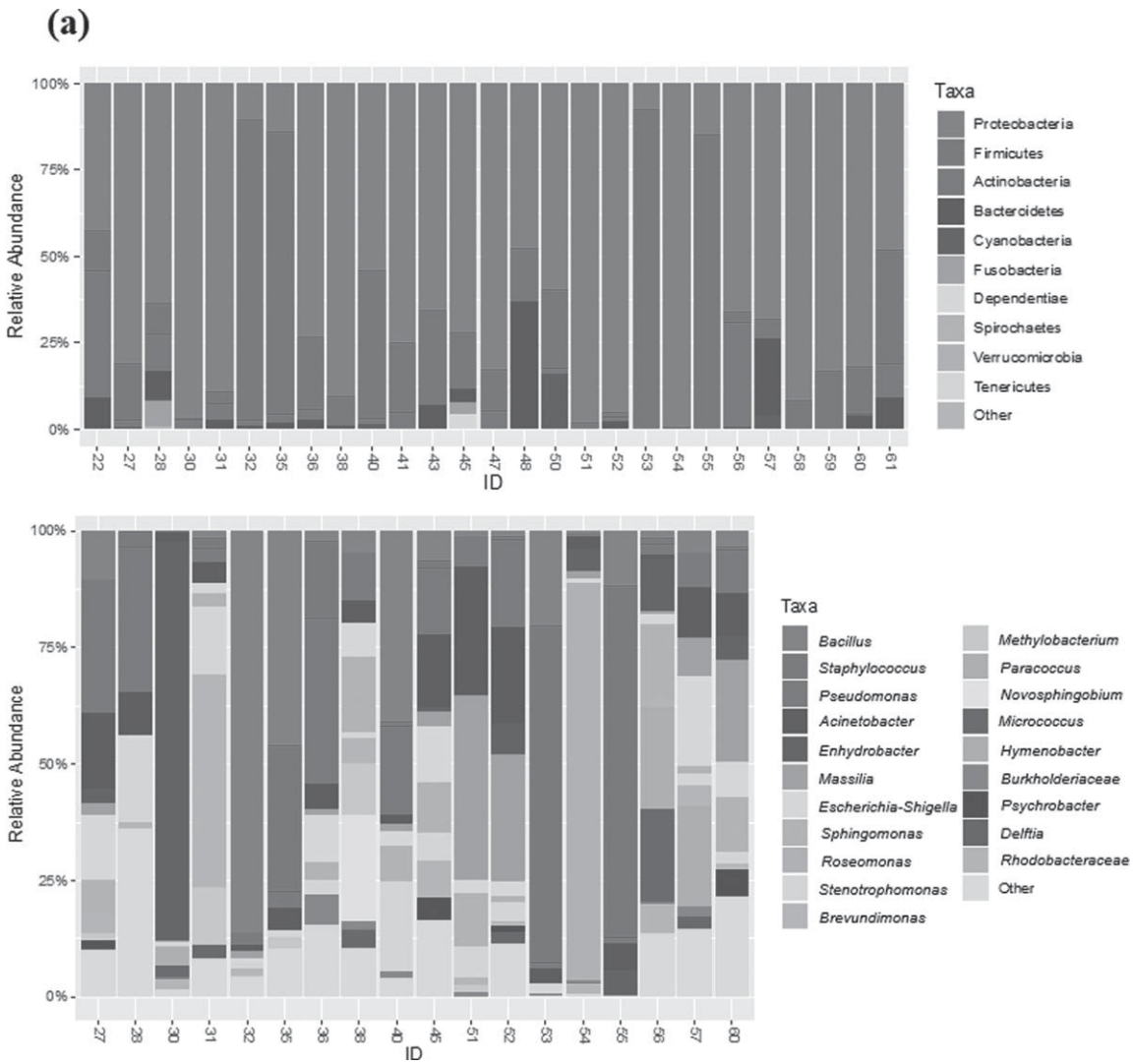
PMI, postmortem interval; CDR, Clinical Dementia Rating.

Supplementary Table 3
Supplementary Patient Demographics Study 2 Cerebellum Samples. Patient demographic information collected from Study 2 patients

Group	Sample ID	Source	Age (y)	Race	Gender	PMI (mhr)	AD Amyloid Plaque	Braak	CDR	pH
Control	72	NBTR	86	W	F	4.67	0	3	0	7
	73	(Study 2)	85	W	F	5	0	2	3	6
	74		84	W	F	18.5	0	1	0	6
	75		83	W	F	6.17	0	1	0.5	7
	76		81	W	F	18.8	4.24	2	0	6
	77		81	W	F	9.5	4.8	2	0	6
	78		83	H	M	16.83	0	0	0.5	6
	79		84	W	M	11.42	0	2	0.5	7
	80		84	W	M	20.92	0	1	1	7
	81		85	W	F	8	0	3	2	7
	82		85	W	M	16.17	0	3	2	6
	83		85	W	F	5	0	0	0	7
AD	62	NBTR	88	W	F	6	4.18	6	4	6
	63	(Study 2)	82	W	M	5.75	11.31	6	2	6
	64		84	W	M	2.33	5.556	6	3	6
	65		88	W	F	3.58	9.68	6	5	6
	66		83	W	F	2.08	27.04	6	3	7
	67		80	W	F	13.5	12.48	6	5	6
	68		84	W	F	1.75	21.76	6	5	6
	69		85	W	F	5.87	12.28	6	4	6
	70		88	W	F	3.25	10.16	6	5	6
	71		75	W	M	10.5	12.48	4	0.5	6
	84		81	W	M	4.75	20	6	4	6
	85		88	W	F	3.5	20.48	6	5	7
	86		84	W	F	2	20.4	6	5	6
	87		83	W	F	4.25	17.94	6	4	6
	88		89	W	F	3	29.2	6	5	6
	89		87	W	F	14.5	25.36	6	2	7
	90		87	W	F	13.33	26.4	6	4	6
	91		87	W	F	1.5	22.32	6	5	6
	92		86	B	F	3.5	7.76	6	2	7
	93		87	W	M	2.75	12.16	6	5	7

PMI, postmortem interval; CDR, Clinical Dementia Rating.

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Supplementary Figure 1. Supplementary Microbiome Distribution per Individual in Study 2. Microbiome enumeration in Study 2 for control (a) and AD (b) subjects. Upper graph depicts the phyla while lower graph depicts genera quantifications.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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A Review of the Brain-Gut-Microbiome Axis and the Potential Role of Microbiota in Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) is a neurodegenerative process characterized by loss of neurons in the hippocampus and cerebral cortex, leading to progressive cognitive decline. Pathologically, the hallmark of AD is accumulation of "senile" plaques composed of amyloid-β (Aβ) protein surrounding neurons in affected regions. Despite extensive research into AD pathogenesis and therapeutic targets, there remains no breakthroughs in its management. In recent years, there has been a spark of interest in the connection between the brain and gastrointestinal tract, referred to as the brain-gut axis, and its potential implications for both metabolic and neurologic disease. Moreover, the gastrointestinal flora, referred to as the microbiome, appears to exert significant influence over the brain-gut axis. With the need for expanded horizons in understanding and treating AD, many have turned to the brain-gut-microbiome axis for answers. Here we provide a review of the brain-gut-microbiome axis and discuss the evidence supporting alterations of the axis in the pathogenesis of AD. Specifically, we highlight the role for the microbiome in disruption of Aβ metabolism/clearance, increased permeability of the blood-brain barrier and modulation of the neuroinflammatory response, and inhibition of hippocampal neurogenesis. The majority of the above described findings are the result of excellent, albeit basic and pre-clinical studies. Therefore, we conclude with a brief description of documented clinical support for brain-gut-microbiome axis alteration in AD, including potential microbiome-based therapeutics for AD. Collectively, these findings suggest that the brain-gut-microbiome axis may be a "lost link" in understanding and treating AD and call for future work.

Keywords: Alzheimer's disease, dementia, gut microbiome, neurodegenerative disorders

INTRODUCTION

Neurodegenerative disease is the term to describe a class of conditions, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), characterized by progressive loss of neurons, primarily in the brain, resulting in symptoms ranging from motor ataxias to cognitive dysfunction.

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Neurodegenerative disease poses a substantial emotional and financial burden on the patient, their family, and society, especially with the increasing aging population [1]. AD is a type of dementia characterized pathologically by cell loss in the hippocampus and cerebral cortex as well as accumulation of insoluble amyloid-β (Aβ) plaques in the interstitium and tau neurofibrillary tangles in neurons. AD is the sixth leading cause of death in the United States, with an estimated incidence of 1–3% and a prevalence of 10–30% of the population > 65 years of age, owing to its mean duration of 10 years [2, 3]. Further, the societal burden of AD is hypothesized to increase tremendously, with the prevalence in the United States increasing from 5.8 million to nearly 14 million and the cost increasing from $290 billion to $1.1 trillion by 2050 [2]. While the pathological hallmarks of AD are well described, the underlying pathogenesis of the disease remains controversial, with multiple hypotheses regarding its etiology proposed [4]. The interaction of the brain and gut microbiome, termed the brain-gut-microbiome axis, has been described in recent years as a model for understanding brain health and disease. Reports of gut microbiome alterations in patients with AD have accumulated a great deal of interest due to their potential to explain various features of the disease process as well as provide a new target for therapeutics [5]. We here provide a review of the evidence supporting a role for alterations along the brain-gut-microbiome axis in the pathogenesis of AD, with a focus on the concordance between these alterations and previously described pathogenic mechanisms. We then conclude with a discussion of the potential for microbiome-targeted therapeutics in the management of AD.

OVERVIEW OF THE BRAIN-GUT-MICROBIOME AXIS

The role of the brain-gut-microbiome axis in health and disease has been a popular source of attention for basic and clinical research, as well as in the media. The concept of an interconnection between the gut and the brain dates as far back as the nineteenth century, with physicians of the time reporting on such conditions as "neurasthenia gastrica" and "autointoxication", in which it was hypothesized that "nervous weakness" may result in impaired digestion, or conversely that impaired digestion may permit accumulation of toxins from the intestines, including microbes, that disrupt nervous system activity [6]. In 1986, Hegstrand and Hine reported significant differences in brain histamine levels between nephrectomized germ-free (GF) and conventionally housed animals, providing some of the first evidence that changes in microbiome composition can influence brain chemistry [7]. This was further supported by the finding that the hypothalamic-pituitary-adrenal (HPA) stress response is exaggerated in GF mice but can be partly corrected with fecal reconstitution using feces from specific pathogen-free (SPF) animals [8]. Continued study of this gut-brain connection has yielded discovery of three distinct, but parallel, pathways for bidirectional communication along the axis: 1) neural, via direct connection through the enteric nervous system (ENS), 2) endocrine, via hormonal signaling, most notably via the HPA system, and 3) immune, via microbial release of inflammatory factors and modulation of mucosal and systemic immunity [9, 10, 11].

Development, structure, and function of the gut microbiome and intestinal barrier

The gut has long been reported to harbor as many as 10^{14} bacterial cells, accounting for the reputed 10:1 ratio of bacterial to human cells often highlighted in the literature. However, recent calculations have determined the total number of bacterial cells to be approximately 3.8×10^{13}, with 10^{11} in the gastrointestinal (GI) tract, placing the above ratio at 1.3:1 [12]. In addition to being quite robust, the human gut microbiome is also highly diverse, with one large sequencing study identifying between 1,000 and 1,150 different bacterial species across the cohort and at least 160 different species in each subject [13]. In contrast to previously held dogma, the development of such a robust and diverse microbiome begins as early as the *in utero* period, supported by the finding that meconium samples, representing material ingested or secreted by the GI tract during fetal life, obtained from neonates demonstrated the presence of conserved microbiota that is unaffected by perinatal, but rather maternal factors [14]. By performing high-throughput sequencing of meconium from term newborns, Gosables et al. identified two distinct types of meconium, the first being enriched with enteric bacteria including the genera *Escherichia* and *Shigella*, while the second was enriched with lactic acid bacteria including the genera *Leuconostoc, Enterococcus, Lactococcus, Staphylococcus,* and *Streptococcus* [15]. Notably, alterations

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in this early microbiota are associated with clinical conditions, including neonatal jaundice [16], premature birth [17], and maternal diabetes [18]. Continued development of the gut microbiome occurs throughout the first years of life, with two major life events dictating maturation to an adult-like composition: 1) mode of delivery (i.e., C-section versus vaginal) and 2) feeding pattern (i.e., breast-feeding versus formula) [19]. While vaginal delivery tends to promote an infant microbiome rich in *Lactobacillus, Prevotella*, and *Sneathia* species, similar to the maternal microbiome due to vertical transfer of vaginal and perianal microbes, C-section yields a microbiome dominated by *Staphylococcus, Corynebacteria,* and *Propionibacterium*, resulting from horizontal transfer of maternal skin flora [20]. The microbiome converges toward its mature adult state by the end of the infant period (3–5 years of life), with factors such as geographical location, home environment, diet, and antibiotic exposure, modifying its final composition [21]. The adult microbiome demonstrates signature diversity along the various regions of the GI tract, and continues to be modified by diet and lifestyle factors [22]. A summary of the changes in the microbiome composition across the lifespan as well as relevant sources is provided in Table 1. The function of the gut microbiome is extensive, with metabolic capabilities including synthesis of vitamins (e.g., vitamin K) [23], synthesis and catabolism of amino acids [24], and formation of short chain fatty acids (SCFAs) which may be used as energy substrates and signaling molecules [25]. Beyond metabolism, the gut microbiome also plays a role in modulating immune system development and function by promoting formation of gut-associated lymphoid tissues (GALT), maintaining the intestinal epithelial barrier, inducing secretory IgA production, and conditioning intestinal mononuclear phagocytes and T-cells [26].

The GI tract is the largest interface between the external world and the body; while it is primarily considered for its role in food and nutrient processing, this exposure is also critical for modulation of immune function. The intestinal epithelium is a dynamic physical barrier that acts as a component of the innate immune system to prevent exposure to harmful antigens and pathogens. Additionally, the epithelium must be selectively permeable to permit translocation of ingested nutrients into the bloodstream. The barrier is composed of a single epithelial layer lining the GI tract lumen, with cells bound to one another by tight junctions, adherens junctions, and desmosomes [27]. Tight junctions, the most apically located intercellular connections, are

Table 1
Composition of microbiome across lifespan

Prenatal		Infant		Adult	
Type A	Escherichia *Shigella*	Vaginal Delivery	*Lactobacillus* *Prevotella* *Sneathia (Increasing distribution of Bifidobacterium and Bacteroides species)*	Oral Cavity	Streptococcus* Haemophilus Prevotella Veillonella Gemella Neisseria Granulicatella
				Esophagus	Streptococcus* *Prevotella* *Veillonella* *Rothia* *Gemella*
Type B	*Leuconostoc* *Enterococcus,* *Lactococcus* *Staphylococcus* *Streptococcus*	C-Section	*Staphylococcus* *Corynebacteria* *Propionibacterium* *(Fewer Bifidobacterium and Bacteroides species)*	Stomach	*Streptococcus* *Helicobacter*** *Prevotella*
				Small Intestine	*Bacteroides* *Clostridium* *Streptococcus*
				Large Intestine***	*Bacteroides* *Prevotella* *Rumnicoccus*
Source: Gosalbes et al. [15]		Source: Yang et al. [20]		Source: Hillman et al. [22]	

Prenatal is subdivided into Types A and B. Infant is subdivided by delivery type (vaginal or Cesarean). Adult is subdivided by region of gastrointestinal tract. *Most common; **High inter-subject variability; *** 70% of all flora.

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composed of multi-protein complexes that regulate paracellular permeability and maintain integrity of the epithelial barrier. Tight junctions are composed of diverse proteins, including occludin, claudin, junctional adhesion molecule (JAM), and tricellulin, which interact with intracellular scaffolds (i.e., zona occludens) to be anchored to the actin cytoskeleton. Disrupted integrity of the intestinal epithelial barrier has been demonstrated in a variety of GI disorders, including inflammatory bowel disease, via alteration of tight junction expression and/or function by inflammatory factors such as IFN- γ, TNF- α, and interleukins [28]. Recent work has demonstrated that intestinal epithelial tight junctions are differentially regulated by the microbiota. Specifically, SCFAs- butyrate and acetate- produced by particular microbiota species are capable of inducing barrier properties in the intestinal epithelium to protect from pathogenic bacteria and toxins [29, 30]. Butyrate has been demonstrated to do so via facilitating tight junction assembly in both AMP-activated protein kinase (AMPK) and hypoxia-induced factor 1 (HIF1)-dependent mechanisms [30, 31]. In addition to the tight junctions between epithelial cells, the layer of mucous produced by goblet cells overlying the epithelium is a critical component of the intestinal barrier. In fact, within the colon, the location with the highest microbiota concentration, the mucous layer is highly complex and organized into three distinct layers: 1) the innermost glycocalyx, composed of membrane-bound mucin proteins, 2) the tightly-linked middle layer, and 3) the low density outer layer formed by middle-layer proteolysis, in which bacteria typically reside [32]. Similar to tight junctions, SCFA-generating microbiota species are protective against pathogenic bacteria via stimulating production of mucus and differentiation of mucus-producing goblet cells [33].

While a healthy microbiome is capable of protecting from pathogenic species via maintenance of the intestinal barrier, dysbiosis, defined as microbial imbalance, may promote barrier disruption and predispose the organism to illness. An important inducer of microbial dysbiosis is antibiotic use, which may cause death of healthy flora and promote the growth of pathogenic species, such as *Clostridium difficile. C. difficile* results in pseudomembranous colitis through the generation of enterotoxins A and B, both of which have been shown to disrupt the cytoskeleton and promote intestinal permeability [34]. Dietary alterations are also intimately linked with alterations in the microbiome, implicating a role for diet in the pathogenesis of metabolic disease beyond the concept of caloric imbalance [35]. Consistent with this, Desai et. al. recently showed that lack of dietary fiber depletes microbial nutrients, thus forcing these organisms to rely on mucus proteins as a nutritional source, resulting in erosion of the mucus barrier. This barrier disruption thus permitted epithelial access and infection with the mucosal pathogen *Citrobacter rodentium* [36]. As described, the interaction between microbiota and the intestinal barrier is critically important in regulation of GI tract immunity and may provide insight into pathogenic mechanisms of a variety of disease processes and targets for future therapy.

Neuroendocrine interactions along the brain-gut microbiome axis

Neuroendocrine interactions with the GI tract occur via two mechanisms: 1) endocrine, via the HPA system, and 2) neural, directly via the ENS. The HPA axis, often considered the "stress response" system, describes the indirect connection between the hypothalamus, pituitary, and adrenal gland via hormone secretion into the bloodstream. Corticotropin-releasing hormone (CRH) is secreted by the hypothalamus into the hypophyseal-portal blood, where it travels to the anterior pituitary to stimulate secretion of adrenocorticotropic hormone (ACTH) into the bloodstream. At the level of the adrenal cortex, ACTH stimulates synthesis of glucocorticoids (i.e., cortisol) by the zona fasciculata which exert systemic metabolic actions [37]. Activation of the HPA axis is well-described in the setting of chronic stress, depression, and anxiety disorders and is programmed by early life events/stressors. Early life stress is associated with heightened activity of the HPA axis and predisposes animals to subsequent neuropsychiatric pathology [38]. Building off the idea that bidirectional communication between the early nervous and immune systems may alter development of neural systems, Sudo et al. demonstrated exaggerated HPA responses, including elevations in ACTH and corticosterone, in GF mice in response to restraint stress compared to similarly exposed SPF mice. Further, reconstitution of the GF mice with *Bifidobacterium infantis* reversed the exaggerated response, highlighting the influence of microbiota on postnatal development of the HPA axis [8]. Beyond its influence directly on the HPA axis, the microbiome plays a role in neurotransmitter, most notably tryptophan, metabolism, which is responsible for

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the generation of the key neurotransmitter serotonin. Absence of such tryptophan metabolizing bacteria is associated with elevated levels of circulating tryptophan, reduced levels of serotonin, and presence of anxiety-like behaviors [39].

Further, the link between the brain, gut, and microbiome is bidirectional. Chronic activation of the HPA axis by stressors can result in altered composition of the microbiome, as demonstrated in a variety of preclinical model of stress such as maternal separation, chronic social defeat, restraint, crowding, heat, and auditory stressors [40]. Alteration of the microbiome by chronic stress, and activation of the immune system, has also been proven to induce colitis; these mechanisms are hypothesized to underly stress-associated gastrointestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [41, 42].

While the influence of the gut microbiome on the HPA axis is via indirect connection through the bloodstream, its influence on the gut's nervous system or ENS is more direct and physical. The ENS is comprised of two distinct ganglion within the wall of the GI tract: 1) the myenteric, or Auerbach, plexus between the inner circular and outer longitudinal layers of the muscularis controls GI motility, and 2) the submucosal, or Meissner, plexus within the submucosal layer innervates GI epithelium. These plexuses are connected by nerve fibers which form mesh-like networks traversing the walls of the GI tract [43]. The ENS is innervated by parasympathetic fibers of the vagus nerve and sympathetic fibers of the prevertebral ganglia. The vagus nerve, which emerges from the brainstem and carries efferents to the enteric ganglia and afferents to the nucleus tractus solitarii, provides the primary bidirectional connection between the CNS and ENS, forming the "hardwire" of the brain-gut axis [44]. In fact, the recent identification of enteroendocrine cells within the gut epithelium, which synapse on vagal neurons, provides a mechanism by which the vagus nerve directly interacts with the GI lumen. Physiologically, this provides a mechanism for luminal nutrient sensation, but may also serve as a direct surface for interaction with the microbiome or microbial nutrients [45]. The interaction between the microbiome and ENS begins in development. ENS neurons are derived embryologically from Sox10-expresing neural crest cells which delaminate from the neural tube and migrate throughout the developing gut tube prenatally [46]. However, a niche of enteric neural stem cells remains within the postnatal gut which maintains the potential for modulation of ENS maturation following colonization with gut microbiota at birth [47]. This potential has been proven in both enteric glial cells and neurons in recent years. Enteric glia normally form extensive networks; however, it has been shown that GF mice have reduced number of mucosal enteric glial cells compared with conventionally housed mice. This lack of enteric glia is rescued by postnatal colonization via gavage feeding of microbiota which promotes formation of glial networks [48]. Similarly, GF mice display neuronal anatomical abnormalities and reduced intestinal transit rate, which is corrected by feeding of microbiota via increased production of serotonin and stimulation of enteric neurogenesis [49]. Once mature, homeostatic maintenance of the ENS is reliant on the presence of enteric neural stem cells that may respond to alterations in the surrounding microenvironment or disease processes. Enteric neurons are known to express Toll-like receptors, suggestive of a role for microbial products in maintenance of the mature ENS [50]. Consistent with this, GF mice and mice lacking the *TLR4* gene display significant delay in GI motility and reduced numbers of nitrergic neurons, while incubation of immortalized enteric neurons with bacterially-derived TLR4 agonist lipopolysaccharide (LPS) promotes expression of the transcription factor NF-κB and cell survival [51]. Furthermore, in culture, bacterial LPS promotes proliferation and delays differentiation of enteric neural stem cells, providing a mechanism by which microbiota may promote ENS homeostasis [52]. These neuroendocrine interactions are summarized in Fig. 1.

MECHANISMS OF BRAIN-GUT-MICROBIOME ALTERATION IN ALZHEIMER'S DISEASE

The direct and indirect connections comprising the brain-gut-microbiome axis described above, and their alteration in a variety of pathologies, raise the question: What is the influence of the axis on the development and/or progression of AD? Despite extensive research into the neuropathology of this devastating disease, little progress has been made in identifying novel targets for therapy. In the subsequent pages we provide a discussion of the evidence for brain-gut-microbiome disturbance in AD and consider the axis as a missing link in AD pharmacotherapy. These mechanisms are summarized in Fig. 2.

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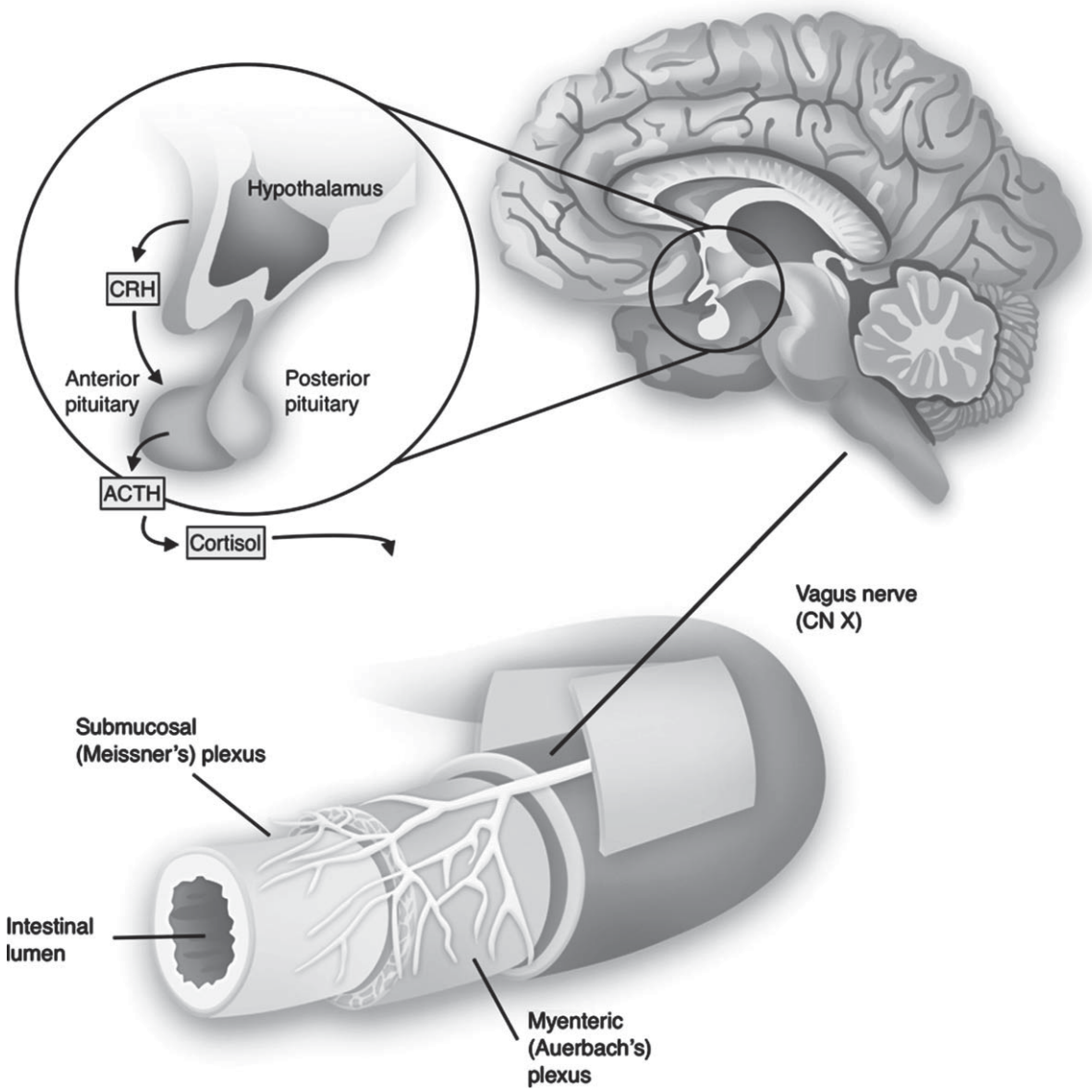


Fig. 1. Neuroendocrine interactions between the brain and gastrointestinal tract. The vagus nerve (CN X) projects from the nucleus and provides parasympathetic innervation to foregut and midgut derived structures. The hypothalamic-pituitary-adrenal axis, classically referred to as the "stress axis", is the primary endocrine connection between the brain and gastrointestinal tract.

Microbial modulation of amyloid-β accumulation and clearance

The amyloid-β (Aβ) hypothesis, based on the neuropathologic findings of amyloid plaques within the brains of AD patients, has been the classic explanation of AD pathogenesis since early reports suggesting altered Aβ metabolism in AD. This is supported by genetic findings of mutations in the amyloid-β precursor protein (AβPP) on chromosome 21 in some AD patients, as well as the increased incidence of AD in individuals with trisomy 21 [53]. A complete review of the Aβ hypothesis is beyond the scope of review. However, to briefly summarize Aβ metabolism: 1) after synthesis of AβPP, it is cleaved into a C-terminal fragment by β- or α-secretase; 2) the C-terminal fragment is then cleaved to various forms of Aβ, most notably $Aβ_{40}$ and $Aβ_{42}$ [54]. $Aβ_{42}$ is considerably more hydrophobic than its counterpart and, despite being the less predominant form, has greater tendency to aggregate and form amyloid plaques [55]. Under normal circum-

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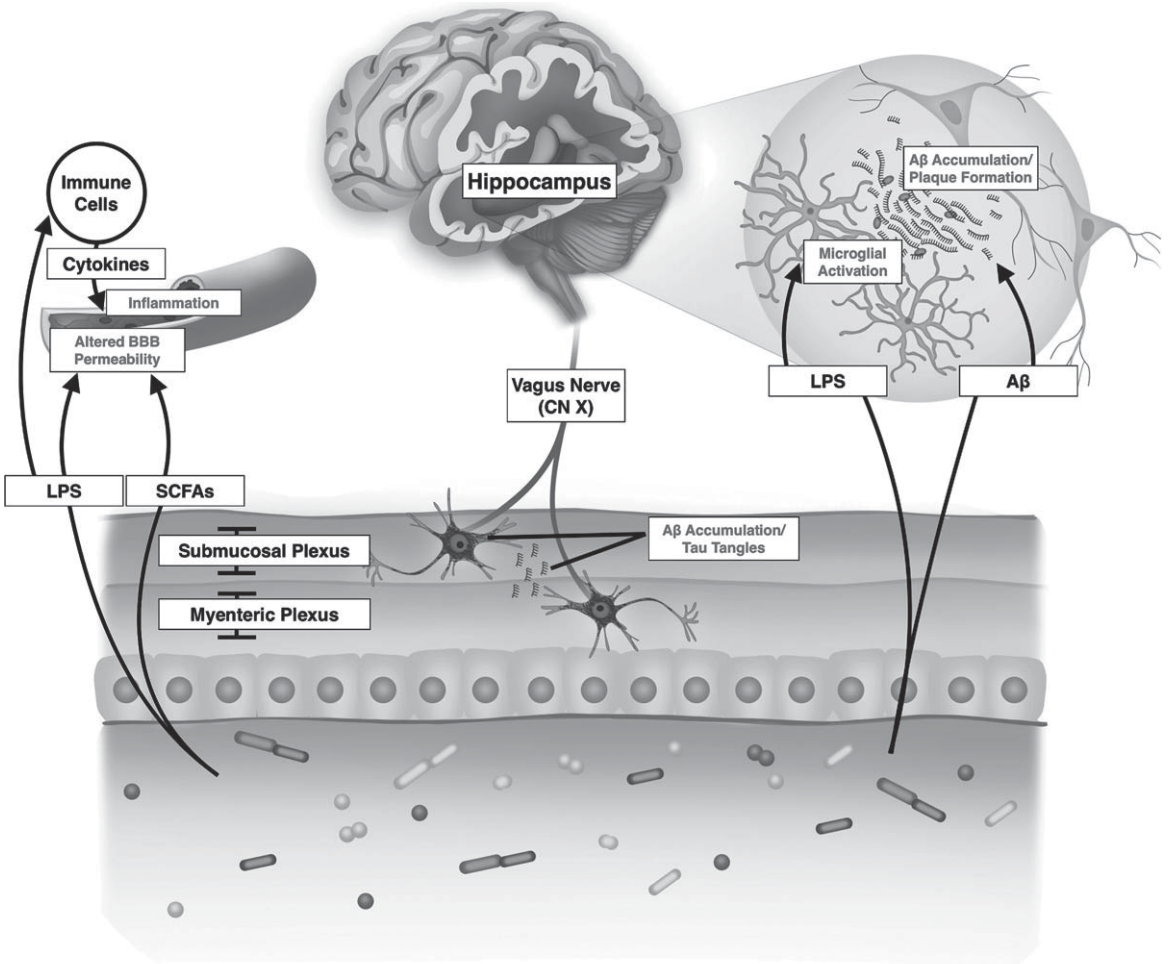


Fig. 2. Summary of bidirectional interactions between the brain, immune system, and gut microbiota via neuroendocrine, immune, and metabolic crosstalk.

stances, as in younger individuals, Aβ is rapidly degraded or removed preventing accumulation and plaque formation. However, with increased age or in pathologic states, the ability to clear these abnormal proteins is reduced [56]. The Aβ plaques are then thought to induce neuronal and synaptic damage, as well as stimulate inflammation via microglia, leading to the characteristic neurodegenerative and cognitive changes [57, 58].

Targeting Aβ, in an effort to prevent formation of neurotoxic plaques within the brain, has been a pharmacological strategy for treatment of AD for over 20 years. Compelling evidence from animal studies have demonstrated that removal of Aβ, typically through immunotherapeutic approaches such as immunization or antibody administration, prevents plaque formation and is correlated with alleviation of the associated cognitive decline [59, 60]. However, to date, there has been no phase III clinical trial that has demonstrated success in achieving primary outcomes using therapy targeting the Aβ pathway [61]. This track record of failure has drawn a great deal of criticism toward the Aβ hypothesis. While it remains a central focus of AD research, and must continue to be studied, there is a general consensus throughout the field that new ideas into the mechanisms and significance of Aβ deposition in AD must be considered. Emerging evidence from the brain-gut-microbiome axis may provide a link understanding Aβ metabolism in AD.

As described earlier, the brain-gut-microbiome axis has a "hard wire" connection in the form of the vagus nerve. This direct connection may permit retrograde neuronal transport of substances from the gut to

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the brain, bypassing the protection of the blood-brain barrier (BBB). This has recently been demonstrated in an animal model in which α-synuclein, the protein component of Lewy bodies that are hallmarks of PD, was transported within the vagus nerve from the gut to the dorsal motor nucleus within the brainstem in a mechanism dependent on microtubules [62]. This provides evidence for the spread of abnormal, potentially pathogenic, proteins via the ENS. While there is no direct evidence of this exact mechanism for Aβ, previous work has demonstrated that Aβ may spread between connected brain regions, thereby accumulating within nodes such as the hippocampal dentate gyrus. Lesioning the connections was sufficient to reduce intra-neurite AβPP accumulation and extracellular Aβ burden [63]. If this mechanism is clinically significant in the pathogenesis of AD, the microbiome may be a source of amyloid protein. Various studies have demonstrated that microbiota species secrete functional amyloid into the GI tract. These proteins were thought to play a role in evasion of the immune system, thus promoting pathogenicity of the secreting organism. For example, the protein Curli, secreted by *Enterobacteria,* including *E. Coli*, is a particularly well described bacterial amyloid which functions in formation of biofilms [64]. Exposure to Curli has recently been shown to enhance accumulation of α-synuclein within the gut and brain of *C. elegans* fed a diet rich in Curli-producing bacteria, suggesting that this bacterial amyloid protein may facilitate α-synuclein aggregation via cross-seeding and priming of the immune response [65]. Similarly, transport of bacterial amyloid and subsequent exposure in the brain may facilitate accumulation of Aβ in AD. In particular, Aβ is known to induce accelerated Aβ deposition and plaque formation in a prion-like seeding mechanism [66]. Recent work has demonstrated that cross-seeding, accelerated aggregation induced by an unrelated misfolded protein, occurs following inoculation of transgenic AD mice with prion protein [67]. As such, in AD, bacterial amyloid might act in a similar fashion to induce the aggregation of Aβ. Another mechanism underlying enhanced Aβ pathology in AD may be related to the release of metabolites from gut microbiome that alter production and/or deposition. For example, it was recently demonstrated that the bacterial metabolite trimethylamine-N-oxide (TMAO) increased with age in both wildtype and transgenic AD mice and that depletion of TMAO decreased hippocampal levels of $A\beta_{42}$ and β-secretase [68].

In addition to its production of amyloid protein that may be transported to the brain and facilitate subsequent Aβ aggregation, microbial factors may alter clearance of amyloid within the brain. Microglia, the brain's resident macrophages, play an important role in homeostatic clearance of Aβ, thus preventing accumulation in physiological states. This response can be stimulated by cytokines such as macrophage colony-stimulating factor (M-CSF), which promote the phagocytosis and lysosomal degradation of Aβ protein in animal models [69, 70]. While this pro-inflammatory response appears to have a protective role in AD pathogenesis, chronic inflammation may actually perpetuate Aβ aggregation. Qiao et al. demonstrated that long-term administration of the bacterial endotoxin LPS markedly accelerated deposition of Aβ [71]. Subsequent work has demonstrated that this may occur through impaired efflux of Aβ via LPS-induced reduction in cerebrospinal fluid, central and peripheral clearance of Aβ, and increased vascular sequestration of Aβ [72]. Closely related to Aβ plaque formation and clearance is hyperphosphorylation of tau protein which contributes to formation of neurofibrillary tangles, a hallmark of AD pathology. In a recent study, Wei et al. demonstrate that vesicles produced from the outer membrane of gut microbiota induce activity of glycogen synthase kinase 3β (GSK-3β) leading to tau phosphorylation and cognitive dysfunction. Notably, their methodology involved extracting these outer membrane vesicles (OMVs) from human AD patients' feces and injecting them into mice in comparison with OMVs from healthy controls, substantiating the clinical relevance of this pathway significantly [73].

Overall, there is significant support for the role of the microbiome in contributing to alterations in Aβ metabolism in AD, including via 1) direct production of amyloid proteins, which may accumulate or cross-seed, and 2) inhibition of mechanisms required for homeostatic Aβ clearance in the brain. While the role and significance of Aβ in AD pathogenesis is still yet to be elucidated, it is apparent that the microbiome may contribute to the neuronal damage of AD in an Aβ-dependent mechanism.

Microbial-mediated alteration of blood-brain barrier permeability and neuroinflammation

Given the lack of evidence supporting the Aβ hypothesis as the sole explanation for AD pathogenesis, more recent research has focused on other mechanisms that may contribute to neurodegener-

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ation in the AD brain. The BBB is present at the level of brain capillaries and is composed, structurally, of tight junctions between endothelial cells. Associated cells, including astrocytes, pericytes, and microglia contribute to integrity of the BBB via regulation of the endothelial barrier [74]. It has long been known that the BBB, which normally acts to protect the brain from exposure to harmful substances in blood (i.e., pathogens, inflammatory mediators, toxins, etc.) is disrupted in AD [75]. In fact, radiological evidence has identified leakage through the BBB in the early stages of AD, suggesting that disruption may contribute to the cascade of pathologic events characteristic of the disease process [76]. Various studies have demonstrated impairment of BBB integrity by Aβ protein in AD models. One study showed that administration of $A\beta_{42}$ to endothelial cells isolated from rat cerebral cortex altered the expression and cellular localization of tight junction proteins, notably promoting cytoplasmic translocation of claudin-5 and zona-occludens-2 [77]. Under normal circumstances, astrocytes contribute to integrity of the BBB by stimulating expression tight junction proteins by endothelial cells via secretion of factors from their characteristic endfeet [78]. However, secondary to β_{42}, astrocytes secrete matrix metalloprotease 9, leading to disruption of the BBB likely through degradation of claudin-5 in endothelial cells [79]. However, we must acknowledge that recent evidence challenges the conception of BBB disruption as a hallmark of AD. In particular, Bien-Lyn et al. demonstrated that impairment of BBB integrity was not observed in various mouse models of AD, including PS2-APP, tau transgenics, and APOE4 knockin mice. Importantly, they also observed no increase in the incidence of infarcts in human AD brains that may be suggestive of widespread BBB disruption [80]. Overall, we believe that there is sufficient evidence to support a role for BBB disruption in AD, although this may not be as widespread as previously thought.

Given the ability for Aβ to induce permeability of the BBB, it is reasonable to hypothesize that a similar effect may occur from secretion of bacterial amyloids. In fact, the concept of microbes and/or microbial factors inducing alteration of the BBB is not new. Some bacteria are capable of directly traversing the BBB, giving rise to CNS infection and/or abscess, via specialized components such as pilli, while others do so via stimulation of an inflammatory response [81]. Specifically, LPS is an endotoxin and pro-inflammatory agent produced by gram-negative bacteria that can potently stimulate BBB disruption in infectious states [82]. However, subclinical alterations in the gut microbiome can also result in variable BBB integrity. For example, in a study comparing GF and pathogen-free mice, it was found that GF mice displayed increased permeability of the BBB due to reduced expression of tight junction proteins occludin and claudin-5. Additionally, treatment of the GF mice with the pathogen-free microbiome rescued the BBB defect, resulting in increased tight junction protein expression and reduced permeability [83]. One mechanism by which altered microbiome composition may influence the BBB is differential secretion of factors by distinct strains of bacteria. LPS is typically associated with pathogenic strains, while commensal or normal flora produce SCFAs. Consistent with this, recent work showed that the bacterial metabolite propionate, an SCFA, protected the BBB from damage via inhibiting oxidative stress in an NFE2L2-dependent mechanism [84].

While this data confirms the ability of varied microbiota composition to influence permeability of the BBB, the significance of this in relation to the neurodegenerative changes characteristic of AD must also be considered. The BBB contributes to isolation of the brain from circulating amyloid protein, thus preventing accumulation of potentially pathogenic amyloid. In pathologic states in which the BBB is disrupted, however, circulating amyloid may enter the CNS. For example, using monkeys, Mackic et al. demonstrated that circulating Aβ can cross the BBB and contribute to formation of amyloid plaque aggregates consistent with AD pathology [85]. In fact, the mechanisms governing transport of Aβ appear to be highly regulated. Two transporters are responsible for cerebrovascular transport of Aβ: 1) low-density lipoprotein receptor related protein-1 (LRP1); and 2) receptor for advanced glycation end products (RAGE). The former is responsible for brain-to-blood efflux, while the latter facilitates blood-to-brain influx [86, 87] Implicating a role for microbial and inflammatory modulation of Aβ transport across the BBB, LPS has been shown to increase Aβ influx and decrease Aβ efflux via decreased expression of LRP1 [88]. This alteration can thus permit accumulation of Aβ, which may act as a seed for subsequent deposition and plaque formation [66]. Similarly, secreted bacterial amyloid in serum may now be able to enter the CNS via the blood, in addition to via the retrograde neural transport above described.

Until recently, the brain was considered to retain immune privilege, a term describing its apparent lack

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of exposure to the inflammatory milieu of circulating blood. In addition to the BBB, which isolates the brain from the periphery, the concept of immune privilege was derived from findings that there is a lack of professional antigen presenting cells (i.e., dendritic cells, macrophages) and low expression of MHC molecules within the CNS. However, in recent years, this concept has been brought to question by the discovery of a meningeal lymphatic system and the presence of mechanisms permitting immune cell entry into the CNS [89]. It seems that, rather than serving as rigid barrier to immune cell entry, the BBB acts in a regulated manner to selectively permit entry of inflammatory cells and mediators. As such, in inflammatory states, which tend to promote BBB disruption (i.e., systemic LPS), entry of inflammatory cells may be permitted [90]. In fact, such systemic inflammation has been associated with increased risk of developing an AD-like phenotype in mice exposed to the viral mimic polyriboinosinic-polyribocytidilic acid [91]. Inflammatory mediators such as complement proteins, chemokines/cytokines, radical oxygen species, and inflammatory enzymes are produced by the brain's resident immune cell, microglia, and have been observed in abundance within postmortem AD brains [92]. Oxidative stress has been extensively studied in neurodegenerative diseases, including AD, and the role for microglia in production of reactive oxygen species provides a potential mechanistic link between inflammation and neurodegeneration [93]. Furthermore, inflammatory infiltrate with bone-marrow derived microglia has been observed in transgenic mouse models of AD, suggesting a contribution of the peripheral immune system, although its role and significance in AD pathogenesis is still being elucidated [94]. Administration of LPS, a potent proinflammatory factor, resulted in increased levels of inflammatory factors, notably IL-1β, IL-6, and TNF-α, as well as Aβ within the hippocampus of rats [95]. Implicating a role for the microbiome in initiation of an innate immune response in AD, Zhao et al. recently reported immunohistochemical evidence of microbiome derived LPS within the perinuclear region of human AD brains. This data suggests that LPS secreted by gram-negative bacilli of the microbiome may leak across an impaired BBB and accumulate within the brain, stimulating an inflammatory response and possible degradation [96].

The role of the microglia in AD pathogenesis has been more widely explored and reported. Original hypotheses suggest that the inflammation seen within the AD brain is merely a secondary response triggered by the neuronal death and degeneration pathologically occurring due to the disease process. However, more recent work indicates that inflammation may be an important driver of AD pathology. As described earlier, microglia contribute to Aβ clearance via phagocytosis and degradation of the abnormal $A\beta_{42}$ protein [69, 97]. Microglia are described as having two activated states: the M1, or classical, is the proinflammatory state characterized by production of neurotoxic proinflammatory mediators; the M2, or alternative, state inhibits inflammation and promotes tissue repair [98]. Stimulation of the M2 state is also associated with enhanced clearance of Aβ and reduced cognitive deficit in an animal model of AD [99]. However, as with most cases of inflammation, microglia act as a double-edged sword. Microglia may be activated into the M1 state by binding of extracellular $A\beta_{42}$ protein to the TLR2 receptor with subsequent release of the proinflammatory mediator IL-8 and associated neuronal damage. Further, deficiency of TLR2 promotes a shift of the microglia to the M2 state and ameliorates the damage [100]. Interestingly, mutations in myeloid cell molecules CD33, a regulator of innate immunity, and TREM2, an innate immune receptor, have been reported to increase risk of AD [101, 102]. Interaction between the microbiome and microglia is considered an important link in the brain-gut-microbiome axis [103]. Microglia are macrophages that localize to the brain during embryogenesis prior to formation of the BBB. Recently, it has been shown that GF mice display altered patterns of microglial development in a sexual dimorphic and, further, treatment of adult mice with antibiotics produce sex-specific perturbations [104]. This suggests that microbial alterations throughout the lifespan may predispose individuals to altered microglial responses and may be important in the pathogenesis of a variety of neurological diseases, including AD. The mechanism for microbial influence on microglial development and function is likely through the secretion of metabolites, including LPS and SCFAs. Supporting this, microglia from mice deficient for the SCFA receptor FFAR2 display a similar immature phenotype as those obtain from GF mice [105]. One mechanism by which microbial dysbiosis may alter microglial activation in the AD brain is through modulation of peripheral immune cells. Supporting this, a recent report demonstrated that alteration of the gut microbiome in a mouse model of AD led to peripheral accumulation of the amino acids phenylalanine and isoleucine,

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which are potent stimulators of differentiation and proliferation of pro-inflammatory T-helper 1 (Th1) cells. These cells can then activate microglia into the M1 phenotype above described. Supporting the clinical relevance of this finding, elevated phenylalanine and isoleucine levels were associated with increased Th1 levels in patients with mild cognitive impairment and treatment with a sodium oligomannate that is currently in Chinese clinical trials attenuated neuroinflammation and reduced associated cognitive decline [106].

Overall, the inflammatory contribution to AD appears to be significant and warrants further study. The impact of the microbiome and microbial factors on the inflammatory cascade is particularly interesting in this respect, as it may provide a foundation for understanding variability in the progression of AD across diverse populations as it relates to exposure, comorbid inflammatory disease, antibiotic use, diet, etc.

Regulation of adult hippocampal neurogenesis

The conventional dogma has long been that development of new neurons is confined to the embryonic and perinatal periods and does not occur in the adult brain. However, this has been largely refuted by the anatomic and molecular findings of life-long neurogenesis within the hippocampus of mammals, including humans [107–109]. Despite the recent report by Sorrells et al. that hippocampal neurogenesis occurs rarely, if at all, past adolescence [110], there remains a great deal of support that neurogenesis proceeds throughout the life span. Most recently, three separate studies demonstrated that neurogenesis, while declining with age, occurs as late as the tenth decade of life, and reduction in neuroblasts and immature neurons is associated with cognitive decline and/or neuropsychiatric/AD [111–113]. This correlates with evidence from animal models that has demonstrated decreased levels of neurogenesis in the pathogenesis of AD, in some cases even occurring prior to the onset of the classical AD pathology [114, 115].

These data suggest that alterations of neurogenesis are critical, potentially even causal events in the development and progression of AD. In accordance with the Aβ hypothesis, soluble Aβ has been shown to impair neuronal proliferation, while oligomeric Aβ promotes the formation of microglia [116]. Brain-derived neurotrophic factor (BDNF) is a growth factor produced in the brain that acts as a key mediator of adult neurogenesis and, notably, facilitates exercise-induced neurogenesis [117]. Levels of BDNF have been observed to be decreased in the brain, blood, and cerebrospinal fluid of individuals with AD; in fact, lower levels of BDNF even correlate with cognitive ability as assessed in the Mini-Mental Status Examination [118]. The mechanism of BDNF reduction may be dependent on exposure to Aβ, as Aβ is capable of reducing mRNA transcripts of BDNF in human neuroblastoma cells [119]. Alterations in the intestinal microbiota are also associated with variation in hippocampal levels of BDNF, with GF mice demonstrating reduced BDNF and exploratory behavior that is rescued following colonization with microbiota from SPF mice [120]. With the known ability of bacteria to produce LPS and amyloids, it is possible that these molecules may mitigate a reduction in BDNF in a manner similar to that of Aβ. Utilizing antibiotic treatment, Möhle et al. showed that alteration in the gut microbiota reduced hippocampal neurogenesis and memory retention, which could be rescued by reconstitution with SPF microbiota, supplementation of probiotics, and BDNF. Interestingly, depletion of $Ly6C^{hi}$ monocytes abrogated the rescue effect of these measures suggesting that this population of monocytes is critical in preservation of brain homeostasis [121]. Macrophages derived from $Ly6C^{hi}$ monocytes have previously been shown to promote functional recovery following stroke in mice [122], and this data may provide a link between microbial alteration and neurogenesis that is crucial to the pathogenesis of AD.

The impact of the microbiome on neurogenesis is critical, not only to AD pathogenesis, but to the regulation of behavior in physiologic settings. More research in this topic is certainly warranted, especially in the context of antibiotic use and its impact on the flora.

CLINICAL CORRELATIONS AND POTENTIAL THERAPEUTIC APPROACHES

The majority of the above described work is taken from *in vitro* or *in vivo* animal studies, which are important, but may not correspond completely to patients at the bedside. As such, we will briefly discuss clinical work that supports the notion that the microbiome is altered in patients with AD. First off, the natural biodiversity of microbiota is well

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known to decline with increased age, with a relative reduction in commensal species, such as *bacteroides*, *bifidobacteria*, and *lactobacilli* and relative increase in opportunists/potentially pathogenic species such as *enterobacteria*, *C. perfringens*, and *C. difficile* [123]. The variation in microbial composition is also dependent on diet and lifestyle; for example, among the elderly, individuals residing in long-term care facilities maintain a microbiome that is significantly different from those living at home [124]. It is likely that the effects are exerted through changes in production of bacterial metabolites, such as LPS and SCFAs. Variation in microbiota provides an additional source of background variation, in addition to genetics and exposures, that may account for the variable courses seen among a multitude of diseases, including AD. Even in familial cases of AD, which are characterized by younger age of onset and typically heightened severity, there remains a considerable deal of variation in the progression that is not accounted for in current models [125]. Background differences in the microbiome, and its influence on Aβ metabolism, barrier permeability, inflammation, and neurogenesis may help provide an understanding of the variable disease course. Perhaps one of the most significant findings in human samples supporting a microbial hypothesis in AD is the finding of a large bacterial load within the cerebral cortex of AD patients using 16 s ribosomal next generation sequencing, a technique considered state-of-the-art for microbiome analysis. In particular, the authors reported enrichment of *Firmicutes* species and *P. acnes* [126]. To the best of our knowledge, there is limited data regarding the variations in the microbial composition of AD patients, with only one study reporting significant differences in gut microbial genotypes of AD patients and controls [127].

With the evidence supporting microbial alteration, an obvious question is: Can the microbiome be targeted in AD therapy? Probiotics, defined as live microbial food ingredients that exert beneficial effects on health, are most notably used in the treatment of GI disease processes including lactose intolerance, diarrhea, and antibiotic-induced side effects. However, future use in other pathologies is promising based on current research [128]. Probiotics have been used with benefit to alleviate stress and improve cognitive function in adults experiencing stress, suggesting a potential use in neurocognitive and/or psychiatric disease [129, 130]. In the setting of AD, probiotics have been used limitedly, with most studies evaluating the effect in animal models. One renowned example is the work of Bonfili et al. which demonstrated that oral supplementation with the probiotic formulation SLAB51 resulted in altered microbiome composition, as assessed by 16 S ribosomal RNA sequencing, as well as reduced inflammatory cytokines. Most notably, this probiotic actually attenuated cognitive decline via reduced Aβ aggregation and restoration of neuronal proteolytic pathways [131]. More recently, our laboratory demonstrated that a novel probiotic formulation (BIOCG) displayed similar neuroprotective effects in a transgenic mouse model of AD through several processes including attenuation of microglial activation, reduction in Aβ load, and preservation of dendritic spine structure and function. This work, which is currently unpublished and in submission, is to our knowledge the first evidence that probiotic administration, and subsequent alteration in microbial composition can alter neuronal physiology via improved long-term potentiation and preserved synaptic spine morphology in a mouse model of AD. As described, clinical data regarding probiotic administration to AD patients is limited. One small exploratory study demonstrated that supplementation with a multispecies probiotic influenced serum tryptophan metabolism, an important modulator of neurotransmitter synthesis which may be disrupted in AD [132]. A larger randomized controlled trial showed that consumption of milk containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* produced significant improvement in Mini-Mental Status Examination scores and improvement on various metabolic measures [133]. This is particularly significant as recent evidence supports the association between metabolic diseases, such as obesity and type 2 diabetes, with the occurrence of AD. In particular, a recent publication has demonstrated that diet-induced perturbation in the gut microbiome alters the shikimate pathway leading to elevated levels of the cytotoxic amine tryptamine [134]. These studies demonstrate that probiotics may be helpful supplements for symptomatic control in AD and management of comorbid metabolic disease. Future research identifying specific strains that are altered in AD or may exert beneficial effects is warranted. At present, there are two registered clinical trials seeking to assess the effect of probiotic supplementation on AD or mild cognitive impairment, one of which is currently recruiting patients.

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CONCLUSION AND FUTURE DIRECTIONS

Overall, our review provides a great deal of evidence, in both preclinical and clinical studies supporting the role of the brain-gut-microbiome axis in AD. A summary of the above described interactions is provided in Fig. 2. Future directions for clinical work include utilizing sequencing to identify AD-specific alterations in the gut microbiome that may provide insight into the mechanisms by which alteration influences AD and potential therapeutic targets. Additionally, large-scale analyses correlating microbial diversity with cognitive status and disease progression in patients with AD may provide helpful prognostic data.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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"Muscle-Gut-Brain Axis": Can Physical Activity Help Patients with Alzheimer's Disease Due to Microbiome Modulation?

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Abstract. Alzheimer's disease (AD) is one of the most common forms of dementia, which cannot be cured at the moment. Therefore, researchers also look for the alternative approaches to its treatment. It is suggested that changes in human gut microbiome mediated by exercise could influence the development and progression of AD and a new term "muscle-gut-brain axis" is introduced. There is much evidence to support this assumption. The gut microbiology is closely related to a wide range of diseases of the nervous system and therefore any negative qualitative and quantitative changes in the composition of the gut microbiota can potentially contribute to the pathophysiology of AD. Research shows that the treatment of intestinal dysbiosis with probiotics/synbiotics/eubiotics can prevent or alleviate the symptoms of these chronic neurological diseases. Studies also point to the positive effects of movement on the health of seniors. A positive correlation can be found between cognitive functions and physical stress, both in the elderly and in AD patients. Even short-term interventions with a relatively low frequency seem to produce positive results, while physical activities can be performed by using relatively simple and cost-effective means. In addition, physical activity can significantly modulate gut microbiome. Thus, it can be concluded that physical activity in humans seems to correlate with gut microbiome, which can prevent the incidence and development of AD.

Keywords: Alzheimer's disease, cognitive disorders, dementia, exercise, microbiome, physical activity, probiotics

INTRODUCTION

Alzheimer's disease (AD) is one of the most common forms of dementia, which cannot be cured at the moment. AD accounts for 60–80% of all dementia cases [1]. It damages neurons and their connections in parts of the brain involved in memory. Thus, the one of the earliest symptoms of AD is memory loss—a sign of cognitive decline, which manifests itself especially in forgetting recently learned information [2]. Later on, other symptoms develop, such as language impairments, apathy, depression, delusions, hallucinations, aggression, and eating, walking, and sleeping problems, which eventually results in inability to execute the tasks of daily living [3].

Unfortunately, due to the changing demographic trends, the epidemiology of AD is growing and AD represents a serious social, economic, and health problem. Currently, there are about 50 million people

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living with AD. However, it is predicted that by 2050 this number should reach 152 million [4]. Although the researchers all over the world attempt to find new drugs for the treatment of AD, so far only four have been approved and used. These include cholinesterase inhibitors, donepezil, rivastigmine, and galantamine, and the NMDA-glutamatergic receptor antagonist, memantine.

The drugs only delay or maintain cognitive decline in patients with AD for some time. In addition, the state of health of most of the patients in a more advanced stage requires professional care, which burdens national budgets. For example, in Europe in 2050, the direct medical costs of care are estimated to be 6,716 mil EUR, with the direct non-medical costs of care at 14,236 mil EUR [5]. Therefore, researchers look for the alternative approaches to the treatment of AD. One of these approaches include physical activities, which are part of a healthy lifestyle, one of the modifying risk factors of AD [3]. Research [6] indicates that physical activities have a positive effect on patients' mental function and the development of dementia. In fact, a lack of physical activity is associated with 20% of AD cases in the UK, USA, and Europe [7].

Thus, the purpose of this article is to explore the correlation between gut microbiome and physical activity/exercise in the development and progression of AD.

METHODS

The methodology is based on a literature review, which was conducted in three main databases: Web of Science, Scopus, and PubMed. The end of the search period is limited by April 2019. The collocated keywords were as follows: microbiome AND Alzheimer's disease, physical activity AND microbiome, exercise AND microbiome, microbiome AND cognition, gut-brain axis and Alzheimer's disease, exercise AND Alzheimer's disease, physical activity AND Alzheimer's disease, physical activity AND cognition, exercise AND cognition. The keywords were combined and integrated in database and journal searches. The terms used were searched using AND to combine the keywords listed and using OR to remove search duplication where possible. The backward search was also conducted, i.e., references of retrieved articles were assessed for relevant articles that authors' searches may have missed.

Altogether 2,721 articles were identified in all these databases. After removing duplicates and titles/abstracts unrelated to the research topic, 435 English-written studies remained. Of these, only 240 articles were relevant for the research topic. These studies were investigated in full and they were considered against the following inclusion and exclusion criteria.

The inclusion criteria were as follows: the period of the publishing of the article was limited by the end search, April 2019; reviewed full-text studies in scientific journals in English were only included; the primary outcomes focused on the correlation of: 1) microbiome and Alzheimer's disease, 2) Alzheimer's disease and physical activity/exercise, and 3) physical activity/exercise and microbiome.

The exclusion criteria were as follows: review and summary articles, meta-analyses, conference abstracts; descriptive studies not including patients with AD; animal studies.

Based on these criteria, 72 studies were included in the final analysis. Figure 1 illustrates the selection procedure.

"Muscle-gut-brain axis": fiction or a new possible term in sport medicine and neurology?

The human gut microbiome consists of billions of cells and its genetic information exceeds host information by about 150 times. This complex biomass may play a role in the emergence and progression of a variety of disorders and diseases, even outside the gastrointestinal system [8]. The microbiome communicates with the brain through the so-called gut-brain axis. This means that on the one hand, the brain can affect the intestines by interfering with their motility, secretion, blood flow, or affect the intestinal immune response. Triggering stimuli can be both psychological (e.g., stress) and physical (e.g., toxins). On the other hand, microbiome through neuronal, immune, metabolic, and endocrine signaling pathways can modulate the brain. Everything is done through the conjunction—the nerve vagus [9, 10]. Factors such as aging, nutrition, and physical exercise have been shown to modify microbes in both a positive and negative sense [11].

AD is one of the most common neurodegenerative diseases of the elderly and leads to irreversible cognitive defect and dementia. A typical feature of AD is the proliferation and deposition of amyloid-β in the brain along with the local inflammatory response at the site of storage [12]. Microbiome seems to be

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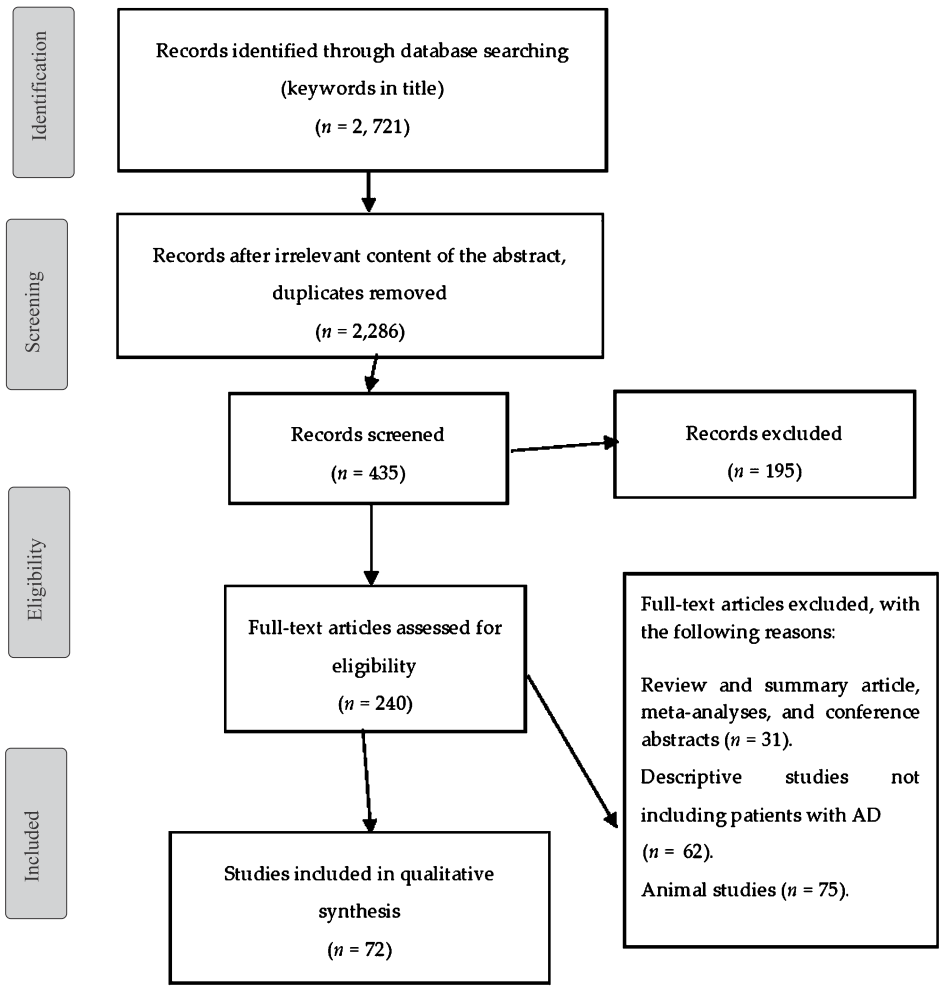


Fig. 1. An overview of the selection procedure.

partly associated with this accumulation, since for many microorganisms of human intestinal content, the formation of proper functional amyloid proteins is typical, including those found in the brain of AD patients [9, 12]. Short-chain fatty acids (SCFAs) have also been shown to be involved in animal models [13].

"Muscle-gut-brain axis"? Based on available data, it is considered that physical activity may reduce AD incidence [14, 15]. Microbiome has been reported to be associated with AD, and exercise is believed to positively influence microbiome [16, 17]. However, there has been no research study yet describing that the changes in microbiome generated by exercises would affect AD. In addition, the term "muscle-gut-brain axis" has not been used in the literature yet. This review seeks to bring an overview of current information.

Microbiome and Alzheimer's disease

Since gut microbiome is closely related to nervous system disease, negative qualitative and quantitative changes in the composition of gut microbiota may potentially contribute to the pathophysiology of AD. For the sake of completeness, it should be noted that the literature refers to the association of human intestinal microorganisms and a variety of neuro(auto)-immune, neurodegenerative, or psychiatric diseases such as multiple sclerosis, neuromyelitis optica spectrum disorders, Parkinson's disease, amyotrophic lateral sclerosis, depression, anxiety, schizophrenia, and autism [18]. Interestingly, neurodegenerative diseases, including AD, have a high rate of gastrointestinal comorbidities, and therefore there is an assumption that

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the treatment of intestinal dysbiosis with probiotics/synbiotics/eubiotics could prevent or alleviate the symptoms of these chronic diseases [19]. However, the views on this issue are not very uniform.

Two recent studies by Paley et al. have been of great interest to intestinal microbioma and AD [20, 21]. The studies link neurodegeneration and cell death induced by tryptophan-derived tryptamine with the presence of gut bacterial Na(+)-transporting NADH:ubiquinone reductase sequence associated with AD. Tryptophan-derived tryptamine presents in human diet and gut microbiome. Tryptamine inhibits tryptophanyl-tRNA synthetase with consequent neurodegeneration in cell and animal models. Thus, gut microbial tryptamine overproduction correlates with AD-associated sequence occurrence. Antibiotic and diet additives induce AD-associated sequence and tryptamine.

An overview of the studies focusing on microbiota and AD is presented in Table 1 (postmortem brain samples) and Table 2 (living subjects).

Alzheimer's disease and physical activity

The issue of the links between the physical activities of man and his health condition is a very hot topic nowadays. Westernization (inappropriate eating habits, excess energy intake, and lifestyle without enough physical activity) is a huge challenge for medical research. Westernization affects all age categories and does not avoid people with disabilities. Like other specific groups, patients with AD also deserve attention and care in connection with their movement mode.

Table 3 below shows a list of studies using aerobic load within the intervention. It contains basic research information including main outcomes. In almost all cases, the detected studies were randomized control trials.

The interventions using force load as a means of working with AD patients are shown in Table 4. The description of the load method differs in the studies—exercises, loads, or repetitions are described in various ways by the authors. This is not surprising because it is not easy to define the exact form of the exercise for such a specific target group, such as AD patients. The balance exercise was included among the strength exercises.

Table 5 lists research studies that used a mixed load in physical activities. The table provides a description of the exercise or the load intensity. In all investigations, a control group was also used, including patients with AD.

A list of studies using alternative approaches is shown in Table 6. These are interventions that differ from commonly used approaches. However, research design was standard, containing control groups. In two research studies, the authors used a case study, which corresponds to the purpose of a given study.

Physical activity and microbiome

In terms of gastroenterological indications, the protective effect of targeted physical activity in humans is associated with a reduction in the risk of gastrointestinal malignancies, gastroesophageal reflux and gastric/duodenal ulcers, liver steatosis, irritable bowel syndrome, and diverticulitis [75].

A number of animal model studies have been reported to indicate that qualitative and quantitative changes in the intestinal flora, under targeted physical activity conditions, can be linked to influencing nutrient absorption, energy distribution, immunity, and gut-brain axis effect [76]. The opposite situation is with experiments on humans. There is an insufficient number of adequately designed controlled trials leading to a difficult assessment of the effect of physical activity on the composition and function of the human intestinal microflora. Interactions such as genetics, nutrition, exercise, individual habits with the environment in which the individual is located also contribute to the possible distortion in data interpretation.

Table 7 provides an overview of individual studies dealing with the effect of physical load/ exercise on gut microbiota.

Considering the results from animal studies, one can reasonably believe that exercise could affect the gut microbiome. This means that exercise and physical activity may induce positive qualitative and quantitative changes in the human gut microbiota, which may appropriately interfere with the onset and development of AD.

DISCUSSION

As it has been mentioned in the introduction, changes in the composition of human microbiome may be associated with the development of a number of gastrointestinal or metabolic diseases, but may also affect central nervous system function. Research reveals that gut microorganisms act on the "gut-brain" axis. The development of the nervous system is a continuous complex process that begins before birth

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Table 1
An overview of the studies focusing on microbiota and Alzheimer's disease: Postmortem samples

Study	N	Pathogen	Main outcome
Hammond et al. [22]	5/5	Chlamydia pneumoniae	Chlamydia pneumoniae antigens + amyloid deposits, and neurofibrillary tangles were present in frontal and temporal cortices of the AD brain.
Poole et al. [23]	10/10	Treponema denticola, Tannerella forsythia, Porphyromonas gingivalis	LPS from periodontal bacteria Porphyromonas gingivalis can access the AD brain during life.
Miklossy et al. [24]	10/4	Borrelia burgdorferi	Aβ and spirochete specific DNA are key components of both pure spirochetal biofilms and senile plaques in AD.
Zhan et al. [25]	24/18	Escherichia coli K99	EC K99 levels were greater in AD compared to control brains; EC K99 was localized to neuron-like cells in AD but not control brains; LPS levels were also greater in AD compared to control brain; LPS colocalized with $A\beta_{1\text{-}40/42}$ in amyloid plaques and with $A\beta_{1\text{-}40/42}$ around vessels in AD brains.
Emery et al. [26]	14/12	Mixture of bacterial populations	Data strongly suggest that AD brains tend to have strikingly large bacterial loads compared to controls - species associated with skin, nasopharyngeal and oral areas such as Firmicutes and most consistently Actinobacteria, especially Propionibacterium acnes (up to 94% of Actinobacteria) are responsible for this.
Zhao et al. [27]	10/8	Bacteroides fragilis, Escherichia coli	Presence bacterial LPS in brain lysates from the hippocampus and superior temporal lobe neocortex of AD brains; mean LPS levels varied from two-fold increases in the neocortex to three-fold increases in the hippocampus, AD over age-matched controls.
Zhao et al. [28]	7/7	N/A	Major source of proinflammatory signals in AD brain may originate from internally derived noxious exudates of the GI-tract microbiome; due to aging, vascular deficits or degenerative disease these neurotoxic molecules may "leak" into the systemic circulation, cerebral vasculature, and on into the brain; this internal source of microbiome-derived neurotoxins may play a particularly strong role in shaping the human immune system and contributing to neural degeneration, particularly in the aging CNS.
Zhao et al. [29]	15/12	N/A	LPS was accumulated in neocortical neurons of AD brain and impaired transcription in human neuronal-glial primary co-cultures.

N, number of subjects (AD/control); LPS, lipopolysaccharide; Aβ, amyloid-beta; GI, gastrointestinal.

and ends in death and is also partly dependent on signals coming from the gut environment, specifically signals from microorganisms. Microbiome and its metabolites are both involved in neuroinflammation but also regulate processes such as blood-brain barrier formation, myelination, neurogenesis, or microglia maturation [84–89]. Microglia maturation has been shown to be dependent on sufficient SCFA and SCFA is produced by gut microorganisms [90]. Furthermore, if the blood-brain barrier formation is affected during its development in terms of its greater permeability, pathogen penetration into the brain and neuroinflammation can be expected [84, 85].

Metabolites and molecules produced by intestinal microorganisms also play a very important role. It is known that γ-aminobutyric acid (GABA) is a major human inhibitory neurotransmitter and is produced by strains of Lactobacillus and Bifidobacterium. GABA is also involved in progenitor neuronal proliferation, synapse formation, and suppression of inflammatory reactions in the body [91, 92]. Another important molecule affected by the gut microbiota is serotonin (5-HT). Up to 95% of the total serotonin in the body is found in the gastrointestinal tract [93]. 5-HT plays an irreplaceable role in the motility function of GIT, but also affects appetite, mood, sleep and cognitive function. Microorganisms are thought to indirectly stimulate cells in the intestine to store and release 5-HT [94].

Moreover, a very specific group are metabolites, the so-called SCFA, including acetic, propionic, butyric, and lactic acids. In addition to being the major energy source of colonocytes (epithelial cells of the colon mucosa), butyrate also induces sprouting dendrites, increases the amount of neurosynapsis, supports learning processes and long-term memory. Butyrate contributes directly to increased motility in the intestine, while propionate reduces bowel motility and facilitates secretion there [95, 96].

Gut microbiome is also very closely associated with systemic inflammatory changes. Microbes stimulates epithelial cells and lymphatic tissue of the intestinal mucosa, releasing amyloid proteins. Microbial dysbiosis and increased intestinal permeability

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Table 2
An overview of the studies focusing on microbiota and Alzheimer's disease: Living subjects.

Study	N	Pathogen	Main outcome
Kountouras et al. [30]	50/30	Helicobacter pylori	Histologic prevalence of HP was 88% in patients with AD and 46.7% in controls.
Kountouras et al. [31]	27/27	Helicobacter pylori	HP-specific IgG antibody levels are significantly increased in cerebrospinal fluid and serum of AD and its titer in cerebrospinal fluid might reflect the AD severity.
Zhang et al. [32]	18/23/18*	endotoxin / LPS	Elevated levels of endotoxin/LPS concentrations in plasma from patients with sporadic amyotrophic lateral sclerosis and AD as compared to healthy controls; levels of plasma LPS showed a significant positive correlation with degree of blood monocyte/macrophage activation in disease groups.
Roubaud-Baudron et al. [33]	53/0	Helicobacter pylori	AD patients infected by HP tended to be more cognitively impaired.
Sparks Stein et al. [34]	35/46/77**	Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Campylobacter rectus, Treponema denticola, Fusobacterium nucleatum, Tannerella forsythia, Prevotella intermedia	Elevated antibodies to periodontal disease bacteria (F. nucleatum and P. intermedia) in subjects years prior cognitive impairment; it suggests that periodontal disease could potentially contribute to the risk of AD onset/progression.
Noble et al. [35]	110/109	Porphyromonas gingivalis, Tannerella forsythia, Actinobacillus actinomycetemcomitans, Treponema denticola, Campylobacter rectus, Eubacterium nodatum, Actinomyces naeslundii	Serum IgG levels to common periodontal microbiota are associated with risk for developing incident AD; high anti-A. naeslundii titer was associated with increased risk of AD; high anti-E. nodatum IgG was associated with lower risk of AD.
Kamer et al. [36]	0/38	N/A	History of periodontal inflammatory/infectious burden, was associated with increased 11C-PIB uptake in Aβ vulnerable brain regions; association between periodontal disease and brain Aβ load; peripheral inflammation/infections are sufficient to produce brain Aβ accumulations.
Akbari et al. [37]	60	Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, Lactobacillus fermentum	After 12 weeks intervention, compared with the control group, the probiotic treated AD patients showed a significant improvement in the MMSE score; positively affects cognitive function and some metabolic statuses in the AD patients.
Cattaneo et al. 60 [38]	40/33/10***	Escherichia/Shigella, Pseudomonas aeruginosa, Eubacterium rectale, Eubacterium hallii, Faecalibacterium prausnitzii, Bacteroides fragilis	Increase in the abundance of a pro-inflammatory Escherichia/Shigella taxon; reduction in the abundance of an anti-inflammatory Eubacterium rectale; this is probably associated with a peripheral inflammatory state in patients with cognitive impairment.
Vogt et al. 95 [39]	25/25	mixture of bacterial populations	Decreased Firmicutes and Bifidobacterium, increased Bacteroidetes in the microbiome of AD participants; correlations between levels of differentially abundant genera and cerebrospinal fluid biomarkers of AD.

N, number of subjects (AD/control); HP, Helicobacter pylori; LPS, lipopolysaccharide; PIB, Pittsburgh compound B (image amyloid-β plaques in neuronal tissue); MMSE, Mini-Mental State Examination Score; *AD patients/sporadic amyotrophic lateral sclerosis patients/healthy control; **AD patients/patients with mild-cognitive impairment/healthy control; ***cognitively impaired patients with brain amyloidosis/ cognitively impaired patients without brain amyloidosis/healthy control.

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Table 3
Aerobic load

Study	N	Age	Duration	Frequency	Intervention	Main outcome
Yu et al. [40]	N/A	60+	6M	3x W, 15–45 min	60–75% HR, bicycle; without CG	Improvement of QOL and of overall cognitive functions.
Vidoni et al. [41]	N/A	55+	182D	N/A	1. treadmill, 40–75% VO2max; 2. stretching and toning exercises, exercises with a large ball, yoga	Pilot study, without any specific results.
Venturelli et al. [42]	21	84 ± 5	6M	4x W, 30 min	30 min of walking, medium intensity	↑6 min walking test; index ADL; ↓MMSE (fewer than in CG).
Tappen et al. [43]	65	87	112D	3x W, 30 min	1. walking; 2. conversation; 3. walking, conversation	↑6 min walking test (3. best).
Sobol et al. [44]	162	N/A	112D	3x W, 1 h.	3×10 min 70–80% HR max	0 chair stand test (30 s), 400 m walk test, up-go-test; ↑VO2max, dual task.
Hoffmann et al. [45]	200	N/A	112D	3x W, 1h	3×10 min 70–80% HR max	0 difference in cognitive tests; ↓neuropsychiatric symptoms.
Friedman & Tappen [46]	30	N/A	70D	3x W, 1h	1. 30 min walking, conversation; 2. conversation	↑MMSE, communicative skills.
Frederiksen et al. [47]	8	N/A	98D	3x W, 1h	3×8 min (50–80% HRmax), bicycle, cross trainer, treadmill	↑power outputs (on machines); hand grip strength, aerobic fitness; ↓chair test (30 s).
Cott et al. [48]	74	82 ± 8	112D	5x W, 30 min	1. walking with communication; 2. talks	↑2 min walking, mental and communicative tests, 2. group performed best; 0 almost in all 1. versus CG
Arcoverde et al. [49]	20	N/A	112D	2x W, 30 min	10 min 40%, 20 min 60% VO2max, 5 min stretching	↑Cambridge cognitive examination, Berg balance test, up-and-go test 0 dual task.
Palleschi et al. [50]*	15	74 ± 1.5	3M	3x W, 20 min	bicycle (70% HRmax)	
without CG	↑MMSE, verbal tests.					
Rolland et al. [51]*	23	N/A	49D	N/A	Aerobic exercises 35 min (walking, bicycle)	↑MMSE, reduction of the risk of falls, behavioral problems 0 ADL.

N, number of patients; M, Month; D, Days; W, weekly; h, hour; HR, heart rate; CG, control group; QOL, quality of life; MMSE, Mini-Mental State Exam; ADL, Activities of Daily Living; ↑, improvement; 0, without any difference when compared with the control group; *nutrition was taken into account.

may induce an inflammatory response in the body by increasing IL-6 levels. This molecule is regularly found in the serum and brain of AD patients [97].

Experiments can be found in the literature where certain gut microorganisms have been associated with the occurrence or development of AD. Some of the studies have been performed on post-mortem samples in AD patients, which in part reduces the plausibility of their causality in the pathophysiology of dementia. The pathogens described include Chlamydia pneumonia [22], Borrelia burgdorferi [24], and Helicobacter pylori (HP) [30, 31]. HP positive patients with AD had lower Mini-Mental State Examination (MMSE) scores, which corresponded to more severe cognitive disorders, and moreover, the degree of disease severity was determined by the titer of significantly elevated levels of HP-specific IgG antibodies found in cerebrospinal fluid and serum [30, 31]. The way bacteria enter the brain of AD patients is still under investigation, but one theory suggests that the permeability of the blood-brain barrier may be increased [98]. Since higher levels of antibodies to periodontitis-causing bacteria have been found in serum in AD patients, another possible theory for entry of microorganisms into the CNS pathway is through the olfactory bulb [34, 35, 99].

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Table 4
Resistance/force load

Study	N	Age	Duration	Frequency	Intervention	Main outcome
Vital et al. [52]	34	N/A	112D	3x W, 1 h	1. exercises on machines (peck deck, leg press, curls), 20 repetitions; 2. walking, talks, relaxation, music	0 differences in cognitive tests (clock drawing test, Brief cognitive battery, Verbal fluency test).
Vidoni et al. [41]	N/A	55+	182D	N/A	1. treadmill, 40–75% VO2max; 2. Stretching and toning exercises, exercises with a big ball, yoga	Pilot study, without any specific results.
Roach et al. [53]	82	N/A	84D	5x W, 1 h	1. power exercises (7–9 repetitions), stand up, push/pull against the nurse, balance exercises, dance positions; 2. walking; 3. talks	The best results in group 1, ↑6 min walking test, Acute care index of function, MMSE.
Lindenmuth & Moose [54]	43	65–98	56D	daily	Exercises while sitting – rotation, isotonic, breathing exercise	↑Cognitive tests.
Garuffi et al. [55]	34	N/A	112D	3x W, 1 h	1. exercises on machines, with dumbbells (3×20 repetitions); 2. social activities	↑ Dynamic balance, walking up the stairs, putting on socks, moving around the house.
Francese et al. [56]	12	N/A	49D	3x W, 20 min	1. exercise with the ball, squeezing the balloon, load on legs; 2. watching videos	↑Physical therapy assessment (power tests), ADL.
Ahn & Kim [57]	23	74.2 ± 6.1	5M	3x W, 1 h	bands strengthening (pressure, pull, bicep, kicking)	↑Chair test (30 s), one leg balance test, gait speed test, 0 up-and-go test.
Yáguèz et al. [58]	27	70.5 ± 5	42D	1x W, 2 h	stretching, circular movements, isometric work, balance exercise, drawing	↑ Attention, visual memory.
Holthoff et al. [59]	30	72.4 ± 4.3	84D	3x W, 30 min	1. passive and active movements	↑ ADL score, ↓ MMSE, neuropsychiatric symptoms in CG.

N, number of patients; M, Month; D, Days; W, weekly; h, hour; HR, heart rate; CG, control group; MMSE, Mini-Mental State Exam; ADL, Activities of Daily Living; ↑, improvement; 0, without any difference when compared with the control group.

The studies also show the association of quantitative changes in human gut microbiota and AD. An excess of pro-inflammatory strains of Escherichia, Shigella, and Bacteroidetes are described [38]. Conversely, strains with anti-inflammatory properties were reduced in AD patients: Firmicutes, Bifidobacterium, and Eubacterium rectale [38, 39]. In general, increased amounts of bacteria correlated with higher AD markers in cerebrospinal fluid. On the other hand, there are bacterial strains that have purely anti-inflammatory properties such as Akkermansia muciniphila and Faecalibacterium prausnitzii [80]. In clinical practice, patients should not forget that many drugs also affect the composition of the gut microenvironment, especially antibiotics, metformin, and proton pump inhibitors [100].

The inclusion of movement programs in the lifestyles of all ages is an undeniable fact. Research shows a complex effect of movement on the health of seniors, for example, Larson et al. [101]. Physical fitness seems to have an impact on the onset of AD, as well as a correlation with its further course [102]. When comparing healthy seniors to patients with AD, positive correlation of cognitive functions and physical tests in both groups, such as Bruce-Keller et al. [103], likewise Burns et al. [104], who complements tasks with dual tasks, or Buchman et al. [105] and their testing the hand grip strength. The interrelation of physical and cognitive components, including bilateral prediction, is reported by Hebert et al. [106]. However, there is some diversity in AD patients. The authors do not agree whether movement interventions have demonstrable benefits in cognition and functional performance parameters [107, 108].

Table 8 lists studies in AD patients with descriptive character. They also investigate the relationship

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Table 5
Mixed load

Study	N	Age	Duration	Frequency	Intervention	Main outcome
Williams & Tappen [60]	45	N/A	112D	5x W, 1 h	1. bodyweight exercises, balance exercises, 20-min walking; 2. walking for 30 min; 3. talks	↑Mood scale, depression.
Teri et al. [61]	153	N/A	3M	daily, 30 min	aerobic, power and balance exercises	↑Physical health (without any specification), depression
Suttanon et al. [62]	40	81.9 ± 5.7	6M	5x W, 1 h	1. balance exercises in the standing, strengthening exercises, walking; 2. educational visits	↑ Functional range, lower score at risk of falling (balance abilities).
Steinberg et al. [63]	27	N/A	84D	N/A	1. tasks for a week: walking, exercises for big muscle groups, bands, wrist and ankles loads, balance exercises; 2. light activities without equipment	↑8 foot walk test, sit-to-stand, Yale Physical Activity Survey, cognitive tests.
Rolland et al. [51]	242	N/A	12M	2x W, 1 h	stretching, walking (20–30 min), bodyweight exercises (legs, sitting down), standing and sitting balance exercises	↑ADL 0 walk speed, up-go-test, one leg balance test.
Roach et al. [53]	82	N/A	84D	5x W, 1 h	1. strength exercises (7–9 opposite), stand up, push/ pull against the nurse, balance exercises, dance position; 2. walking; 3. talks	The best results in group 1, ↑6 min walking test, Acute care index of function, MMSE.
Pitkälä et al. [64]	210	N/A	12M	2x W, 1 h	1. exercise set by a specialist (not specific); 2. exercise in the center - for endurance, balance, strength	↓1. and 2. Functional independence measure, short physical performance battery, but less than in CG.
Pedroso et al. [65]	21	77.9 ± 7.1	4M	3x W, 1 h	balance exercises, aerobic exercise, exercise with weights, cognitive tasks with verbal response	↑MMSE, Berg balance test, cognitive functions, 0 up-and-go test.
Nascimento et al. [66]*	20	N/A	6M	3x W, 1 h	mostly aerobic load (60–80% HR max), compound exercises (15–20 repetitions), balance and rhythm exercises	Smaller worsening in neuropsychiatric and functional tests. 0 instrumental activities.
de Andrade et al. [67]	30	77 ± 6.3	112D	3x W, 1 h	20-min aerobic activity, 35-min dual task (balance exercise, exercise with weights, stretching, cognitive tasks	↑Sit-to-stand (30 s), Berg functional scale, ↑cognitive functions (also dual task) ↓timed up-and-go test (improvement in CG).
Arkin [68]	24	78.8 ± 8	70D	2x W, 1 h	20–30-min aerobic activity (bike, walking), 20–30-min strength exercises on machines	↑6 min walking test, the strength of the upper and lower body.
Öhman et al. [69]**	210	≥65	12M	2x W, 1 h	1. exercise at home, 15-min bicycle, nordic walking, dance, 15-min exercise with dumbbells, 15-min dual tasking, 15 min: walk up the stairs, lifting things from the ground; 2. in a group, 15 min: rower, bike, walking, 15 min: exercise in the gym, 15 min: lifting things from the ground, climbing the ladder, balance exercises 15 min: dual tasking; 3. nothing	↑ clock drawing test in group 1, ↓ verbal fluency and MMSE in both groups.
Vreugdenhil et al. [70]	40	51–89	112D	dennę	Exercises at home - 10 exercises, 30-min walk	↑Balance test, up-go-test, sit-to-stand, cognitive tests.

N, number of patients; M, Month; D, Days; W, weekly; h, hour; CG, control group; MMSE, Mini-Mental State Exam; ADL, Activities of Daily Living; ↑, improvement; ↓, worsening; 0, without any difference when compared with the control group; *only females; **nutrition was taken into account.

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Table 6
Alternative movement approaches

Study	N	Age	Duration	Frequency	Intervention	Main outcome
Abreu & Hartley [71]	1	N/A	84D	2x W, 1 h	Based on salsa dance	↑Up-and-go test, Berg balance test, 6-min walk test, reduction of the number of falls in the six-month follow up period.
Sobel [72]	50	82	N/A	2x W, 45 min	1. Bingo + talks; 2. walking/ exercises while sitting + talks	↑Word naming test, word list recognition (in group 1).
Santana-Sosa et al. [73]	16	76 ± 4	84D	3x W, 1 h	exercise accompanied by music, walking, light stretching, mobility, resistance exercise with expander, 3×15 repetitions	0 activities of daily living, up-go-test, flexibility, ↑arm curl, chair stand test (30 s), 2 min step test.
Myers et al. [74]	1	N/A	N/A	N/A	aquatic therapy	↑Physical tests.
Öhman et al. [69]	210	≥65	12M	2x W, 1 h	1. exercise at home, 15-min bike, nordic walking, dance, 15-min exercise with dumbbells, 15-min dual tasking, 15 min: walking up the stairs, lifting things from the ground; 2. in a group, 15 min: rower, bike, walking, 15 min: gym exercises, 15 min: lifting things from the ground, climbing the ladder, balance exercises, 15 min: dual tasking; 3. nothing	↑ Clock drawing test in group 1, ↓ verbal fluency and MMSE in both groups.

N, number of patients; M, Month; D, Days; W, weekly; h, hour; ↑, improvement; ↓, worsening; 0, without any difference when compared with the control group.

Table 7
Human studies: Effects of exercise on gut microbiota

Study	N	Age	Main outcome
Clarke et al. [77]	40/23+23*	23–36	Exercise increases gut microbial diversity; protein consumption positively correlates with microbial diversity and creatine kinase.; athletes and low BMI group higher proportions of the genus Akkermansia.
Estaki et al. [78]	39	18–35	Cardiorespiratory fitness is correlated with increased microbial diversity in healthy humans; increased microbiota diversity and butyrate production is associated with overall host health.
Barton et al. [79]	40/46**	N/A	Microbial-derived SCFAs are enhanced within the athletes; differences in fecal microbiota between athletes and controls - more evident at the functional or metabolic level.
Bressa et al. [80]	19/21***	18–40	Higher abundance of health-promoting bacterial species in active group (incl. Faecalibacterium prausnitzii, Roseburia hominis and Akkermansia muciniphila); body fat percentage, muscular mass and physical activity significantly correlated with gut microbiota composition.
Paulsen et al. [81]	12****	18–70	Gut microbiota composition is correlated with positive changes in cardiorespiratory fitness level.
Stewart et al. [82]	10/10*****	N/A	Gut microbiota and bacterial profiles from patients with long-standing T1D in good glycemic control and high physical fitness levels are comparable with those of matched people without diabetes.
Yang et al. [83]	71	19–49	Cardiorespiratory fitness is associated with gut microbiota composition, independent of age and carbohydrate or fat intake.

N, number of subjects; *elite athletes/low+high BMI controls; **professional male athletes/healthy controls; ***active/sedentary group; ****breast cancer survivors; *****T1D patients/controls.

between physical parameters, physical activities and the course of resp. AD status. In some cases, it is indicated if a group of healthy seniors was used for comparison.

The interventions where **aerobic load** is used are quite frequent. It is usually a means of walking (with an accompaniment), walking on a treadmill or a stationary bicycle. In some studies, the effort is more accurately captured by the heart rate [44, 45, 47, 50] or by VO2max [41, 49]. This is generally a moderate intensity, with the maximum reported to 80%. To intensify the effect on cognitive abilities when

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Table 8
Descriptive studies (without the intervention)

Study	N	Age	Duration	Note	Main outcome
Winchester et al. [109]	104	65+	12M	Monitoring of movement activities	The most common activity is walking, 2 h weekly ↑ better MMSE, mood state.
Scarmeas et al. [102]	357	65+	N/A	N/A	The importance of physical activity before AD, the relationship of physical activity with the later course of AD.
Hebert [106]	367	65+	48M	N/A	Decrease in cognitive function predicts impairment of upper and lower limb functions.
Coelho et al. [110]	21/18	N/A	N/A	1. patients with AD; 2. healthy people	In patients with AD, there is an increase in the brain derived neurotrophic factor in aerobic load.
Burns et al. [104]	31/31	65+	N/A	1. patients with AD; 2. healthy people	AD - lower aerobic fitness, lower values in physical performance test (picking up a pencil, filing a book, walking 50 feet, writing a sentence).
Buchman et al. [105]	877	N/A	N/A	N/A	Hand grip strength force associated with slower AD development.
Bruce-Keller et al. [103]	50/50	N/A	N/A	1. patients in the initial stage of dementia; 2. healthy people	Positive correlation between cognitive functions and physical tests in both groups.
Perry & Hodges [111]	24	N/A	N/A	N/A	There is no connection between memory and physical activity in patients with AD.

N, number of subjects; M, Month.

walking, talking with a patient is usually performed as well [43, 46, 48]. It seems that these studies do not put emphasis on the performance, but on the movement as such. Frequency of exercise is most often 3 times a week, in the range of 30–60 min. The choice of walking as a means is a logical step; it is a natural compound movement and is a sign of some patient's self-sufficiency. Therefore, it is also used for testing (ADL, 2-min or 6-min walking test, up-and-go test). Winchester et al. [109] confirm this as the most common activity. It is not possible to state an absolute positive influence of aerobic load on the monitored cognitive and functional parameters. Nevertheless, there is evidence that an independent aerobic activity may affect cognitive functions in AD patients. On the other hand, there is evidence that the measurements also worsened the measured values: MMSE [42], neuropsychiatric symptoms [45], and chair-test [47].

Like aerobic activities, **resistance exercises** are generally appropriate. The resistance/strengthening exercises of the AD has different forms: body weight exercises [61], exercises on machines [52], exercises with dumbbells [55], exercises with expander [73], with the ball [56]. Only Yáguèz et al. [58] and partly Francese at al. [56] use isometric muscle exercise, in other cases isotonic movements are conducted. Furthermore, the balance exercises are used in Roach et al. [53]. This is not a direct effect on the strength development, but these coordination abilities are related to strength assumptions. The interventions were mostly 3–5x per week. An interesting exception is the study by Lindenmuth and Moose [54], who themselves did the exercises with patients daily. Unfortunately, the lack of detail in the exercise in this study makes it difficult to interpret. This is also true for Holthoff et al. [59] and Francese et al. [56] The form of exercise is not presented. The expected positive results in tests with a strength component such as chair test, stairway walking were confirmed by Ahn and Kim [57], Garuffi et al. [55], or Frances et al. [56]. However, cognitive and AD-specific tests such as ADL and MMSE were chosen to verify the effectiveness of the intervention. In the vast majority of studies, progress can be reported in some of the measured parameters (2-min walking test, hand grip strength, or up-and-go test). As with the aerobic load, there are studies in which the authors either did not achieve statistically significant deviations from the control group or even worsened, for example, in chair test, 400 m walking test [52, 57, 59]. In addition, it appears that it is not easy for certain patients in advanced AD who have experienced significant degradation of exercise functions to accurately define

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the load. In these cases, the caregiver's work is very important since s/he can regulate resistance for the patient.

In therapeutic interventions, the use of **mixed physical activities** is no exception. This category includes activities where aerobic and strengthening exercises were also used. Their ratio varies, but mostly it is 20–30 minutes of aerobic activity combined with a resistance exercise [60, 67, 112]. Unfortunately, in some cases the description of activities is too superficial; there is no numerical expression or a specific selection of exercises [61, 62, 64, 65]. Only in one study by Pitkälä et al. [64] was there worsened results or insignificant deviations from the control group. However, this is a longitudinal study where degressivity in the traits of interest can be expected due to the age of the patients and the nature of the disease. In addition, in Nascimento et al. [66], both female groups worsened; the control group, however, was found to be worse than the experimental one. Other studies always show some improvement in the chosen test [53, 63, 67].

Compared to the usual procedures in the selection of physical activities, there exist **alternative approaches** which, due to the results, may represent other suitable pathways for patients with AD. Abreu and Hartley [71] used Salsa as a medium; Myers et al. [74] used aquatic therapy; both were case studies. Sanatana-Sosa et al. [73] use simultaneously with resistance training and walking, exercises accompanied by music. Similarly, Öhman et al. [69] applies dance as one of the methods. In the study by Sobel et al. [72], bingo was selected for one of the groups. Again, in terms of outputs, the results were mixed, but in each study, there were some improvements in the monitored tests. Thus, research shows that it is advisable to choose a holistic approach to the health of the individual in order to affect a greater number of factors influencing wellness and general fitness. Thanks to music, the environment or the suitably chosen attitude of nurses, the emotional and social component of the healing process will be significantly influenced.

Table 9 summarizes the possible limitations of the findings of individual studies dealing with the relationship between the physical activity and AD.

Table 9
Limitations of the findings in the detected studies

Connection with the disease	Connection with the physical activity
Small sample sizes in some studies	a lack of the analysis of the previous movement activities
Short duration of the intervention	for different stages of AD, the same intervention cannot be applied and comparable results cannot be expected
Large age variation of the research sample, or age not shown	stable results can be seen as a positive effect at an advanced age
Different AD stages (sometimes not even mentioned in the study)	slowing down cognitive degradation can also be considered a positive outcome in AD

Despite the lack of human studies (but enough data from animal models), physical activity appears to be significantly modulating the gut microbiome [71]. It can thus be concluded that the state of physical activity in humans seems to correlate with the composition of gut microorganisms [113–115]. In general, it has been found that greater microbial diversity is associated with "excess health". A more diverse microbiome is also able to respond better to repeated slight fluctuations [116]. In this context, exercise was observed to induce a more diverse composition of human gut microbiome [117]. The diversity of microbiome was also associated with cardiovascular condition in studies. Microbiomes of individuals with higher VO2max levels tend to produce more butyrate [78], which belongs to SCFA with anti-inflammatory effect [118]. Barton et al. [79] report that the gut microbiome of athletes promoted a more intense metabolism of macronutrients (especially carbohydrates and proteins) and higher SCFA concentrations were found compared to the sedentary control group.

SCFA is believed to serve as a source of energy for various tissues, to improve insulin sensitivity, and to alter central nervous system morphology [119–121]. Inflammation plays a very important role in the human body in connection with AD. In animal models of older age, a decrease in the expression of inflammatory mediators and apoptotic markers in intestinal lymphocytes is described by physical exercise [122].

The intestinal microbiome is influenced by many lifestyle factors and by the environment in which we live [123]. One of the components is a movement activity, which acts on the organ system and has an overlap into the function of the gastrointestinal tract. In view of the focus of this article, research studies that have used similar interventions as those found in AD patients have been explored. Bressa et al. [80] observed a group of premenopausal women for 6 weeks and found a clear difference between the active and control groups in the positive microbiome change. The active group had to meet the minimum

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requirements of 3 hours of physical activity per week. The effect of exercises on the structure of microbiome in 18–70 women who have had breast cancer is described by Paulsen et al. [81]. The analyzed group had to undergo at least 60 minutes of moderate intensity physical activity per week for 6 months. The result was a correlation in microbiome structure and cardiorespiratory fitness of the research group. On another sample of 19 obese women, microbiome modifications were demonstrated during a 6-week intervention. The activity was to ride a stationary bike 3 times a week with low to medium intensity for 40–50 minutes [124]. Allen et al. [16] also selected moderate intensity and aerobic load. After a 6-week program (3 times a week, 30–60 minutes of walking on a belt or riding a stationary bike), she notes changes in SM composition and retrograde development after exercise. In summary, regular aerobic physical activity has a confirmed effect on the composition and function of SM [76].

Overall, research shows that exercise can increase the number of beneficial bacteria, generally enhance the diversity of microbiomes, and also increase the amount of commensal species. All these effects are clearly beneficial to the host and improve his health. The available data summarized in this paper strongly support the fact that, among other well-known internal and external factors, exercise appears to be a significant factor that can cause changes in terms of both qualitative and quantitative composition of the gut microbiome. It is well known that a stable and diverse microbiome is indispensable for the normal function of the gastrointestinal tract, which in turn positively affects the gut-brain axis and contributes overall to a healthy person's condition.

A major limitation of studies monitoring the effect of exercise on the gut microflora is their cross-sectional design, factors that can temporarily alter the intestinal microflora (diet, antibiotics, obesity, impaired bowel barrier function, etc.); however, they are not evaluated in most of the detected studies.

Conclusion

AD is one of the most common neurodegenerative diseases of the central nervous system. It occurs primarily in the elderly and leads to incurable cognitive impairment and dementia. It is estimated that there are currently around 50 million people living in the world suffering from AD. However, the real assumptions are expected to reach 152 million by 2050. The medical condition of most patients at an advanced stage requires professional care that burdens national budgets. For example, direct health care costs are estimated to be EUR 14, 236 million in Europe between 2050 and 616 million euros in direct non-medical care costs [3]. With a slight exaggeration, one can argue that the professional public is alert and a large number of research teams are trying to find a solution.

One of the great issues of coexistence is the human intestinal microbe. After entering the general "microbiome" in database Pubmed, 57,002 publications can be found, of which 1,515 are clinical trials and 1,345 are on humans (as of July 6, 2019). There is no doubt that the composition of the human intestinal microbiome correlates with the neurological functions due to the gut-brain axis and therefore, in many articles, microbiome appears to be a promising target for anti-aging therapeutic interventions. As the number of patients grows, public budget administrators try to reduce the cost of potential treatment. On a general level, most medical recommendations can see a return to human nature, i.e., a healthy lifestyle that includes appropriate nutrition and movement. From the evidence we have provided on AD, movement, and microbiome, it can be seen that the trends of diversion from consumerism have their own merits, and human intestinal microbiota could be the link. Microbiome is associated with AD. AD is positively influenced by physical activity. And physical activity has a beneficial effect on the human intestinal microbiome. It is a comprehensive complex of interactions, which could result in benefits for AD patients, but also for the professional public and national budgets.

This study separately presents everything that has been done to humans in these areas, but it does not have an experimental character; its purpose is to serve as a theoretical basis for the future implementation of adequate human research that is still lacking. It might seem like speculation, but the opposite is true. Recent experimental studies in animal models strongly support the link between cognitive defect, exercise, and microbiome. Research published by Abraham et al. [125, 126] clearly demonstrates through experiments that "exercise and probiotic treatment can decrease the progress of AD and the beneficial effects could be mediated by alteration of the microbiome". Similar statements can be found in a review by Ticinesi et al. [127], which also uses the term "muscle-gut axis". In fact, Ticinesi et al. [127] and our study are the first two studies discussing these links.

Based on the positive results of these research studies, it is possible to propose specific procedures that

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should be applied when working with AD patients. Due to the large differences between patients (stage of AD, age, fitness level, or economic background), it is not possible to determine the exact procedure, but the following general points can be recommended:

- long-term intervention, at least 3-4 times a week;
- activity both aerobic (30–60 minutes mean intensity, including walking), and
- strength (20–30 minutes containing exercises with own body, resistance exercise with
- expander or light external load), they can be combined;
- include dual task (collection of items, walking and talking, stand/walk with full
- glass, etc.);
- create socially (family members, trained caregivers) and emotionally (music,
- movement in nature, decoration) pleasant environment;
- creatively use the possibilities (material, financial, etc.) for activities such as dance,
- rhythmic activities, bingo, or similar games.

In conclusion, the interdisciplinary approach, i.e., the relationship between patient, doctor, and physiotherapist/personal trainer should be paramount in these situations. The research indicates that the complexity of AD patient-microbiome interactions and the potential for affecting them have a profound effect on AD therapy, respectively prevent the development of this disease.

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Short-Chain Fatty Acids and Lipopolysaccharide as Mediators Between Gut Dysbiosis and Amyloid Pathology in Alzheimer's Disease

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Abstract.

Background: Metagenomic data support an association between certain bacterial strains and Alzheimer's disease (AD), but their functional dynamics remain elusive.

Objective: To investigate the association between amyloid pathology, bacterial products such as lipopolysaccharide (LPS) and short chain fatty acids (SCFAs: acetate, valerate, butyrate), inflammatory mediators, and markers of endothelial dysfunction in AD.

Methods: Eighty-nine older persons with cognitive performance from normal to dementia underwent florbetapir amyloid PET and blood collection. Brain amyloidosis was measured with standardized uptake value ratio versus cerebellum. Blood levels of LPS were measured by ELISA, SCFAs by mass spectrometry, cytokines by using real-time PCR, and biomarkers of endothelial dysfunction by flow cytometry. We investigated the association between the variables listed above with Spearman's rank test.

Results: Amyloid SUVR uptake was positively associated with blood LPS ($\text{rho} \geq 0.32$, $p \leq 0.006$), acetate and valerate ($\text{rho} \geq 0.45$, $p < 0.001$), pro-inflammatory cytokines ($\text{rho} \geq 0.25$, $p \leq 0.012$), and biomarkers of endothelial dysfunction ($\text{rho} \geq 0.25$, $p \leq 0.042$). In contrast, it was negatively correlated with butyrate ($\text{rho} \leq -0.42$, $p \leq 0.020$) and the anti-inflammatory cytokine IL10 ($\text{rho} \leq -0.26$, $p \leq 0.009$). Endothelial dysfunction was positively associated with pro-inflammatory cytokines, acetate and valerate ($\text{rho} \geq 0.25, p \leq 0.045$) and negatively with butyrate and IL10 levels ($\text{rho} \leq -0.25$, $p \leq 0.038$).

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Conclusion: We report a novel association between gut microbiota-related products and systemic inflammation with brain amyloidosis via endothelial dysfunction, suggesting that SCFAs and LPS represent candidate pathophysiologic links between the gut microbiota and AD pathology.

Keywords: Brain amyloidosis, inflammation, lipopolysaccharide, microbiota, short chain fatty acids

INTRODUCTION

Alzheimer's disease (AD) pathology is the most common cause of neurodegenerative dementia, featuring extra neuronal accumulation of amyloid-β (Aβ), and intraneuronal deposition of hyperphosphorylated tau. However, despite great progress in understanding the dynamics of the molecular pathology, treatments targeted at AD pathology and its modifiers have not yet resulted in a disease modifying therapy.

There is clear evidence that AD is characterized by the presence of a pro-inflammatory status both in the periphery, in term of higher blood levels of pro-inflammatory cytokines, and in the brain, in term of activated microglia, the resident innate immune cells of the central nervous system (CNS). Moreover, Aβ has recently been recognized as an antimicrobial peptide, as part of the innate immune system [1] and, while monomeric Aβ shows little antimicrobial activity, its capability of aggregation allows to form antimicrobial pore-forming structures [2].

Based on this, researchers have recently proposed a potential role for the gut microbiome in the initiation and exacerbation of AD pathology and several preclinical and clinical studies indeed support this hypothesis. For example, in 2016, for the first time, Minter and colleagues reported that antibiotic-induced perturbations in the gut microbiota (GMB) diversity influence neuroinflammation and amyloidosis in a murine model of AD [3]. Subsequently other studies by using the sequencing of bacterial 16S ribosomal RNA from fecal samples of APP transgenic mice revealed significant differences in the GMB composition compared to that of control wild type mice [4, 5]. Interestingly, in the paper of Harach and collaborators, the authors found also that cerebral Aβ was significantly reduced in germ-free APP transgenic mice and that their recolonization with microbiome coming from conventionally raised APP transgenic mice increased cerebral Aβ pathology, and this increase was less effective when wild type mice microbiota was used. Preclinical data have been also supported by clinical data, where the presence of alterations in the composition of the gut microbiome have reported in AD patients as compared to control individuals [6, 7]. Our research group in 2016 demonstrated an increased abundance of proinflammatory *Escherichia/Shigella* and decreased abundance of anti-inflammatory *Eubacterium rectale* in patients with cognitive impairment and brain amyloidosis and also an association of their abundances with peripheral inflammation [8]. A subsequent study that used a 16S sequencing approach revealed that the GMB profile in AD patients is characterized by a reduced microbial diversity, decreased abundance of *Firmicutes* and *Bifidobacterium* and an increased abundance of *Bacteroidetes*; interestingly, the relative bacterial abundance correlated with the increase of cerebrospinal fluid markers of AD pathology [7].

There are several pathways through which the gut bacteria may influence brain functioning and promote neurodegeneration in the context of AD pathology. One of these biological processes is represented by inflammation: microbes are known to influence the development and regulation [9] of the immune system and, therefore, may alter the interaction between the immune and the nervous systems. The gram-negative membrane protein lipopolysaccharide (LPS), which has pro-inflammatory properties, was found in amyloid plaques and around vessels in AD brain [10]. Moreover, LPS levels have been found significantly higher in plasma samples of AD patients as compared to controls [11], and a positive association was found between LPS levels and degree of blood monocyte/macrophage activation. Importantly, a pro-inflammatory status has been identified as among the most important determinants of endothelium dysfunction [12]. Indeed, changes in the gut microbiome composition could lead to alterations in the functionality of endothelial cells, which are key cellular players of inflammatory reactions and strictly interact with the soluble factors involved in the regulation of each of the different steps of the inflammatory reaction; such endothelial dysfunction could then promote or enhance the

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spread of the inflammatory status from the gut to the blood and to the brain. The GMB is also known to produce several metabolites through fermentation on non-digestible carbohydrates with neuroprotective properties [13] that directly or indirectly affect brain functions. Among these, short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are known to beneficially modulate the peripheral and central nervous systems and have been already suggested to play a central role in AD [14]. In particular, it has been hypothesized that SCFAs may attenuate AD by serving as substrates for energy metabolism [15] and providing an alternative energy source to counteract the brain hypo-metabolism that contributes to neuronal dysfunction in AD [16]. Recent evidence also suggests that SCFAs may help to modulate maturation and function of microglia in the brain [17]. More recently, it was also shown that butyric acid may provide therapeutic benefits for AD through epigenetic mechanisms of action by inhibiting histone deacetylase and normalizing aberrant histone acetylation [18]. This evidence suggests that the production of SCFAs in the gut may represent another biological mechanism through which the gut microbiome may influence AD.

In this study we aimed at evaluating whether inflammatory mediators and bacterial products are potential pathogenic links between the GMB and amyloid pathology in AD via endothelial dysfunction. To this purpose, we tested the associations of brain amyloidosis with blood levels of LPS, SCFAs, cytokines, and several markers associated with endothelial dysfunction in a cohort of elderly subjects with cognitive performance ranging from normal to dementia.

MATERIALS AND METHODS

Study participants

Participants were Italian community-dwelling persons of 50 to 85 years of age recruited from a large Italian study on amyloid imaging in patients with cognitive complaints, the Incremental Diagnostic Value of [18F]-Florbetapir Amyloid Imaging [INDIA-FBP] study [19]. Inclusion criteria were cognitive abnormality, age between 50 and 85 years, and availability of an informant (spouse, adult child, or another knowledgeable informant). Cognitively impairment was defined as follows: 1) presence of cognitive complaints reported by patients or proxy or by the doctor; 2) presence of no intracranial metabolic or psychiatric causes of cognitive impairments; 3) presence of abnormal scores in $\geq$2 cognitive tests; and 4) history of progression of cognitive symptoms. The clinical evaluation included the collection of medical cognitive, functional, and drug history, and physical and neurological examination. The cognitive assessment of each participant included the Mini-Mental State Examination (MMSE) and the Alzheimer's Disease Assessment Scale, cognitive portion (ADAS-Cog). In the context of this parent study, 150 patients and controls who were not under antibiotic and anti-inflammatory treatment over the past 3 months or had been diagnosed with major depression or other psychiatric disorders were proposed to contribute samples of stools and blood. Accepting patients signed an ad-hoc informed consent. Venous blood was sampled from an antecubital vein between 08:00–10:00 h am and collected in EDTA tubes (BD Vacutainer Systems, Franklin Lakes, NJ).

[^{18}F]-Florbetapir amyloid PET

As previously reported [8], amyloid PET was performed at the Nuclear Medicine Service of Spedali Civili and Fondazione Poliambulanza in Brescia with GE Discovery 690 and Siemens Biograph 40 m PET–computed tomography scanners, respectively. PET was a 10-min (two 5-min frames) 3-dimensional acquisition, 50 min after the injection of an intravenous bolus of 370 MBq (10 mCi) of ^{18}F-Florbetapir [20]. Attenuation correction was calculated based on the co-acquired computed tomography. PET images were reconstructed onto a 128 $\times$ 128 matrix with slice thickness of 3–3.3 mm, using a 2- to 3-mm Gaussian post-reconstruction filter. Standardized uptake value ratios (SUVRs) were calculated in the frontal, parietal, temporal, anterior cingulate, posterior cingulate, and precuneus regions of interest as well as global measure.

Inflammatory mediators

Isolation of total RNA was performed using the PAXgene blood miRNA kit according to the manufacturer's protocol (PreAnalytiX, Hombrechtikon, CHE). RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrophotometer (NanoDrop Technologies, Delaware, USA) and by using the Agilent BioAnalyzer (Agilent Technologies) and samples were stored at –80°C until processing. Gene expression analyses on candidate genes was

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performed in RNA samples from blood circulating cells (obtained by using PaxGene tubes) using semi-quantitative real-time PCR and included a panel of inflammatory mediators known to be involved in AD. In particular, the gene expression measurement was performed for the pro-inflammatory mediators IL1β, NLRP3, IL6, CXCL2, CXCL10, TNFα, and IL18 as well as for the anti-inflammatory mediators IL10 and IL4. Each sample was assayed in duplicate, and each target gene was normalized to the expression of three reference genes, glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and beta-2-microglobulin, using Taqman Assays on a 384 wells Real Time PCR System (Biorad). The expression levels of each target gene were normalized to the geometric mean of all three reference genes, and the Pfaffl method was used to determine relative target gene expression of each gene in patients as compared with controls. The primer sequence for each of the genes is available on request to the authors.

GMB-related products

LPS was measured in plasma by ELISA (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientifics) and SCFAs levels (i.e., acetate, propionate, valerate, and butyrate) using gas chromatography (GC) according to the following procedure. Briefly, after thawing at room temperature (RT), 400 microliters of plasma were deproteinized by the addition of 50 microliters of 16% (W/V) metaphosforic acid and mixed by vortexing for 30 s. After, an incubation at 60°C for 30 min was performed to completely precipitate the protein fraction. Successively, all samples were centrifuged at 8000 rpm for 30 min, the supernatant was collected and filtered at 0.2 micron. The SCFA determination was performed using the GC DANI 1000 (Dani instruments S.p.A. Monza Italy) equipped with split-splitless injector and HP-FFAP megabore water compatible capillary column (length 30 mt with 0.53 mm internal diameter; Agilent Technologies INC, CA, USA). Iso-valerianic acid was used as internal standard. The GC conditions were Injector 200°C and F.I.D. (detector) 260°C. Column temperature setting was: initial temperature 85°C, hold time 0.5 min, PRGM1 column heating rate 7°C/min, PRGM1 final column temperature 135°C, hold time 0.1 min, PRGM2 column heating rate 25°C/min, PRGM2 final column temperature 160°C, hold time 4.0 min. For the concentration of each acid, three standard solutions have been prepared: 1) low 80 ppm; 2) medium 120 ppm; 3) high 200 ppm) with 100 ppm of I.S. for each one. Response Factor calculation was determined as follow:

$$RF = \text{I.S. peak area} \times [\text{V.F.A.}] / \text{V.F.A.} \times [\text{IS}]$$

The concentration of each VFA was determined by applying the following formula:

$$[\text{VFA}] = 100 \times \text{VFA peak area} \times \text{VFA RF} / \text{I.S. peak area}$$

The results were expressed in micromoles/liter.

Endothelial dysfunction markers

The LEGENDplex™ Human Adhesion Molecule Panel multiplex assay (Cat.#740945, BioLegend) was used to measure the plasma level of the following cell adhesion molecules (CAMs): intracellular CAM 1, 2 and 3 (ICAM-1, ICAM-2, and ICAM-3, respectively), vascular CAM (VCAM-1), platelet endothelial CAM 1 (PECAM-1), activated leukocyte CAM 1 (ALCAM-1), epithelial CAM (EpCAM), neural CAM (NCAM), endothelial selectin (E-selectin), platelet selectin (P-selectin), leukocyte selectin (L-selectin), platelet endothelial CAM 1 (PSGL-1), and CD44. Briefly, the surface of each fluorescent bead set is conjugated with specific capture antibodies so that they can be considered as capture beads. The fluorescent capture beads are incubated in 1:50 diluted human plasma samples containing the target molecules for 2 hr at room temperature in a V-bottom 96 well plate in continuous shaking in the dark. Successively, after 2 wash steps, a biotinylated detection antibody mix (25 microliters) is added to each well and incubated for 1 h at RT in continuous shaking. In this way, each detection antibody will bind to its specific analyte bound on the capture beads (capture bead-analyte-detection antibody sandwich). Then, a 25 mL volume of streptavidin-phycoerythrin (SA-PE) solution is subsequently added and incubated for 30 min at RT. The binding to the biotinylated detection antibodies will provide a fluorescent signal (FL2 channel) intensities in proportion to the amount of bound analytes. After a final wash step, the 96-well reading was performed using a Beckman-Coulter Cytoflex flow cytometer equipped with a plate reader. For each well, a minimum of 6,000 events was recorded. Since the beads are differentiated by size and internal fluorescence (FL4 channel), analyte-specific populations can be segregated using forward and side scatters properties followed by FL4 channel fluorescence analysis (each bead set has a

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different FL4 intensity). The PE fluorescent signal quantified will be proportional to the concentration of tested adhesion molecules according to the standard curve generated in the same assay. The flow cytometry data were uploaded in FCS-3.0 file format on the LEGENDplex™ Cloud-based Data Analysis Software (Dec 05, 2019, BioLegend) for analyte quantification. Each sample included was tested in duplicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (v 8.1.1) (GraphPad Software, San Diego, CA, USA). Unadjusted associations of the variables listed above were assessed with Spearman's rank correlation, the nonparametric version of the Pearson correlation which reduces the influence of extreme values. The effect of age and global cognition on the correlation coefficients and *p*-values was calculated using the Partial and Semi-Partial (Part) Correlation ("ppcor") R package (v 1.1) [21]. Significance was set at $p < 0.05$ (two-tailed).

RESULTS

Demographic and clinical characteristics were as expected for this population with the exception for the low prevalence of hypertension (Table 1). Considering the broad range of age and cognitive performance of the cohort included in the present study, we first explored the association between age and global cognition with brain amyloidosis, blood levels of LPS, SCFAs, cytokines, and adhesion molecules (Supplementary Figure 1). Age was associated with the pro-inflammatory cytokines IL6 and TNFα and the adhesion molecules VCAM-1, CD44, and ICAM-3 ($0.22 \leq \text{rho} \leq 0.40$, $p < 0.046$). Global cognition, measured using MMSE and ADAS-cog, wan mainly associated with valerate, IL1β, CXCL2, and IL10, several adhesion molecules as well as with all the markers of brain amyloidosis ($0.25 \leq \text{rho} \leq 0.42$, $p < 0.040$).

Brain amyloid load is associated with circulating GMB-related products

In order to improve our understanding on the relationship between brain amyloid deposition and GMB, we measured the plasma levels of the Gram-negative membrane protein LPS and four SCFAs produced by the intestinal bacteria, namely acetate, propionate, valerate, and butyrate. Association of circulating GMB-related products with global and regional cortical amyloid load was evaluated using Spearman's rank test. Elevated levels of LPS were found associated with greater amyloid pathology in all the regions considered (Fig. 1). In particular, LPS resulted stronger associated with amyloid load in frontal, posterior, anterior cingulate cortex, and precuneus ($0.44 \leq \text{rho} \leq 0.49$, $p < 0.001$) than in temporal and parietal cortex (rho = 0.35 and 0.032, $p = 0.006$ and 0.011, respectively). Among the SCFAs, a positive relationship between acetate and valerate as well as a negative association of butyrate with brain amyloid deposition was observed (Fig. 1). High levels of acetate and butyrate were stronger associated with

Table 1
Demographic and clinical features of the 89 study participants

Socio-demographics	Mean (SD) or N (%)	Min-Max
Age (y)	69.6 (6.9)	54.1–86.0
Female	48 (54%)	n.a.
Education (y)	8.7 (4.1)	4.0–18.0
Body mass index[a]	25.3 (3.8)	17.0–37.1
Weight loss/gain	22 (25%)	n.a.
Florbetapir (standardized uptake value ratio)	1.11 (.22)	0.69–1.72
Cognitive performance		
Mini-Mental State Examination	22.8 (5.4)	2.00–30.0
ADAScog	16.2 (10.21)	4.0–55.6
Cognitive stage		
Unimpaired/ MCI/ dementia	16/39/34 (18/44/38%)	n.a.
Risk factors		
Hypertension	46 (52%)	n.a.
Diabetes	13 (15%)	n.a.
Vascular diseases	17 (19%)	n.a.
Hypercholesterolemia	39 (44%)	n.a.
History of stroke	7 (8%)	n.a.
Drugs		
Acetylcholinesterase inhibitors	6 (7%)	n.a.
Memantine	0	n.a.
Antidepressants/ hypnotics/ anxiolytics	34 (38%)	n.a.
Antipsychotics	5 (6%)	n.a.
Food supplements	11 (12%)	n.a.
Drug abuse	0	n.a.
Alcohol abuse[b]	3 (3%)	n.a.
Sleep patterns[c]	4.16 (4.80)	n.a.

[a]weight/height^2 and measured in kg/cm^2. ADAScog, Alzheimer's Disease Assessment Scale–Cognitive Subscale. [b]Alcohol abuse referred to the past, not currently in progress. [c]The following five night sleep symptoms were evaluated: difficulty falling asleep, waking up frequently in the night, early morning awakenings, not feeling rested in the morning, difficulty staying awake and need to take a nap during the day. For each symptom, respondents were asked to estimate frequency of the symptom during the past month on a five-point scale: 0 = never; 1 = 1–3 days; 2 = 4–7 days; 3 = 8–14 days; 4 = 15–21 days; 5 = 22–31 days [88].

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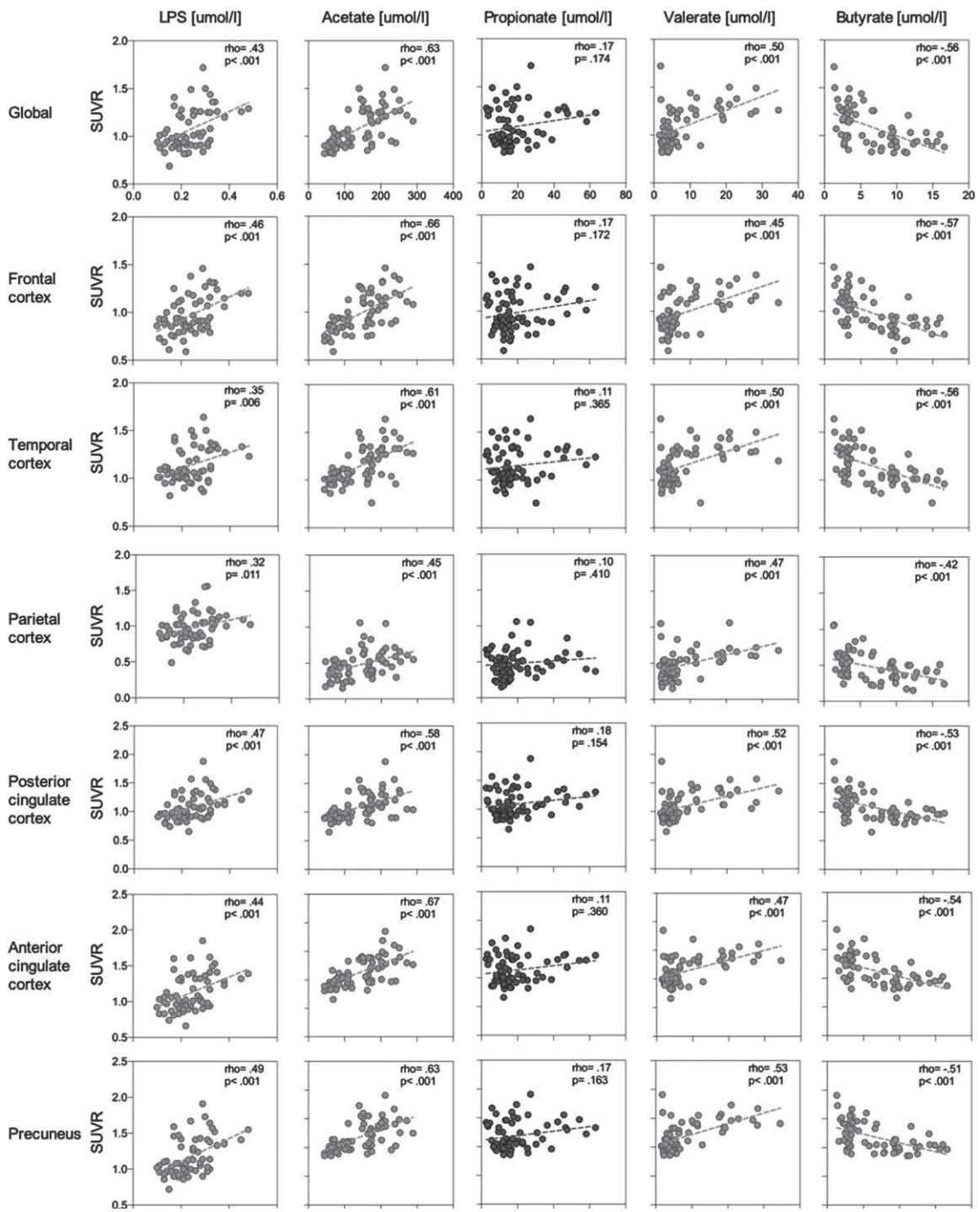


Fig. 1. Association matrix of circulating GMB-related products (LPS and SCFAs) with cortical amyloid load (SUVR). Spearman's rank correlation analyses included PET and SCFAs data for 67 subjects, PET and LPS data for 61 subjects. Two-tailed significant correlations are shown in purple. SUVR, standardized uptake value ratio of florbetapir.

frontal, temporal, posterior, anterior cingulate cortex, and precuneus ($0.58 \leq \text{rho} \leq 0.66$, $p < 0.001$ for acetate; $-0.57 \leq \text{rho} \leq -0.51$, $p < 0.001$ for butyrate) than parietal cortex ($\text{rho} = 0.45$, $p < 0.001$ for acetate and $\text{rho} = -0.42$, $p < 0.001$ for butyrate) while valerate did not show any region-specific association

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($0.45 \leq \text{rho} \leq 0.53$, $p < 0.001$). In contrast, propionate was not associated with PET biomarkers ($0.07 \leq \text{rho} \leq 0.18$, $p \geq 0.182$). Similar findings were obtained after controlling the Spearman's Rank Correlations for age or global cognition measured using MMSE (Supplementary Figure 2).

Brain amyloid load is associated with circulating inflammatory mediators

Next, we evaluated the RNA gene expression from blood circulating cells of several pro- and anti-inflammatory cytokines and we investigated their relationship with brain amyloid pathology. Elevated expression of 4 out of 7 pro-inflammatory cytokines, namely IL1β, NLRP3, IL6, and CXCL2 were associated with higher amyloid load at both global and regional level (Fig. 2). The stronger association between cortical amyloid deposition and inflammation was found in the posterior cingulate cortex ($0.38 \leq \text{rho} \leq 0.43$, $p < 0.001$) and the weaker association in the parietal cortex ($0.25 \leq \text{rho} \leq 0.35$, $0.001 \leq p \leq 0.023$). Among the anti-inflammatory cytokines, high expression of IL10 but not IL4, was found consistently associated with low amyloid deposition in all the regions considered ($-0.31 \leq \text{rho} \leq -0.26$, $p \leq 0.018$). Removing the outliers did not affect the correlation analysis between amyloid and IL1β, CXCL2, and IL10. Similar findings were obtained adjusting the Spearman's Rank Correlations for age or global cognition measured using MMSE (Supplementary Figure 3).

Circulating GMB products are weakly associated with peripheral inflammation

As both LPS and SCFAs are known to modulate inflammatory response, we tested the association between peripheral GMB-related products and inflammatory mediators. High levels of LPS, acetate and valerate were correlated with greater gene expression levels of at least one of the pro-inflammatory cytokines IL1β, NLRP3, CXCL2, and IL18 ($0.23 \leq \text{rho} \leq 0.35$, $p \leq 0.095$, Fig. 3). Moreover, high levels of acetate were weakly associated with lower expression of the anti-inflammatory cytokine IL10 ($\text{rho} = -0.24$, $p = 0.060$). Conversely, elevated butyrate levels were associated with lower levels of the pro-inflammatory NLRP3 and IL6 ($\text{rho} = -0.24$, $p = 0.060$ and $\text{rho} = -0.31$, $p = 0.016$, respectively) and of the anti-inflammatory IL4 ($\text{rho} = -0.24$, $p = 0.068$) as well as with higher levels of TNFα ($\text{rho} = 0.24$, $p = 0.066$). Propionate did not show any association with inflammatory biomarkers ($0.02 \leq \text{rho} \leq 0.21$, $p \geq 0.104$). Similar findings were obtained adjusting the Spearman's Rank Correlations for age but not for global cognition where p-values higher than 0.1 were reported for LPS and IL1β, valerate and IL18, and acetate and IL10 (Supplementary Figure 4).

Endothelial dysfunction is associated with circulating GMB products, inflammatory mediators, and brain amyloid load

To verify our hypothesis that GMB products and systemic inflammation compromise blood-tissue barrier integrity leading to vascular damage and AD pathogenesis, we measured the plasma levels of a panel of adhesion molecules. The upregulated expression of such molecules is one of the phenotypic features of endothelial dysfunction. Among the GMB products, the SCFAs acetate and valerate were found consistently associated with endothelial damage (Fig. 4). Indeed, elevated levels of acetate and valerate were moderate or strongly associated with high levels of almost all the endothelial markers considered ($0.25 \leq \text{rho} \leq 0.46$, $p \leq 0.045$). In contrast, butyrate seemed involved in the maintenance of the endothelial integrity, as suggested by its negative associations with NCAM, P-Selectin, and PSGL-1 ($-0.35 \leq \text{rho} \leq -0.25$, $p \leq 0.038$). LPS was weakly associated only with P-Selectin ($\text{rho} = 0.23$, $p = 0.099$).

Overall, elevated levels of adhesion molecules were associated with high expression of proinflammatory cytokines ($0.26 \leq \text{rho} \leq 0.42$, $p \leq 0.040$) and low expression of the anti-inflammatory IL10 ($-0.29 \leq \text{rho} \leq -0.26$, $p \leq 0.027$). IL4, usually considered an anti-inflammatory cytokine, showed a correlation profile similar to that identified for the pro-inflammatory cytokines as it was positively associated with 6 out of 13 adhesion molecules ($0.27 \leq \text{rho} \leq 0.40$, $p \leq 0.036$). Finally, elevated levels of CD44, NCAM, ICAM-2, P-Selectin, E-Selectin, PECAM-1, PSGL-1, ICAM-1, and ICAM-3 were associated with amyloid deposition in almost all the cortical regions considered ($0.25 \leq \text{rho} \leq 0.52$, $p \leq 0.042$). Similar findings were obtained adjusting the Spearman's Rank Correlations for age or global cognition measured using MMSE (Supplementary Figure 4).

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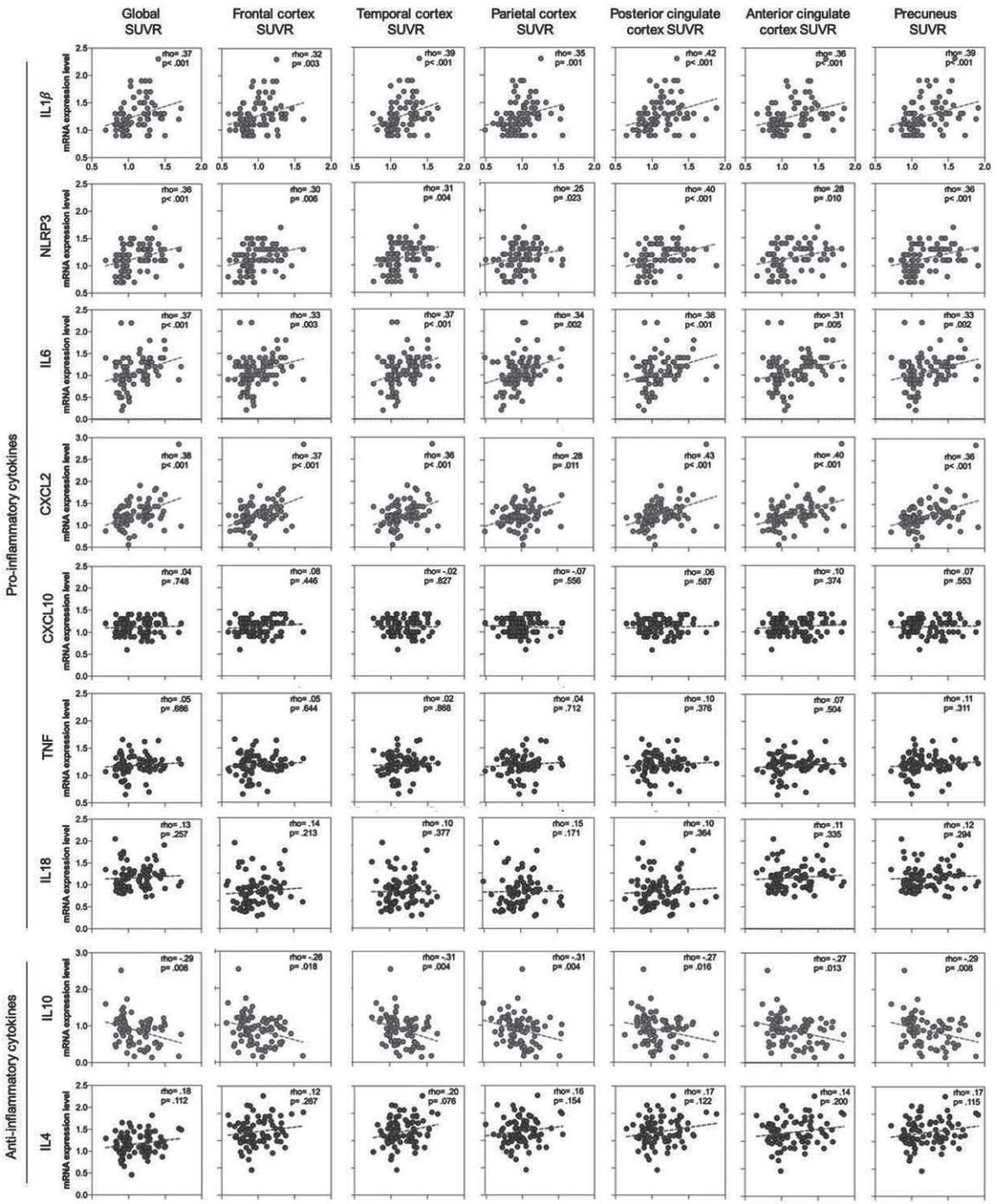


Fig. 2. Association matrix of circulating cytokines with cortical amyloid load. Spearman's rank correlation analyses included PET and inflammatory cytokine data for 83 subjects. Two-tailed significant correlations are reported in purple. CXCL2, Chemokine (C-X-C motif) ligand 2; IL, interleukin; NLRP3, NOD-, LRR- and pyrin domain-containing 3; SUVR, standardized uptake value ratio; TNF, tumor necrosis factor.

DISCUSSION

This is a cross-sectional study investigating the association among a number of circulating markers of GMB metabolism, systemic inflammation, and endothelial dysfunction, that might mediate the effect of the GMB on brain amyloidosis and thus be involved in the pathophysiology of AD (Fig. 5). The

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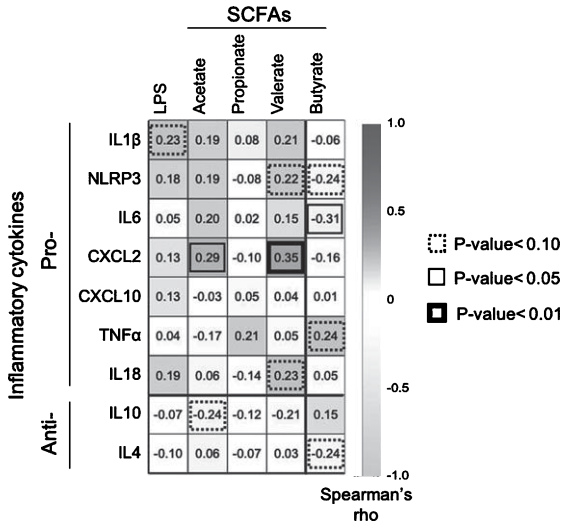


Fig. 3. Association matrix of circulating GMB-related products and cytokines. Columns represent GMB-related products; rows represent inflammatory mediators. Spearman's rank correlation analyses included data for 61 subjects. Figure denotes Spearman's rank correlation coefficient values. These are also represented on a yellow to purple color scale to facilitate the appreciation of association patterns. Significance is denoted by dotted and solid cell borders. CXCL2, Chemokine (C-X-C motif) ligand 2; IL, interleukin; LPS, lipopolysaccharide; NLRP3, NOD-, LRR- and pyrin domain-containing 3.

associations that we found confirm current knowledge on GMB metabolism and support the novel hypothesis of an effect of GMB-related products and systemic inflammation on brain amyloidosis, via endothelial dysfunction. Of course, the cross-sectional nature of our study prevents us to draw causal inferences. However, the strength of the associations (i.e., the size of the rho correlation coefficient) is coherent with the pathophysiological hypothesis.

Endothelial dysfunction in AD

The blood-tissue barrier is mediated by endothelial cell-cell adhesions as tight and adherents junctions as well as other CAMs, including PECAM-1 [22]. Several CAMs are also involved in leukocyte transendothelial migration by binding their leukocyte ligands and allowing activated leukocytes entry into the tissues [22]. Extracellular stimuli such as inflammatory mediators and SCFAs affect the expression of CAMs, influence the endothelial activation and thus, the blood-tissue permeability [23–25]. Increasing evidence suggests that several CAMs are involved in the pathophysiological processes of AD. However, the link of CAMs with microbiota and inflammation as a possible underlying mechanism of cerebral amyloid pathology has not been studied till now. *In vitro* studies demonstrated that Aβ and LPS increased the expression of ICAM-1 and the PECAM-1 [26, 27]. These molecules, found increased in the plasma AD patients [28, 29] and in AD plaques [30], mediate the transendothelial migration of leukocytes across the blood-brain barrier (BBB) [31, 32] and have been proposed to initiate the endothelial signaling cascade and influence the progression of neuroinflammation [32]. Increased levels of E-Selectin and P-selectin has been also reported in the plasma of AD patients [33, 34]. The former has been associated with vascular changes [34], the latter with fast cognitive decline [33]. Moreover, AD platelets show a significant increase in surface expression of P-selectin, that represents a marker of granule secretion [33, 35]. This pre-activated state of circulating platelets in AD patients has been proposed to contribute to dementia progression by triggering perivascular inflammation, induction of vasoconstriction, and consecutive brain hypoperfusion in addition to contributing to the peripheral Aβ pool [33]. The expression of the NCAM, considered an indicator of neurogenesis and neuronal plasticity, is altered in the brain and cerebrospinal fluid of AD patients compared with controls [36, 37]. The decrease of NCAM expression has been associated with cholinergic system alterations in the prefrontal and temporal cortex of humans [38] and inversely associated with phosphorylated tau, the other hallmark of AD pathology, in the murine entorhinal cortex [39]. *In vitro* studies using glioblastoma cells shown that CD44 induced neurodegeneration through the phosphorylation of tau [40] and attenuated the activation of the Hippo signaling pathway [41], recently found altered in several AD-related brain regions and proposed as an early event in AD development [42]. Furthermore, increased CD44 gene expression has been reported in lymphocytes derived from AD patients [43].

The effect of SCFAs and LPS on amyloid pathology: Direct and indirect via endothelial dysfunction and systemic inflammation

Direct evidences of the impact of SCFAs and LPS on amyloid aggregation come from *in vitro* and preclinical studies. Butyrate, but not acetate, inhibited the *in vitro* aggregation of soluble and neurotoxic $A\beta_{42}$ aggregates by interfering with the initial protein-protein interactions [14]. LPS potentiated the

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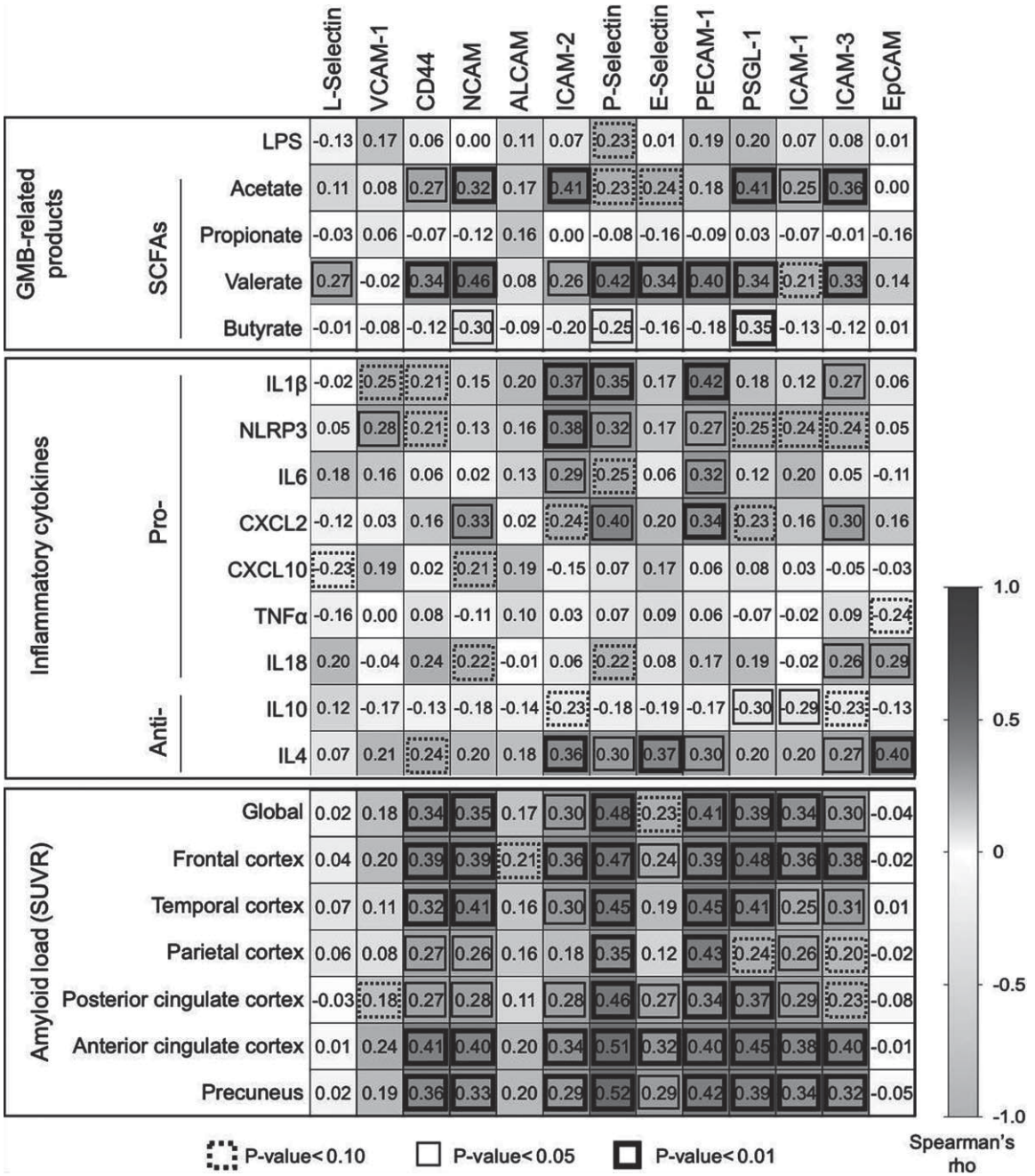


Fig. 4. Association matrix of circulating markers of endothelial dysfunction with circulating GMB-related products and cytokines and cortical amyloid load. Columns represent adhesion molecules; rows represent GMB-related products, inflammatory mediators and cortical SUVR uptake. Spearman's rank correlation analyses included data for 67 subjects for endothelial dysfunction markers, SCFAs and amyloid load, 61 subjects for endothelial dysfunction markers and cytokines, 52 subjects for endothelial dysfunction markers and LPS. Figures denote Spearman's rank correlation coefficient values. These are also represented on a yellow to purple color scale to facilitate the appreciation of association patterns. Significance is denoted by dotted and solid cell borders.

Aβ fibrils formation *in vitro* [44] and, when directly injected into the ventricles of rats, reproduced many of the inflammatory and pathological features seen in AD brains [45].

An indirect contribution of SCFAs and LPS on AD development via tissue-blood barrier impairment (gut-blood barrier (GBB) first and then the BBB) has been also proposed. Considering the gut

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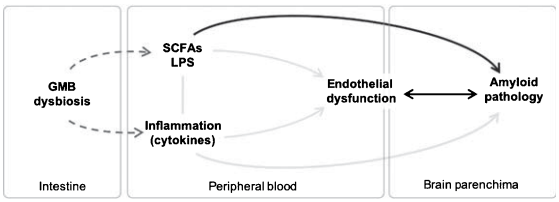


Fig. 5. Possible pathophysiological mechanisms for the GMB in AD. Straight arrow tones denote the strength of the association based on rho mean absolute values of the significant ($p < 0.05$) correlations: light grey, rho 0.30–0.35; dark grey, rho 0.4; black, rho > 0.5.

interface of the GBB, butyrate is important for maintaining the colonic epithelium. Indeed, it has been shown to enhance barrier function by supplying energy to intestinal epithelial cells and by increasing junctional integrity [46]. Conversely, LPS increased intestinal tight junction permeability [47]. At the brain interface of BBB, SCFAs, mainly butyrate, increased the expression of tight junction proteins in the frontal cortex and hippocampus of mice [48]. Systemic LPS and its components, known to be able to infiltrated the brain [49], induced BBB structural and functional alterations including the activation of the transcription of pro-inflammatory and cytotoxic pathways in astrocytes [50] and breakdown of inter-cellular tight junctions [51]. Considering the vascular interface of the barriers, butyrate acts as histone deacetylase inhibitor, transcriptional modulator, and anti-inflammatory molecule on microvascularity [52] and, conversely to acetate, decreased the endothelial Nlrp3 inflammasome assembly and IL1β production formation and activation in endothelial cells [53]. LPS activation of endothelia cells results in the production of various proinflammatory mediators, including IL6, and ultimately cellular injury [54]. Butyrate and LPS as well as acetate act as anti- and pro-inflammatory stimuli, respectively, also on innate and adaptive immune response. Butyrate promotes an antibacterial activity in intestinal macrophages and restricts bacterial translocation [55] while acetate mediates the production of cytokines (IL6, CXCL1, and CXCL2) by intestinal epithelial cells [56]. Butyrate ameliorated the pro-inflammatory response of immune cells to antigen stimulus as LPS by expanding the Treg cell populations [57] and by reducing the expression of pro-inflammatory cytokines (i.e., IL-6) in human mature dendritic cells [58].

The effect of cytokines on amyloid pathology: Direct and indirect via endothelial dysfunction

In addition to the pathways described above, cytokines might have a direct effect on amyloid aggregation or indirect via endothelial dysfunction. Increased local and systemic expression of cytokines have been reported in AD patients [59]. Cytokines as IL-1β and TNF-α are produced by microglia and astrocytes in response to exogenous and endogenous insults [60] or are selectively transported into the brain by the BBB [61]. Inflammatory mediators as IL1, IL6, IL18, TNF-α, and TGF-β1 affect AβPP expression level [62] and metabolism [63] possibly facilitating its amyloidogenic processing and increasing Aβ production and deposition. Moreover, chemokines produced in response to amyloid deposition, are responsible for the recruitment of peripheral immune cells, such as monocytes [64], neutrophils, and T cells [65], favoring the extent of local inflammation. These findings can be interpreted in the light of the emerging role of $A\beta_{42}$ as a physiological mediator of the innate immune system [1] and as an antimicrobial peptide with functions in the cerebral innate immune system [66]. Thus, its production in the brain may be a protective response to bacterial and viral infections [67]. In line, synthesized small-molecule inhibitors targeting *Porphyromonas gingivalis*, the keystone pathogen in chronic periodontitis identified in the brain of AD patients, reduced the brain bacterial load, blocked $A\beta_{42}$ production, reduced neuroinflammation, and rescued neurons in the hippocampus of infected mice [68]. Besides, it is also plausible that amyloid deposition in the brain is the results of age-related defects in the immune signals that monitor the molecular mechanisms implicated in its production, degradation, and clearance.

It is well known that BBB endothelial cells respond to inflammatory stimuli by generating vasoactive substances and through the modification of tight junction structures that increased barrier permeability [69]. Specific inflammatory mediators have been shown to be associated with the evolution of the BBB disruption [70]. Moreover, IL1β induced changes in BBB permeability [71] and the pathological relocation to the BBB of a chemokine (CXCL12) linked to leukocyte infiltration into the CNS [72]. IL-6 has been shown to disrupt the integrity of the BBB in rat brain endothelial cells [73], while NLRP3 deficiency ameliorated cerebral injury in mice after ischemic stroke by reducing infarcts and BBB damage [74].

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The theory of inflammaging describes the low-grade, chronic, systemic inflammation in aging, in the absence of overt infection ("sterile" inflammation) [75]. While the features of inflammaging remain "normal" or "subclinical" in many elderly individuals, a portion of individuals (postulated to have a "high responder inflammatory genotype") may shift to age-associated diseases [76]. This shift has been proposed to serve as a prodrome or an exacerbating factor for development of AD [77]. Among the major identified sources of inflammaging, there are endogenous host-derived cell debris, amyloids, free radicals, immunosenescence as well as products and metabolites produced by the GMB [78]. Thus, our results fit well the hypothesis that poses inflammaging as the link between the gut microbiome alteration, that triggers and sustains systemic inflammation, and the inflammation leading the onset of the pathological hallmarks of AD [79].

This study has a number of limitations. First, we have not studied the effect of *APOE* genotype on the reported associations. The allele $\varepsilon 4$ of *APOE* is a known risk factor for brain amyloidosis and AD. While the effect of *APOE* genotype on GMB composition is unknown, the role of host's genome on the selection and growth of gut bacteria is well known [80], and might be responsible for some of the variance of the effects observed in the current study. Second, we have not taken into consideration oxidative stress, known to be contributes to endothelial damage [81–83], Aβ accumulation [84] and detected in the early stage of AD, even prior to the marked Aβ accumulation [85, 86]. Interestingly, GMB appears to be an electron acceptor and, by shaping the redox potential of the gut, influences the permeability of the intestine [87]. Third, the present study shares several aspects with a recent paper on the GMB in patients with AD [8]. However, differently from that work, here we have studied [^{18}F]-Florbetapir uptake as continuous variable, gaining in sensitivity, and included the evaluation of functional GMB-related variables such as LPS and SCFAs. Last, some of the studied markers were not available for all study participants. The research programs that contributed the data to the current study are still ongoing and recruiting participants, and future studies with a larger study population will allow to verify or falsify the current findings and also to evaluate other important biomarkers (i.e., *APOE* genotype, tau pathology, and oxidative stress biomarkers) not considered in the present manuscript.

In conclusion, we report a novel association of GMB-related products and systemic inflammation with brain amyloidosis possibly via endothelial dysfunction, suggesting that SCFAs and LPS represent candidate pathophysiologic links between the GMB and AD pathology. Reduction of butyrate together with increased acetate, valerate and LPS levels may compromise the tissue-blood barrier integrity, cause and sustain low-grade systemic inflammation and, ultimately, enter the CNS and facilitate the AD pathological cascade. Based on these evidences, examination of gut microbiome could present potential avenues and opportunities to discover and develop specific microbial signatures that are associate with the prognosis of AD progression. Ultimately, interventions that modulate the gut microbiome and enrich beneficial bacteria and bacterial metabolites may be helpful in ameliorating, preventing or slowing neurodegeneration.

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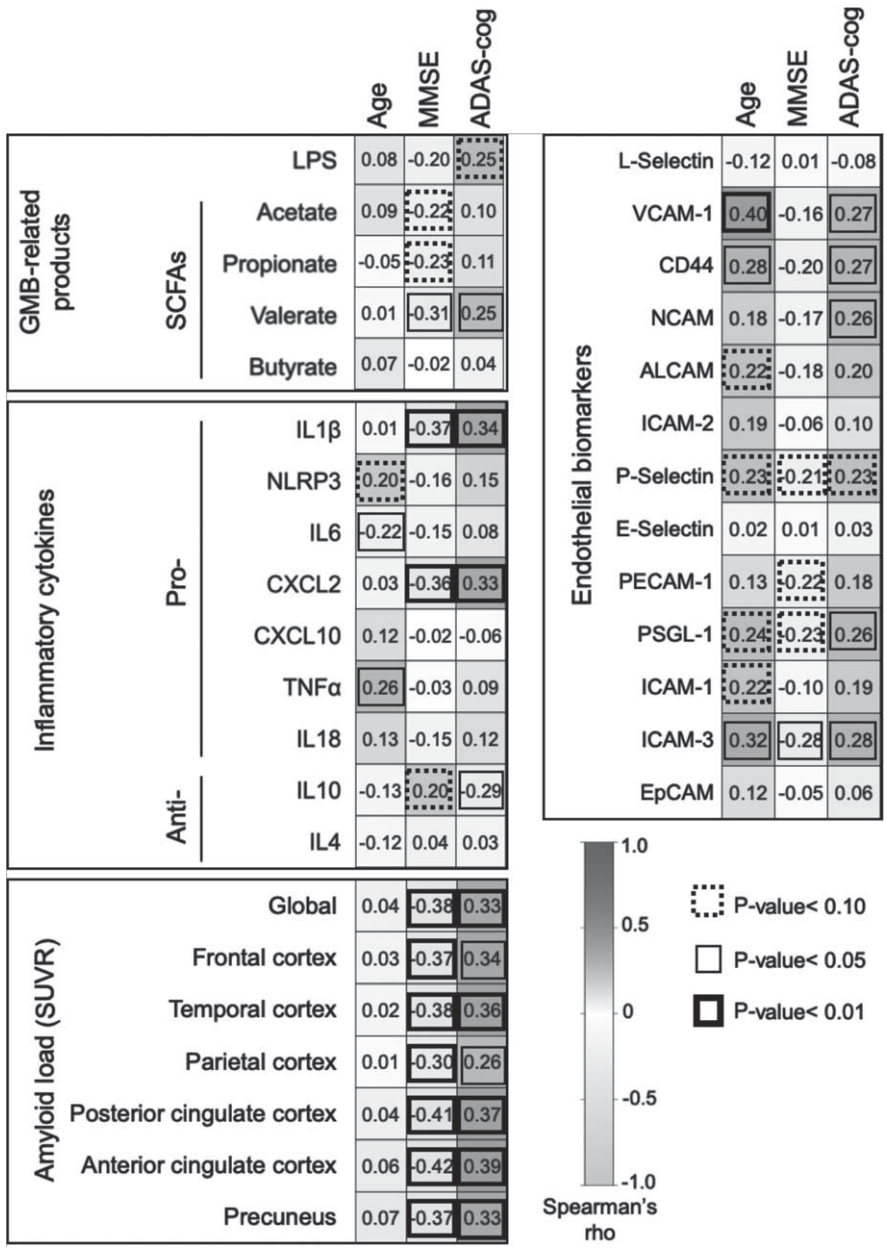
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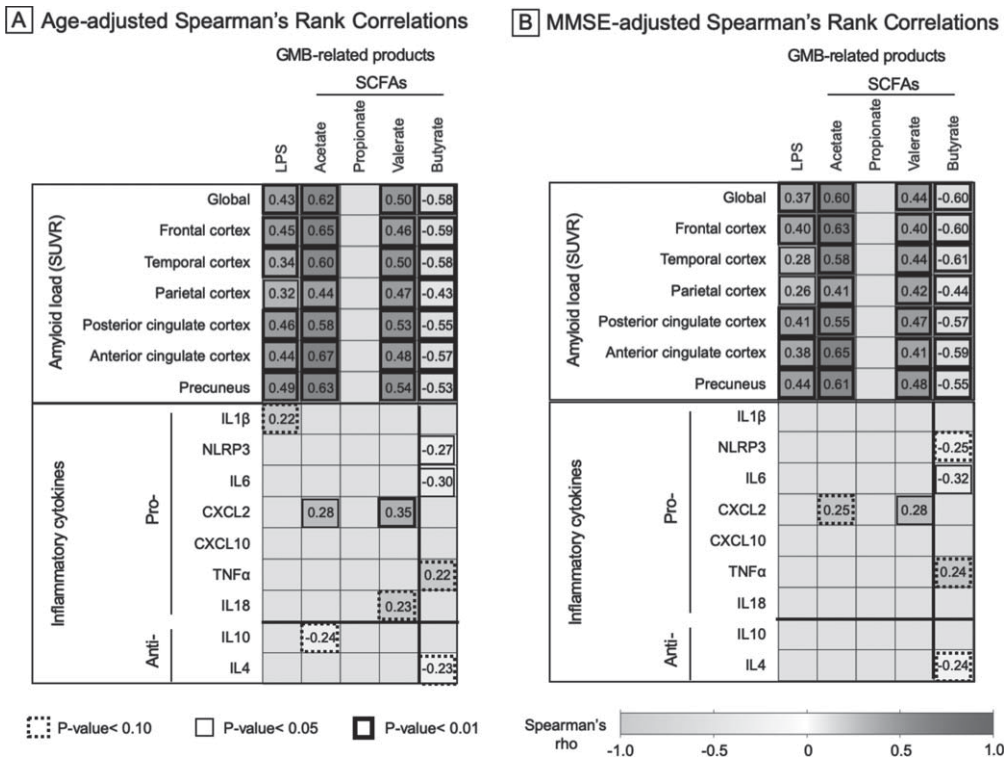
SUPPLEMENTARY MATERIAL

Short-Chain Fatty Acids and Lipopolysaccharide as Mediators Between Gut Dysbiosis and Amyloid Pathology in Alzheimer's Disease



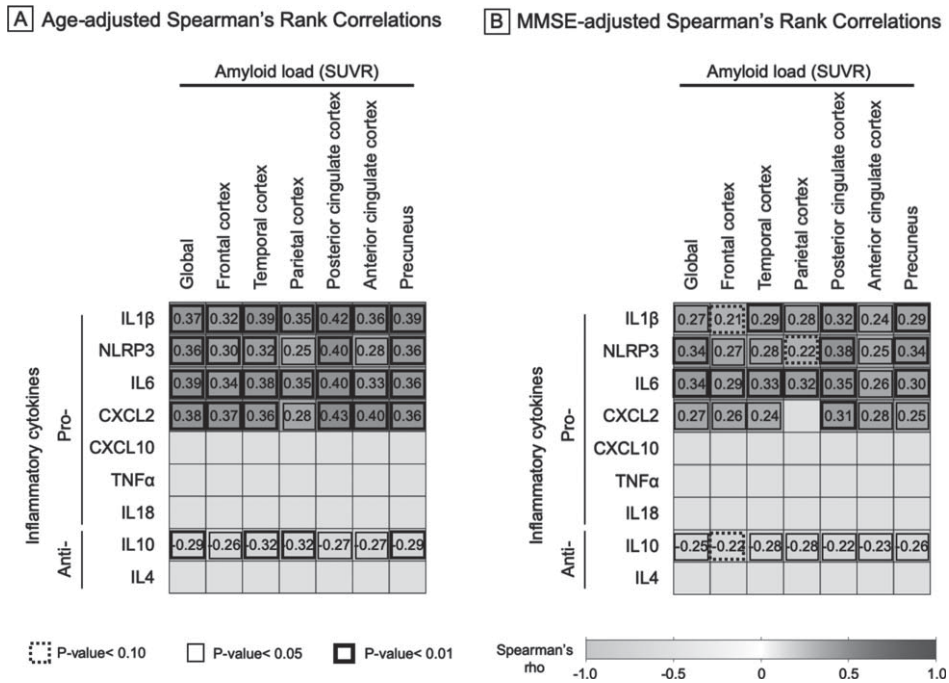
Supplementary Figure 1. Association matrix of age and global cognition with circulating endothelial biomarkers, GMB-related products, cytokines and cortical amyloid load. Columns represent age and measures of global cognition (MMSE and ADAS-cog); rows represent GMB-related products, inflammatory mediators, cortical SUVR uptake and endothelial biomarkers. Spearman's rank correlation analyses included data for 67 subjects for age, global cognition, endothelial dysfunction markers, SCFAs, 83 subjects for age, global cognition and cytokines, 61 subjects for age, global cognition and LPS, 89 subjects for age, global cognition and amyloid load. Figures denote Spearman's rank correlation coefficient values. These are also represented on a yellow to purple color scale to facilitate the appreciation of association patterns. Significance is denoted by dotted and solid cell borders.

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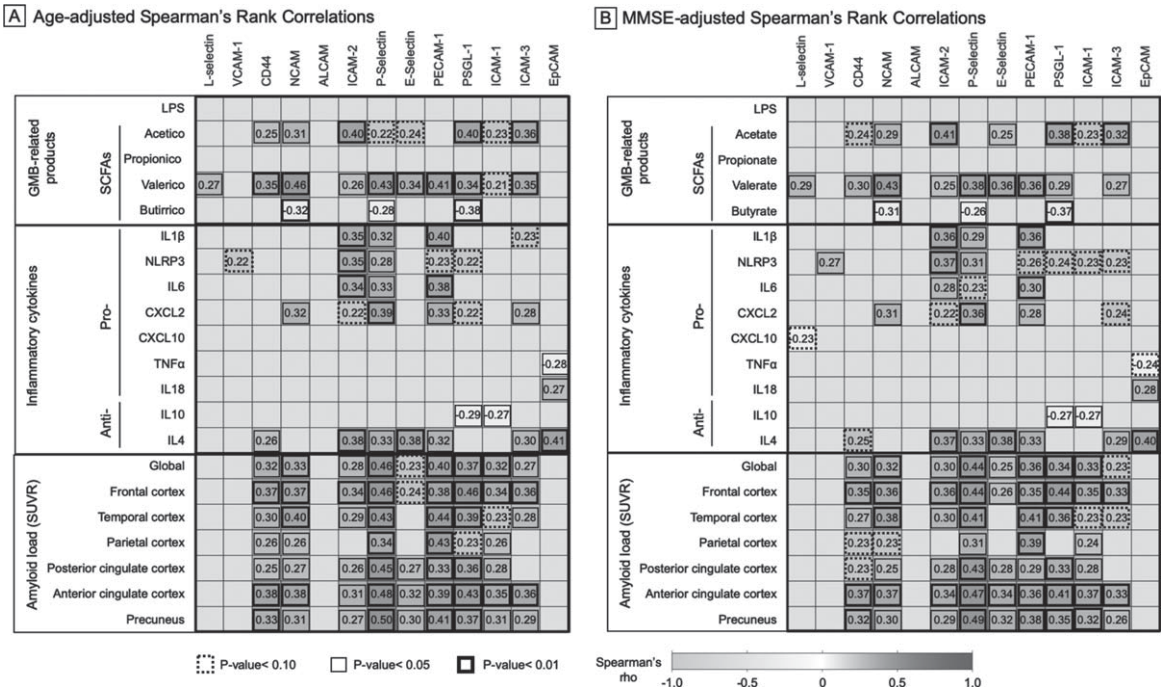
Supplementary Figure 2. Age-adjusted (A) and MMSE-adjusted (B) Spearman's Rank Correlations of circulating GMB-related products with cortical amyloid load and cytokines. Adjusted Spearman's rank correlations were performed only between variables found associated ($p < 0.10$) in Figures 1 and 3. Columns represent GMB-related products; rows represent cortical SUVR uptake and inflammatory mediators. Spearman's rank correlation analyses included data for 61 subjects except for SCFAs and amyloid load, where 67 subjects were included. Figures denote Spearman's rank correlation coefficient values adjusted for age (A) or global cognition measured using MMSE (B). These are also represented on a yellow to purple color scale to facilitate the appreciation of association patterns. Significance is denoted by dotted and solid cell borders.

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Supplementary Figure 3. Age-adjusted (A) and MMSE-adjusted (B) Spearman's Rank Correlations of circulating cytokines with cortical amyloid load. Adjusted Spearman's rank correlations were performed only between variables found associated ($p < 0.10$) in Figure 2. Columns represent cortical SUVR uptake; rows represent inflammatory mediators. Spearman's rank correlation analyses included data for 83 subjects. Figures denote Spearman's rank correlation coefficient values adjusted for age (A) or global cognition measured using MMSE (B). These are also represented on a yellow to purple color scale to facilitate the appreciation of association patterns. Significance is denoted by dotted and solid cell borders.

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Supplementary Figure 4. Age-adjusted (A) and MMSE-adjusted (B) Spearman's Rank Correlations of endothelial dysfunction with circulating GMB-related products and cytokines and cortical amyloid load. Adjusted Spearman's rank correlations were performed only between variables found associated ($p < 0.10$) in Figure 4. Columns represent adhesion molecules; rows represent GMB-related products, inflammatory mediators and cortical SUVR uptake. Spearman's rank correlation analyses included data for 67 subjects for endothelial dysfunction markers, SCFAs and amyloid load, 61 subjects for endothelial dysfunction markers and cytokines, 52 subjects for endothelial dysfunction markers and LPS. Figures denote Spearman's rank correlation coefficient values adjusted for age (A) or global cognition measured using MMSE (B). These are also represented on a yellow to purple color scale to facilitate the appreciation of association patterns. Significance is denoted by dotted and solid cell borders.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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New Approaches to Profile the Microbiome for Treatment of Neurodegenerative Disease

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Abstract. Progressive neurodegenerative diseases represent some of the largest growing treatment challenges for public health in modern society. These diseases mainly progress due to aging and are driven by microglial surveillance and activation in response to changes occurring in the aging brain. The lack of efficacious treatment options for Alzheimer's disease (AD), as the focus of this review, and other neurodegenerative disorders has encouraged new approaches to address neuroinflammation for potential treatments. Here we will focus on the increasing evidence that dysbiosis of the gut microbiome is characterized by inflammation that may carry over to the central nervous system and into the brain. Neuroinflammation is the common thread associated with neurodegenerative diseases, but it is yet unknown at what point and how innate immune function turns pathogenic for an individual. This review will address extensive efforts to identify constituents of the gut microbiome and their neuroactive metabolites as a peripheral path to treatment. This approach is still in its infancy in substantive clinical trials and requires thorough human studies to elucidate the metabolic microbiome profile to design appropriate treatment strategies for early stages of neurodegenerative disease. We view that in order to address neurodegenerative mechanisms of the gut, microbiome and metabolite profiles must be determined to pre-screen AD subjects prior to the design of specific, chronic titrations of gut microbiota with low-dose antibiotics. This represents an exciting treatment strategy designed to balance inflammatory microglial involvement in disease progression with an individual's manifestation of AD as influenced by a coercive inflammatory gut.

Keywords: Antibiotics, Alzheimer's disease, microbiome, neurodegenerative disease, neuroinflammation

INTRODUCTION

People are living longer now than ever before [1, 2]. With increasing aging populations in some of the most populous countries in the world, there is an unfortunate concomitant increase in those suffering from age-related, chronic diseases, such as dementia

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[2, 3]. Dementia is an umbrella term for brain diseases characterized by gradual cognitive decline of mental faculties, including impairment of memory, reasoning, judgment, and language, that are beyond the normal scope of healthy aging [4]. The most prevalent type of dementia, and the subject of this review, is Alzheimer's disease (AD), a progressive neurodegenerative disorder defined by the extracellular deposition of amyloid plaques largely composed of amyloid β-protein (Aβ) and intracellular neurofibrillary tangles of hyperphosphorylated tau protein [5, 6]. The plaques and tangles that are the pathological hallmarks of AD delineate it further as a protein misfolding disease, where aberrant protein structures and aggregates have been shown to be synaptotoxic to neurons and associated with the debilitating memory deficits of AD [5, 7]. Curiously, the vast majority of AD presents sporadically, and the extent of plaque deposition does not always dictate whether a person will exhibit memory deficits [7]; facts both of which have led many to explore other peripheral factors that lead to neurodegeneration [8–11].

The peripheral factor of interest in this review is the gut microbiome and the extent to which its dysbiosis and metabolite production may influence neurodegenerative disease progression. We shall explore the emerging connections between age-related immune dysregulation and inflammation, neurodegenerative disease, and the gut. This review will also highlight the necessity for AD staging and associated AD microbiome/metabolite profiles as justifications for implementing the low-dose, chronic use of targeted antibiotics and other potential drugs to titrate toxic gut bacteria. This approach may enhance the innate immune effect of protective, peripheral microglia cells over time to treat neurotoxic mechanisms associated with the progression of AD before irreversible decline sets in.

Aging and immune function

The greatest risk factor for developing AD is age [12]. Aging is also associated with a chronic state of low-grade inflammation called "inflammaging" that is characterized by higher concentrations of circulating pro-inflammatory mediators such as IL-6 and C-reactive protein [13]. Persistent, dysregulated inflammatory responses are thought to be responsible, in part, for the elderly's increased susceptibility to infection [14]. An aging person's immune system typically reacts less robustly to environmental stimuli than a younger person's does, with altered B and T cell development and differentiation resulting in an overall decrease in functional specific immunity [14]. The decline in lymphocyte diversity has also been associated with chronic inflammation, as well as with viral and bacterial infections, particularly by the expansion and over-stimulation of memory T cells and exhaustion of the naïve T cell pool [15]. Additionally, a cohort of 40 AD patients were discovered to have higher proportions of activated, late-differentiated $CD4^+$ $CD25^+$ T cells and decreased portions of a naïve T cell pool, potentially due to chronic stimulation of the immune system [16]. It is important to note that a generalized dysregulation of aging immune function does not singularly guarantee the development of dementia as gene expression and promotion of lymphocyte diversification can be dependent on diet, exercise, hormone regulation, chemical exposure, and pathogen infection [17, 18].

Due to the difficulty in separating age-causative inflammation from exogenous factors that induce inflammation, it remains unclear to what extent which inflammatory mediators relative to others will result in harmful systemic inflammation nor is there a known, definitive source of inflammaging [19]. During an acute inflammatory response, such as to an injury or infection, the innate immune system regulates the activation of an adaptive immune response that normally resolves after pathogen clearance that is followed by wound-healing mechanisms. Chronic inflammation is thought to be the result of chronic stimuli, such as cellular debris of damaged host cells or other damage-associated molecular patterns (DAMPs), that the adaptive immune system recognizes and upregulates an innate immune response for removal [20]. A modest decrease in number of circulating peripheral dendritic cells in aging may contribute to less robust adaptive immune responses as we age [21–23]. This effect is worsened with corticosteroid administration, marked by reduction in IFN-α production and a decrease in number of circulating dendritic cells [24]. Reduction in the body's ability to adequately detect and address emergent infections may prolong inflammation through impairment of T cell differentiation to quiescent states [15], suggesting that the aging body is inclined to exhibit chronic inflammation due to dysregulation in the immune system.

There is no known, definitive cause of AD. However, in light of the multifactorial nature of the disease, it may be that AD is the cumulative result of a decades-long inflammatory response to CNS stressors. There is emerging evidence of viral and

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microbial presence in the AD brain and potential connections of gut microbiome diversity to neuroinflammation, amyloidosis, and neurodegeneration. For example, multi-omic experiments found that human herpesvirus HHV-6A and HHV-7 are more prevalent in AD brain compared to controls, and that multiple viruses, most notably HHV-6A, are linked to AD risk genes including ones related to processing amyloid-β protein precursor (AβPP) [25]. Importantly, amyloid β-protein (Aβ) results from the proteolytic cleavage of AβPP and Aβ fibrils are suggested to have broad spectrum anti-microbial properties capable of agglutination and disrupting cellular membranes to induce ion dyshomeostasis [26]. Further, low-grade herpesvirus, fungal, and bacterial infections have been associated with seeding Aβ deposits in the brain, suggesting that amyloidosis could be a generalized, innate immune response to the persistent invasion or incorrect perception of pathogens within the CNS [26–28]. The sources of peripheral inflammation are myriad but the progression of AD neurodegeneration is increasingly linked to heightened levels of circulating cytokines that stimulate further cytokine secretion in the CNS, by way of signaling via the vagus nerve or by directly crossing the blood-barrier to activate microglia and astrocytes toward a proinflammatory state [29]. Proinflammatory microglia activation is associated with other neurodegenerative diseases, and recent studies with transgenic ALS model mice show that peripheral macrophages and spinal cord microglia have distinct gene expression regulation patterns and that modulation of peripheral macrophages shift microglia away from damaging pro-inflammatory behavior to neuron and synapse support [30]. Many more studies will be required to better understand the interplay between the surveillance mechanisms at play in the CNS that contribute to neurodegeneration. Identifying how peripheral factors may influence inflammatory mechanisms for a particular neurodegenerative disease will provide much needed insight for potent, targeted therapeutics and low-dose, chronic treatments that could modulate harmful microbes.

Early neurodegenerative disease biomarkers and efforts to profile the microbiome

Owing to the fact there are no efficacious treatments available to slow or stop the progression of neurodegeneration that can develop over decades, an important proactive approach to combating dementia will involve identification of early biomarkers associated with increased risk for disease before symptomatic onset occurs. A cohort study of 3,565 individuals found that the relationship between cerebrospinal fluid (CSF) levels of Aβ and tau by categorization into amyloidosis and neurodegenerative NIA-AA profiles was profile-dependent and that higher $A\beta_{42}$ CSF levels are related to earlier stages of AD [31]. This further supports evidence that Aβ pathology may precede and influence tau pathology in AD [32–34]. A longitudinal study of 2014 participants within the Alzheimer's Disease Neuroimaging Initiative database developed a stochastic Markov model to predict the time it takes to present with clinical AD based on four broad biomarker categories, including: cognitive functional score; CSF and plasma levels of Aβ, neurofilament light (NfL), and tau; and normalized MRI volumetric measurements of the brain [35]. Although the study could not identify single biomarkers that delineate the clinical progression of AD for an individual, NfL was identified as a potential early marker for neurodegeneration [35]. Additionally, population CSF levels of $A\beta_{42}$ increased at higher rates years before tau CSF levels did, before the symptomatic onset of AD [35]. Biomarker studies are widely important to help uncover how a disease presents itself, but age-related diseases that develop over long timeframes vastly complicate this process. CSF biomarkers observed at late stages of disease may define how the brain has compensated for extensive, irreversible neuronal damage but may not fully allude to earlier, deterministic neuropathologic mechanisms. Biomarkers present in serum may reveal information that is difficult to delineate from other concomitant health issues so it will be necessary to exploit a diverse array of biological sampling to capture a broad picture of a given neurodegenerative state so that targeted, efficacious treatment becomes a reality.

Determination of a neurodegenerative disease microbiome profile, through identification of pathogenic microbes and metabolic pathways, represents an appealing way to tailor a treatment plan based on a respective patient's manifestation of dementia. The general concept of microbiome profiling has been thoroughly explored in animals and in small human cohorts. Some early efforts were made to identify the stable, healthy human microbiome composition into clustered groupings called enterotypes. The enterotype concept was introduced by comparative 16S rRNA metagenomic analysis of fecal samples from 22 individuals from 4 countries when three

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enterotypes emerged, each with *Bacteroides*, *Prevotella*, and *Ruminococcus* genera most abundant, respectively [36]. As the healthy human gut composition is complex, it is important to identify beneficial commensal organisms from pathogenic ones, particularly for the context of chronic disease. It has been noted that grouping together microbiome constituents into distinct clusters of species, as is done with enterotypes, may unfortunately over-simplify and overlook the contribution of lesser abundant species to the health of the host, or even mask the inter-variability of an individual's enterotype constituents to another of the same enterotype [37]. This is further complicated by confirmed diagnosis of AD occurring at autopsy which is obviously much too late for early intervention. Inclusion parameters to determine the composition of the AD gut microbiome must be multi-factorial to account for any differences related to gender, age, cognitive function, medication use, *APOE* genetic markers, brain imaging, and biomarkers.

Treatment of neurodegenerative disease is hindered largely in part by a lack of analytical biomarker testing that may predict disease progression early on, most importantly before the advent of cognitive decline. To broadly address this need in the context of general health, a comparative study of 4,347 publicly available human fecal metagenomes produced a microbiome signature of 50 species associated with the healthy human gut, determined by the Gut Microbiome Health Index, and may predict whether an individual possesses a healthy microbiome based on the overall abundance of health-prevalent species relative to health-scarce species [38]. Even though the results of these kinds of studies vary considerably, a major asset is the data is very often publicly available and future efforts to determine a metabolic microbiome profile for the staging of neurodegenerative disease will be made through new and novel computational methods and statistical analyses to assess the immense interstudy data that is rapidly accumulating. An observational study is planned with cohorts of at least 250 subjects diagnosed with mild and moderate stage AD for stool collection and blood sampling analysis. Preparation of DNA libraries from stool samples for shotgun metagenomic sequencing, PCR amplification of 16S rRNA, and unbiased mass spectrometry-based metabolomics of blood samples are robust methods to determine microbial and protein composition from complex, human-derived media [39–42]. The study is proposed to be adequately powered (unpublished data, Elmaleh 2020), utilizing detectable effect sizes from the human microbiome project [43] for relative abundances among rare and common taxa. Although currently in early stages of progress, the study will broadly expand the ability to determine AD staging as it relates to gene-specific microbial and human metabolite profiles. It remains very hopeful that the pooling of such data generated, its creative analysis, and collaboration from many research groups will uncover the inter-related variables most associated with neurodegenerative processes, such as human metabolomic profiling of prodromal AD, that occur before and during the development of AD.

Figure 1 provides an overview for an approach that allows for targeted, chronic titrations of the gut microbiome based upon a specific neurodegenerative disease profile. An individual's profile is generated to connect the extent of symptomatic decline through cognitive testing, such as global Clinical Dementia Rating-sum of boxes (CDR-SOB) and Mini-Mental State Examination (MMSE), to biological markers observed in circulating metabolites and constituents of an individual's fecal microbiome. With a large enough cohort, cross-comparisons of individual profiles can be made to identify commonalities among specific neuroactive metabolites and microbiota to diagnose and treat early-stage dementia. In the proceeding sections, we will review the connections between aging, inflammation, the microbiome and neuroactive metabolites, and current work in this burgeoning field to highlight the potential use of chronic, low-dose antibiotics to treat neurodegenerative disease.

Aging, microbiome, and inflammation

The body's CNS is protected from exposure to peripheral media very selectively through the blood-brain barrier (BBB). Considering AD as a cumulative result of neuroinflammation-induced neurodegeneration is bolstered by the increase in permeability of the BBB as we age. Magnetic resonance imaging experiments have shown that age-related BBB deterioration begins in the hippocampus and worsens with mild cognitive impairment and in AD [44]. Numerous entities have been associated with BBB permeability including Aβ vascular deposition with decreased expression of tight junction claudin-1 and claudin-5 proteins and increase in matrix metalloproteases MMP-2 and MMP-9 [45], neuron-derived perivascular tau protein [46], reactive oxygen species [47], and short chain fatty acids (SCFA) produced from gut microbiota [48].

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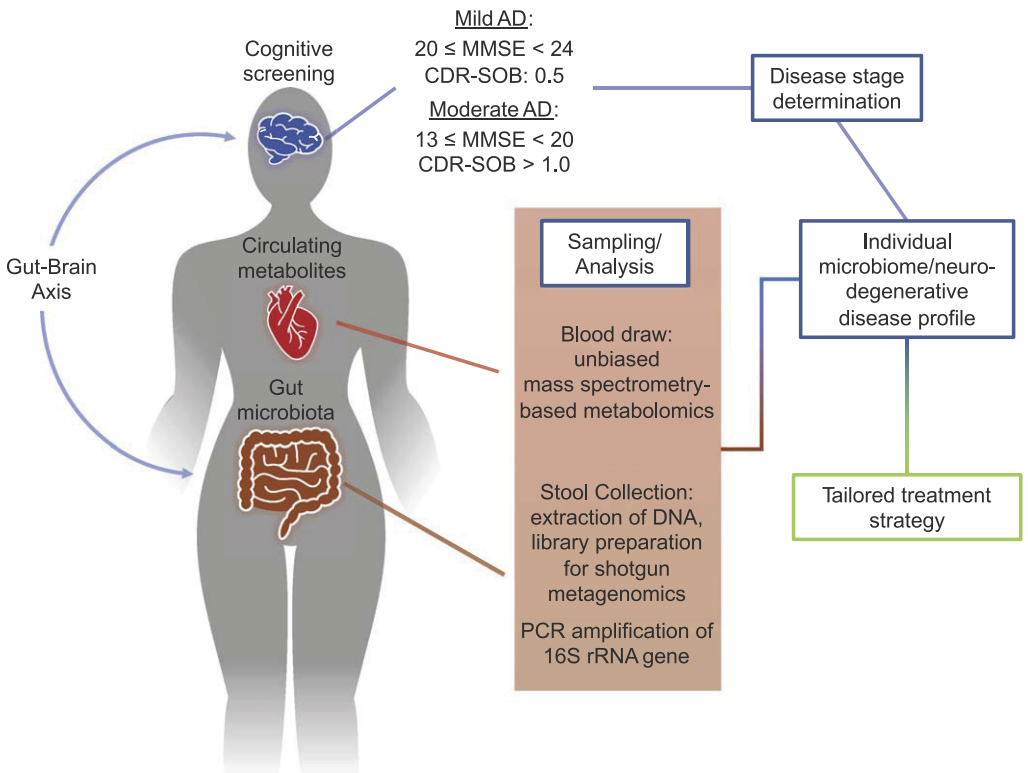


Fig. 1. Safe, chronic titrations of bacterial metabolites as treatment for neurodegenerative disease. Intercommunication along the gut-brain axis by pathogenic resident microbes in the gut are able to influence the progression of neurodegenerative processes. Multi-omic analysis of circulating blood metabolites and feces, coupled with dementia screening, can develop an individual's neurodegenerative profile at their respective stage of disease. This approach, when married with a large sample set, may uncover specific microbial species and metabolite levels relevant to a particular stage of cognitive decline. Using the generated profile, a tailored combination treatment plan can be constructed that addresses an individual's presentation of disease. MMSE, Mini-Mental State Examination; CDR-SOB, Clinical Dementia Rating – sum of boxes.

A more permeable BBB could be associated with heighten immune responses in the CNS. Microglia provide immune surveillance within their microenvironment in the CNS and are essential to the maintenance and health of neurons. In the aging brain, microglia may be morphologically de-ramified and "primed" toward a pro-inflammatory state, as evidenced by increased MHC II receptor and mRNA expression of TNF-α, IL-6, and IL-1β, as well as anti-inflammatory cytokines IL-10 and TGFβ1 upon lipopolysaccharide (LPS) challenge [49, 50]. Interestingly, LPS administration in aged mice increased expression of IL-10 in primed microglia but did not result in decreased production of pro-inflammatory IL-1β [50], a result that may be explained by an age-associated decline with IL-10 and TGFβ signaling pathways within aging microglia and astrocytes [51]. Astrocytes normally support BBB endothelial cell integrity and are involved in the recruitment and restriction of immune cells from the periphery into the CNS through cytokine and chemokine secretion after injury [52]. Transgenic *APOE* mouse models of AD show that astrocyte-derived *APOE4* involves the inflammatory cytokine cyclophilin A (CypA) to induce BBB dysfunction through activation of a CypA—NF-κB—MMP-9 signaling pathway that causes neurovascular deficits and may initiate neurodegeneration [53].

Rethinking AD as more than just a neurodegenerative disease has brought about efforts to further delineate pathological from commensal or symbiotic residents of the gut by identifying their physiological abundance and exploring the contributions to metabolism and how their dysfunction may play a role in pathogenesis. Fecal transplant studies in animals have shown that the microbiome can exhibit substantial neurocognitive effects after transplantation, even to some degree restoring cognitive impairment (Table 1).

The composition of the healthy human gut is highly variable, with thousands of possible bacterial species represented and potentially many more due to the

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Table 1
Fecal transplantation in AD animal models

AD Model	Transplant Method	Transplant Effect	Reference
Male APP/PS1 (APPswe/PS1dE9) mice	Prior to transplant, APP/PS1 mice were given 3-day antibiotic regimen of vancomycin, ampicillin, and metronidazole. Fecal solutions prepared daily from non-transgenic wild type mice were administered intragastrically to APP/PS1 mice daily for 4 weeks	Cognitive improvement gauged by MWM and ORT Decreased amyloid deposition in brain, levels of $A\beta_{40}$ and $A\beta_{42}$, and tau phosphorylation Improved synaptic plasticity with increased synapsin 1 and PSD-95 Significantly recovered phyla *Bacteroidetes* predominance and corresponding butyrate levels post-transplant	[172]
$ADLP^{APT}$ mice (exhibits both Aβ deposition and tau pathology; systemic inflammation via increased lipocalin 2 expression and gut dysbiosis)	Intragastric fecal transplant from WT mice 5 days per week for 16 weeks Second group: ABX antibiotic cocktail [152] administered to $ADLP^{APT}$ mice prior to 4-week fecal transplant from untreated WT or $ADLP^{APT}$ mice	Reestablishing healthy gut microbiota improved gut barrier integrity and reduced brain amyloid burden and tau pathology Improved short-term (Y-maze) and long-term (Contextual fear-conditioning) memory Reduced gut and systemic inflammation Possible mitigation of CNS immunological priming with decrease in Iba-1-positive microglia and GFAP-positive astrocytes in frontal cortex	[173]
AD rats: Male SD rats with left lateral ventricle injection of pre-aggregated $A\beta_{42}$ solution This study examined the effect of XAN, a triterpenoid from *Xanthoceras sorbifiolia*, to modulate gut microbiota composition to improve AD pathology, despite XAN's minimal BBB permeability	3 days post $A\beta_{42}$ injection through day 19, rats were given daily intragastric fecal transplants from rats already 17–18 days into sham group (no $A\beta_{42}$ injection) or from rats already 17–18 days into a high-dose group of intragastric XAN treatment	Rats given fecal transplants from XAN-treated AD rats improved learning and memory based on Y-maze and MWM tests and decreased hippocampal neuron loss compared to AD rats and AD rats given fecal transplants from sham group XAN was associated with gut microbiota *Firmicutes*/*Bacteroidetes* modulation	[174]
Two-month-old pseudo germ-free C57BL/6 mice	C57BL/6 mice given 1000 mg/L each of ampicillin, neomycin sulfate, and metronidazole in drinking water for 14 days ad libitum prior to daily gavage of fecal transplants from either 7-month-old SAMP8 or SAMR1 mice for 14 days	Mice with fecal transplant from SAMR1 mice but not from SAMP8 mice improved cognitive behavior and escape latency in MWM Abundances of 14 bacteria from six phylogenetic levels implicated with cognitive function and fecal transplant including marked changes in phylum *Firmicutes* decrease (SAMP8), order *Bacteroidales* increase (SAMP8 and SAMR1), and genus *Parasutterella* increase (SAMP8) relative to control	[175]
Three-month-old male SAMP8 mice This study examined the effect of chronic noise stress on microbiota-gut-brain axis	Post 30-day noise exposure, feces were collected from SAMP8 control mice (<40 dB) and SAMP8 mice in high-noise exposure group (98 dB), prepared and transferred to male 3-month-old SAMP8 mice	Mice receiving fecal transplant from high-noise exposure group had deficient epithelial integrity seen with decrease in CLDN1 and ZO-1 expression; also, higher Aβ deposition in hippocampus compared to mice receiving fecal transplant from SAMP8 control mice	[176]

ABX, antibiotic cocktail including kanamycin, gentamicin, colistin, metronidazole, and vancomycin; $ADLP^{APT,}$ AD-like pathology with amyloid and neurofibrillary tangles; APP/PS1, amyloid precursor protein/presenilin-1; BBB, blood-brain barrier; CLDN1, claudin 1; dB, decibels; GFAP, glial fibrillar acidic protein; Iba1, ionized calcium-binding adaptor molecule; MWM, Morris water maze; ORT, object recognition test; PSD-95, postsynaptic density protein 95; SAMP8, senescence-accelerated mouse prone 8; SAMR1, senescence-accelerated mouse resistant 1; SD, Sprague-Dawley; WT, wildtype; XAN, xanthoceraside; ZO-1, tight junction protein 1.

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Table 2
Prevalent taxa along the gastrointestinal tract

Organ	Mouth/Nose	Stomach	Small Intestine		Colon
			Duodenum	Jejunum & Ileum	
Taxa	*Corynebacterium* *Bifidobacterium* *Staphylococcus* *Streptococcus* *Aggregatibacter* *Lactobacillus* *Fusobacterium* *Porphyromonas* *Actinomyces* *Prevotella* *Haemophilus*	*Lactobacillus* *Streptococcus* *Staphylococcus* *Enterobacteriaceae*	*Lactobacillus* *Streptococcus* *Staphylococcus* *Enterobacteriaceae*	*Bifidobacterium* *Bacteroides* *Lactobacillus* *Streptococcus* *Enterobateriaceae*	*Bacteroidesm* *Eubacteriu* *Clostridium* *Peptostreptococcus* *Streptococcus* *Bifidobactrium* *Fusobacterium* *Lactobacillus* *Enterobacteriacae*
Counts	$\sim 10^9$ CFU/mL	10^1–10^3 CFU/mL	10^1–10^3 CFU/mL	10^4–10^7 CFU/mL	10^{10}–10^{11} CFU/mL
pH	Nose: 5.5–6.5 Mouth: 6.6–7.3	1–3	6.7		7
pO_2	~atmospheric	77 mm Hg	33 mm Hg	33 mm Hg	<33 mm Hg

CFU/mL, colony-forming units per milliliter. Compiled from references [55, 177–181].

individual diet and geographical environment, genetics, and immune response [54]. The population and density of gut flora is spatially diverse along the GI tract, which is likely dependent on the pH, oxygen partial pressures, and digestive role of a particular compartment, as well as commensal and symbiotic relationships with the host [55]. A large study of fecal samples from 1,000, age and sex stratified, healthy individuals analyzed by 16S rRNA gene sequencing found that species diversity is largely dominated by the two phyla *Firmicutes* and *Bacteroidetes*, and that gut microbiota composition is significantly determined by an individual's environment and demographics [56]. Table 2 includes some of the most abundant taxa along the GI tract.

In the case of many acute pathological infections, it is tempting to find and accuse a specific microbe as having a causative, aberrant role in any one of these conditions, and understandably so, as the bulk of the microbiome is made up of bacteria, and a lesser extent fungi and parasites, many of which have known and negative connotations in regards to human health [57–59]. These detrimental attributes may be due in part to over expression of would-be normally commensal bacteria relative to others in the gut that can trigger robust inflammatory responses. The relative population of a mucin-degrading microbe, *Ruminococcus gnavus*, can increase in Crohn's disease flare ups, secreting a glucorharmnan polysaccharide that induces significant TNF-α release from dendritic cells through direct activation of the toll-like receptor TLR4 [60]. A fecal microbiome metagenomics study of 20 ALS patients and 20 cognitively normal individuals discovered an increase in *Bacteroidetes* and decrease in *Firmicutes* abundance compared to controls [41]. Another study comparing fecal microbiome of 25 AD patients to those of 25 cognitively normal, age-matched individuals identified a relative increase in *Bacteroidetes* and decreased *Firmicutes* and *Bifidobacterium* in AD [61]. A transgenic Parkinson's disease (PD) mouse study found the presence of gut microbiota was a prerequisite for motor deficits and the promotion of inflammatory microglia activation in an α-synuclein dependent manner, highlighting the interdependence of gut microbes and the neurodegenerative processes in the brain [62]. Interestingly, distinct gut microbiota alterations in *Blautia*, *Akkermansia*, *Escherichia-Shigella*, and *Desulfovibrio* in APP/PS1 AD model mice preceded onset of amyloid deposition and microglia-related neuroinflammation [63], suggesting further that neurodegenerative processes could be influenced by activity within the gut and early biomarker analysis may have diagnostic potential for disease later in life.

Additionally, the inter-individual variability of microbiome populations can be extensive among inflammatory disease states as well as among healthy individuals. Inflammatory bowel disease exhibits significant heterogeneity with dominant microbiota varying from individual to individual but can be of similar diversity levels for an individual regardless of inflammatory status, a factor that has been attributed to epigenetic factors like host DNA methylation and gene expression [64]. The gut microbiome collectively functions like another organ, aiding in

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digestion, elimination of harmful microbes, and the critical maintenance of the gut mucosa that are all essential for proper immune function [65]. The chronic dysregulation of microbiota populations, or dysbiosis, can subsequently impact the body's ability to fight off infections without initiating a larger scale inflammatory response, which has been attributed to inflammatory bowel disease and chronic low-grade systemic inflammation [65]. Shotgun metagenomics analysis of fecal samples of the extremely old (e.g., over 105 years of age) in comparison to other, younger age groups have increased expression of genes involving xenobiotic degradation and altered carbohydrate and lipid metabolism, suggesting that healthy aging may be related to the body's regulation of gut microbiota in response to environmental pollution stressors and dietary habits [42]. Advanced aging is often accompanied by increased weight loss and malnutrition, both of which are increased risk factors for developing AD [66]. *Methanobrevibacter smithii* is the dominant Euryarchaeon constituent of the human gut microbiota and is known to affect polysaccharide digestion by consuming bacterial fermentation end products, increasing the energy utility of ingested food [67]. Populations of methanogenic archaea have higher relative diversity in the gut microbiome of older individuals [68]. Anorexic individuals have increased *M. smithii* abundance compared to normal healthy adults [69] likely due to the body promoting *M. smithii* survival. Increased gut populations of methanogenic archaea are associated with increased adiposity, weight gain, and obesity [70], all of which are related to higher serum levels of pro-inflammatory biomarkers [71]. This systemic inflammation may further trigger primed microglia in progressive disorders like AD-related dementia, developing over decades and presenting differently for every person, and has subsequently made the identification of particular microbes that are directly related to generalized dementia inherently challenging.

The microbiome's role in key neurochemical and neurometabolic pathways associated with stress and progressive neurodegenerative disorders is emerging. Microbial community sequencing approaches, notably 16S rRNA gene sequencing of fecal samples, have been used to identify altered relative microbe population distributions in the gut with several inflammatory conditions including inflammatory bowel disease [72], irritable bowel syndrome [73, 74]; as well as neurodegenerative and psychiatric disorders including Parkinson's disease [75], AD [61, 76], autism spectrum disorder [77–79], schizophrenia [80], and major depressive disorder [81]. It is clear that peripheral microglia and macrophages are involved in the neurodegenerative process. However, these preliminary indication studies need a more substantive determination of the components of the microbiome genetic profiles that modify the CNS and increase or decrease neuroinflammation. It cannot be overstated that the volume of data that emerges from these kinds of studies are immense and careful methodology for its analysis is required. In our planned study, we intend to profile the gut microbial community in early AD subjects with CDR global score of 0.5 and in subjects with AD onset and correlate them with other human metabolic information to uncover specific lines of taxa and gene functions within the microbiome that is associated with both early and moderate disease stages. In contrast to other studies, this study will incorporate at least 250 subjects to allow a significant statistical difference for a more accurate indication of the specific AD staging profile. The identification of such a profile should lead to a tailored treatment design that would be based on the microbial and/or human metabolic profile.

Neuroactive metabolites of the gut

Gut microbiota are in many ways able to influence the CNS directly and indirectly, largely through the secretion of endotoxin and production of a range of metabolites (Table 3). Though some of the neuroactive metabolites provided in Table 3 may be endogenously produced by human metabolism or by microbiota, production of constituents of the tryptophan metabolic pathway is inherently dependent on dietary consumption of the essential amino acid tryptophan precursor that cannot be produced metabolically in humans. It is also of note that neuroactive catecholamines such as dopamine and norepinephrine are enzymatically produced from non-essential L-tyrosine [82]. Dopamine is produced in the cytosol in regions of the midbrain and forebrain and stored in synaptic vesicles until released into the synapse [82]. Dopamine is converted to norepinephrine by dopamine β-hydroxylase with cofactor ascorbate [82]. Both dopamine and norepinephrine, along with neurotransmitters serotonin and γ-aminobutyric acid, can also be produced by gut microbiota to influence host behavior and physiology [83].

The cellular envelope of most gram-negative bacteria is lined with endotoxin, also known as LPS, that contributes to bacterium structural stability and

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Table 3
Neuroactive metabolites produced by gut microbiota and other sources

Metabolites	Sources	Microbiota Taxa Producers	Neuroactive properties	References
γ-aminobutyric acid (GABA)	Microbiota and human metabolism	*Bifidobacterium* spp. *Bacteroides* spp. *Parabacteroides* spp. *Eubacterium* spp. *Lactobacillus* spp.	Primary inhibitory neurotransmitter in the CNS; regulates neuron proliferation, differentiation, and synapse formation	[83, 182]
Norepinephrine	Microbiota and human metabolism	*Bacillus* *Escherichia coli* K-12 *Saccharomyces* *Clostridium* spp.	Neurotransmitter involved in arousal and wakefulness, memory retrieval, restlessness, and attention	[183–185]
Dopamine	Microbiota and human metabolism	*Streptococcus* *Enterococcus* *Escherichia*	Neurotransmitter with broad associations in the body including movement, memory, sleep, and arousal	[82, 186]
Acetylcholine	Microbiota and human metabolism	*Lactobacillus* spp.	Neurotransmitter ubiquitous in central and autonomic nervous systems, as well as roles in activation of skeletal muscles for voluntary movement	[187–189]
Lipopolysaccharide (LPS)/endotoxin	Microbiota	Gram negative bacteria	Strong promoter of neuroinflammation and toxicity to dopaminergic neurons via microglia activation; Increases caspase-11 and IL-1β expression	[190]
Short chain fatty acids (SCFA) Acetate, propionate, butyrate	Microbiota and fermented foods	*Bacteroides* *Clostridia* *Akkermansia muciniphila* *Bifidobacterium* *Lactobacillus* *Ruminococcus* *Prevotella* *Roseburia*	Promotes microglia homeostasis, modulate inflammation signaling in microglia and astrocyte culture Propionate can indirectly promote intestinal gluconeogenesis as a ligand for FFAR3 in periportal afferent nerves Butyrate, and to lesser extent other SCFA, can inhibit histone deacetylase activity, an attribute associated with cognitive improvement in mice	[191, 192]
Components of Tryptophan Metabolic Pathway				
Indole	Microbiota	*Clostridium sporogenes* *Escherichia coli* *Proteus vulgaris* *Paracolobactrum coliforme* *Achromobacter liquefaciens* *Bacteroides* spp.	Overproduction in gut may lead to increased levels of indole oxidation neuro-depressant products, oxindole and isatin, in the brain	[100, 193]
Indole-3-propionanic acid (IPA)	Microbiota	*Clostridium sporogenes*	Neuroprotective anti-oxidant against oxidative damage by Aβ, SOD dysfunction, and lipid peroxidation	[194, 195]

(Continued)

Table 3
(*Continued*)

Metabolites	Sources	Microbiota Taxa Producers	Neuroactive properties	References
Tryptamine	Microbiota	*Streptococcus thermophilus* (NCFB2392) *Clostridium sporogenes* *Ruminococcus gnavus*	Modulation of intestinal motility and homeostasis by interaction with enteric nervous system Can induce the release of serotonin	[100, 196]
Indole-3-acetic acid (IAA)	Microbiota and human metabolism	*Clostridium bartlettii* *Eubacterium hallii* *Bacteroides eggerthii* *Bacteroides thetaiotaomicron*	Increased accumulation of IAA in serum associated with MMSE-determined cognitive decline Activation of pro-inflammatory AhR/p38MAPK/NF-κB pathway in human endothelial cells, and increase in ROS production	[197–199]
Serotonin (5-hydroxytryptamine or 5-HT)	Microbiota and human metabolism	*Escherichia coli* K-12 *Lactococcus lactis* subsp. *Cremoris* (MG1363) *Lactococcus lactis* subsp. *lactis* (IL1403) *Lactococcus plantarum* (FI8595) *Streptococcus thermophilus* (NCFB2392) *Enterococcus faecalis* (NCIMB775) *Morganella morganii* (NCIMB10466) *Klebsiella pneumoniae* (NCIMB673)	Neurotransmitter with roles in gastrointestinal regulation, blood clotting and vasoconstriction/dilation, and a wide range of influence on mood, anxiety, sleep, satiety and cognition	[101, 184, 196, 200–202]

AhR, aryl hydrocarbon receptor; FFAR3, free fatty acid receptor 3; IAA, indole acetic acid; IL-1β, interleukin 1 beta; MMSE, Mini-Mental State Exam; NCFB, National Collection of Food Bacteria; NCIMB, National Collection of Industrial, Food and Marine Bacteria; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38MAPK, p38 mitogen-activated protein kinase; SCFA, short chain fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase.

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membrane vesicle trafficking and can be excreted into the local cellular environment through bacterial outer membrane vesicles and also upon rupture of the bacterium cell wall [84]. The toxicity of LPS is thought to be due to the exaggerated host immune response to LPS rather than any inherent toxicity. The outermost domain of the LPS is composed of a glycan polymer O-antigen moiety that is specific for a given bacterium and is what the immune system develops antibodies against. The LPS inner domain composed of the relatively conserved Lipid A moiety is responsible for the toxicity associated with LPS and is recognized by the immune system through the formation of the TLR4-MD-2 heterodimer complex, although efficient activation of the complex is dependent on the number of lipid chains and conservation of both phosphate groups within Lipid A [85]. TLR4 is expressed by many cells of the immune system and TLR4 activation by LPS promotes the secretion of many pro-inflammatory cytokines [86] through a diverse set of potential signaling pathways including activation of NF-κB, MAP kinase, and IRF5 [87].

LPS can not only activate pro-inflammatory signaling pathways in the gut but also in the CNS [88]. Murine experiments show BBB permeability of LPS alone to be quite low and LPS breaches likely occur through non-BBB, peripheral LPS receptors [89]. It is not yet known exactly what mechanism LPS uptake into the CNS occurs, but experiments with primary human neuronal-glia co-cultures show that LPS accumulates within neuron parenchyma and impairs neuronal transcription products such as NfL more so than astroglia products such as glial fibrillary acidic protein (GFAP) [90], suggesting that LPS may interrupt neuronal homeostatic structural maintenance and post-injury repair processes, contributing to neurodegeneration transcriptionally as well as through neuroinflammation. Interestingly, heightened systemic LPS levels have been found to promote cognitive deficits in C57BL/6J mice by activating NF-κB signaling pathway to induce neuroinflammation as evidenced by increased TNF-α, IL-1β, prostaglandin, and nitric oxide [91], and increased retinal neurodegeneration in predisposed P23H rats [92]. Peripheral administration of a single dose of LPS in mice experienced increased TNF-α levels in the brain that was sustained over 10 months despite serum and liver levels reverting to baseline [93]. Experiments with organotypic hippocampal slice cultures from C57BL/6Babr mice show synaptophysin levels, a protein ubiquitously expressed in synapses, drop upon LPS administration, a process that is directly mediated by microglia and inflammatory cytokine IL-1β [94]. Cytokine treatment of IL-4, IL-6, IL-13, IL-1β, TNF-α, and IFN-γ to a variety of cell types have been associated with deterioration of endothelial cell tight junction proteins [95], suggesting that LPS may breach the BBB under chronic, systemic inflammatory conditions to further promote neuroinflammation. *Porphyromonas gingivalis* oral exposure to AβPP transgenic mice experienced increased amyloid deposition in hippocampus and cortex, heightened brain levels of pro-inflammatory cytokines TNF-α and IL-1β, and increased levels of LPS in the brain and serum, suggesting that periodontitis caused by *P. gingivalis* may induce neuroinflammation that brings about pathologic mechanisms related to AD [96]. It is clear that exposure to bacterial LPS induces a wide range of cascading effects that can be detrimental to neuronal function long-term.

Dysregulated tryptophan metabolism by the gut microbiome

Tryptophan is an essential amino acid whose metabolic pathway is dysregulated in AD, often associated with kynurenine arm of the tryptophan metabolic pathway. Decreasing circulating levels of tryptophan are shown to worsen cognitive decline of AD patients [97]. This is potentially related to increased immune activation with increasing age, leading to increased inflammation, upregulation of kynurenine through indoleamine 2,3-dioxygenase (IDO) activity and as much as a 50% decrease in available tryptophan for serotonin production [98,99]. Availability of tryptophan could also be due to the ability of the resident microbiome to metabolize tryptophan. Indole-3-propionic acid (IPA) is a product of microbial tryptophan metabolism that induces the expression of tight junction proteins, such as occludins, and can inhibit NF-κB signaling to reduce production of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β following LPS exposure [91].

Approximately 90% of the essential neurotransmitter serotonin in the human body is produced in the gut and many constituents of the gut microbiome are able to induce serotonin production by stimulating enterochromaffin cells in the intestine to promote gut homeostasis and regulate intestinal peristalsis [100]. Serotonin is important for cross-communication between the central and enteric nervous system and is regulated through, and aids in maintaining brain, nerve health, intestinal

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movements, and regulates behavioral effects such as emotion, stress response, and appetite [101]. As such, underproduction or lack of access to serotonin is associated with mood and depressive disorders, and the interplay between serotonergic system and cholinergic, dopaminergic, GABAergic system play important roles in memory retention and cognition [102].

Upregulation of the kynurenine metabolic pathway occurs in AD patients, with increased indoleamine 2, 3 dioxygenase (IDO) activity that also leads to increases in quinolinic acid in the hippocampus [103]. Quinolinic acid is an NMDA glutamate receptor agonist with high selectivity for the NR2B subunit (GRIN2B) in the forebrain, and high concentrations are associated with neuron excitotoxicity and may initiate disruption of neuronal cytoskeleton homeostatic mechanisms [104, 105]. Additionally, quinolinic acid stimulates lipid peroxidation in an Fe^{2+} dependent manner which may be abated by iron chelation therapy [104]. Intraperitoneal injection of *E. coli* LPS into C57Bl/6J mice increased the brain concentration of quinolinic acid approximately four-fold [106]. It is possible that bacterial secretions such as LPS originating in the gut that are potently pro-inflammatory could initiate a wide range of neurotoxic effects if the source of inflammation is sustained.

Challenges with analysis of the microbiome and current clinical trials

Despite the incredible amount of data that is produced, the appeal of identifying the functional proteome of gut microbiota (metaproteomics), the constituent taxa and gene functions of the gut microbiota (metagenomics), and the degree to which constituent genes are expressed (metatranscriptomics) is the potential to holistically relate the functional behavior of the gut microbiome and its production of metabolites (metabolomics) to host health, importantly with regards to neurodegenerative disease progression [107]. High resolution mass spectrometry-based quantitative metabolomics platforms are commonly used to identify a biological sample's metabolite composition [108]. Robust quantitative metaproteomic methods have been utilized to determine gut microbiome proteomes from fecal samples in colorectal cancer [109], but the complex nature of exploring the microbiome has brought about many challenges and implementation strategies with omics workflows that have been reviewed at length elsewhere [110–113]. Comparative investigations into the microbiome constituent differences via fecal samples is quickly becoming a robust platform to understand inter-individual variability [114]. Challenges associated with the collection of fecal samples to determine microbiota composition are numerous and the distribution observed can be method-of-collection and storage-dependent [77]. For example, approximately 99% of bacteria within the colon are obligate anaerobes and can be sensitive to exposure to oxygen [67]. Thus, the environment in which feces are collected, the kits used for feces collection, storage conditions, time lapsed from collection, and its analysis in atmosphere can all have downstream effects on what microbiota distributions are observed in addition to variances and specificity in genomic sequencing methods [115, 116]. Full-length 16S rRNA high-throughput sequencing may be eventually required instead of less computationally-intensive short-read approaches to sort polymorphisms in the bacterial 16S gene to identify specific strains of the same species [116]. We foresee this aspect may be critically important for some individuals as different strains of the same bacterial species can elicit widely inconsistent adaptive immune responses [117], potentially aiding in the identification of inflammatory and neurodegeneration-related microbiota.

Perhaps the most significant challenge with collecting vast amounts of multi-omics data is in its interpretation. Fortunately, this downside is well recognized, and many researchers have begun to implement strategies to draw out inter-disciplinary connections between human and bacterial metabolism and disease. The Virtual Metabolic Human (VMH) database is one such interactive utility that links information of myriads of known metabolites, metabolic reactions, human and microbial genes, Mendelian diseases, specific microbes, and foods [118]. Others have expanded the VMH concept for virtual reconstruction of whole-organ metabolism in relation to the host microbiome to potentially model healthy inter-organ and co-metabolism dynamics to better predict effective therapeutics for a particular individual based on sex, sex-specific and shared organ types, bodily fluid and dietary metabolites, and host/microbe genes [119]. These virtual models can also be tailored to a specific disease. Mechanistic information of how multiple upstream disorders, including interconnections between β-cell dysfunction and insulin resistance, could be elucidated with VMH that result in symptomatic diabetes, providing an exciting opportunity to explore

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combination treatment strategies [120]. Genome-scale metabolic modeling has also been applied to AD and found several metabolic pathways were downregulated, including folate metabolism and mitochondrial trafficking [121], the latter of which is strongly associated with late-onset AD [122].

It will also be critical to have a simple-to-follow and unambiguous collection protocol to reduce compliance errors, especially if AD patients were to be asked to perform the collection and the surrender of stool samples independently. Because the microbiome is so variable, obtaining an individual's baseline composition is exceedingly difficult. This inherently demands large cohort studies to determine what the healthy baseline is across many people for adequate comparison to a diseased or imbalanced microbiome [123]. As such, multiple considerations need to be taken into account for the particularly challenging nature of microbiome molecular analyses, and many best-approaches are continuously evolving with ongoing research and findings. In addition to sample collection and storage, these challenges can include facets of experimental design, metadata collection, data generation and processing, and data analysis and statistics. Choices in microbial molecular assays used, as mentioned above, are also challenging as they have independent and complementary benefits, but multi-omic studies are becoming increasingly common [124]. For longitudinal comparison of future, large-participant studies of the microbiome, the standardization of collection and storage methods for stool samples as well as robust comparison of multivariable statistical tools will be critical to determine the metabolomic, metagenomic, and transcriptional differences that may relate to neurodegenerative disease progression. Multiple exploratory and interventional clinical trials are currently exploring connections between the gut microbiome and AD (Table 4).

The results of additional clinical trials exploring the effect of dietary supplements that have the capacity to modulate gut dysbiosis may provide further insight for improvement of gut-related cognitive decline. An ultimately underpowered randomized, double-blind controlled trial with the multinutrient Souvenaid found no significant impact on cognitive performance in prodromal AD patients as evaluated by a neuropsychological test battery over 24 months, but the observed cognitive decline post-trial was much improved over estimates [125–127]. Interestingly, increased plasma levels of docosahexaenoic acid (DHA), a brain-abundant omega-3 fatty acid ingredient in Souvenaid, are reported to increase gut microbiome alpha diversity that is linked to decreased gut inflammation [128] but phosphatidylcholine DHA, and not DHA, was associated with a reduced risk of developing dementia [129, 130]. The hormone melatonin commonly used as a dietary supplement as a sleep aid is an antioxidant whose production in the brain is reduced with aging and in AD [131]. Melatonin can increase gut microbiota *Firmicutes*:*Bacteroidetes* ratio and goblet cell differentiation in a TLR4-dependent manner in mice, and has been associated with decreasing Aβ-induced neuroinflammation *in vivo* [132]. Resveratrol is an orally available polyphenol derived from many common foods and reduces amyloid plaque formation in Tg19959 AD mice model [133]. Resveratrol is well-tolerated in humans and was demonstrated to reduce pro-inflammatory MMP9 in CSF [134, 135] and has been found to modulate gut microbiota populations in various animal models as well as in humans [136]. Other dietary supplements associated with modulation of the gut microbiome and neuroinflammation include curcumin [137], alpha-tocopherol (vitamin E) [138, 139], and vitamin D [140, 141].

Required cohort sizes

As seen in Table 4, most microbiome cohorts studied to date were relatively small, and in most cases include only a few dozen patients. This may have limited discoveries. For example, in recent work [142], we showed that by profiling both the microbiome and serum metabolite levels of 491 healthy participants, we were able to apply machine-learning algorithms to predict metabolite levels in held-out individuals on the basis of their gut microbiome composition. We obtained statistically significant predictions for hundreds of metabolites, in some cases explaining more than 50% of the observed variance in the metabolite levels. We further validated these microbiome-related predictions by showing a high replication rate in two geographically independent cohorts from the U.K. and Denmark that were not available to us when we trained the algorithms. These data also allowed us to reveal specific bacterial interactions with metabolites. Unlike in other settings, using standard statistical power analysis to compute the required cohort size in advance may be challenging, as we need assumptions on the size of the effect that various microbiome parameters will exhibit in order to apply the statistical power calculation methodologies. Given the large interpersonal variability that exists in the microbiome

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Table 4
Clinical Trials Exploring Gut Microbiome and/or Metabolic Profiling with AD in Humans

Disorder	Identifier/References	Study	Status/Results
AD and mixed AD (MMSE $\leq$ 26)	NCT03167983 Sponsor: Medical University of Graz [203]	Non-interventional To study associations with gut microbiome composition, permeability of the gut, and stage of dementia Total participants: 23 patients with dementia, 18 age-, sex-matched controls with no cognitive impairment Stool and serum samples: evaluated microbiome composition via 16S rRNA gene sequencing and bioinformatics via QIIME 2 and Calypso 7.14 tools	Increased BBB permeability with AD: higher serum diamine oxidase levels Increased inflammation with AD: higher soluble CD14 levels Health-related taxa: 3 species of family *Lachnospiraceae* (genus *Lachnospiraceae* NK4A136 and *Lachnospira*) Mild Dementia- related (MMSE = 20–26): *Faecalibacterium prausnitzii* Moderate Dementia-related (MMSE = 10–19): *Lactobacillus amylovorus* and its higher taxonomic levels Severe Dementia-related (MMSE = 0–9): *Clostridium clostridiforme*, *Streptococcus salivarius*
AD-like dementia, mild cognitive impairment (MMSE = 21–26)	NCT3847714 Sponsor: Medical University of Graz	Intervention: Probiotic supplement Omni-Biotic Stress Repair (*Bifidobacterium bifidum* W23, *B. lactis* W51, *B. lactis* W52, *Lactobacillus acidophilus* W22, *L. casei* W56, *L. paracasei* W20, *L. plantarum* W62, *L. salivarius* W24, *Lactococcus lactis* W19, 7.5 $\times$ 109 CFU/g twice daily dissolved in water) Control: 3000 mg placebo powder, twice daily Total participants: 58 Outcome Assessments: Abundance of butyrate-producing bacteria Biomarkers for gut permeability including diamine oxidase in serum, zonulin in stool Biomarkers for inflammation including calprotectin in stool, soluble CD14 in serum, lipopolysaccharide binding protein in serum, bacterial peptidoglycan in serum, and bacterial DNA in serum	Trial is ongoing
AD-like amnestic MCI (CDR = 0.5) or subjective MCI	NCT03991195 Sponsor: Xuanwu Hospital, Beijing Collaborators: First Hospital of Tsinghua University; Beijing Normal University	Intervention: 3-month course of *Bifidobacterium* supplement Total participants: 90 Randomized, double blind, placebo-controlled (placebo group has CDR = 0) Outcome assessments: Measurement of cognitive impairment from baseline to 3 months via MoCA and AVLT Changes in gut microbiota at 3 months between supplement and placebo group measured by high-throughput 16S rRNA sequencing Structural and Functional MRI-based, Diffusion Tensor Imaging and FNIRS-based brain network changes in amnestic MCI individuals	Trial is ongoing

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Individuals at least 70 years of age with subjective cognitive complaint, CDR-FTLD ≤ 0.5, MMSE > 21	NCT04265482 Sponsor: Geroscience Center for Brain Health and Metabolism	Non-interventional Study aims to follow an exclusively Chilean cohort in Santiago, Chile, (the GERO cohort) over the course of at least 3 years to accumulate a bank of neurocognitive and psychological data, as well as a bank of biological samples including plasma, serum, whole blood, buffy coat, peripheral mononuclear cells and stool at 0, 18, and 36 months from start of study, stored long-term for a wide range of experiments, including microbiome studies Total participants: 300 Outcome assessments: Assess rate of functional decline and progression to clinical dementia; longitudinal change in biomarkers and neuroimaging; Evolution of health-related quality of life assessment (EQ3D); cardiovascular events and mortality rates	Trial is ongoing
Mild to moderate AD patients age 70–90 (MMSE > 15). Patients with rapid cognitive decline are defined as having a 3-point loss in MMSE score over 1 year prior to start of study	NCT03487380 Sponsor: Nantes University Hospital	Non-interventional This study aims to explore the fecal microbiome differences in patients with AD and those with rapid cognitive decline from cognitively normal patients Total participants: 300 (100 mild-moderate AD; 100 AD with rapid cognitive decline; 100 cognitively normal) Outcome assessments: shotgun metagenomic sequencing of the three study groups to analyze functional and taxonomic differences between AD microbiome and control; creation of a prediction score for rapid cognitive decline based on microbiome analysis and MMSE, quality of life, and MNA questionnaires	Trial is ongoing
Mild to moderate AD patients ($10 \leq$ MMSE ≤ 23) age 55–85	NCT03856359 Sponsor: Duke University Collaborator: Bausch Health Americas, Inc.	Open-label Phase II clinical trial Intervention: rifaximin antibiotic 550 mg twice daily for 3 months Total participants: 10 Outcome assessments: Cognitive assessment via ADAS-cog at baseline, 3 months, 5 months Serum inflammatory biomarkers including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and TNF-α from baseline, 3 months, 5 months Change in serum neuronal markers, such as NfL, from baseline, 3 months, 5 months Analysis of shift of pro-inflammatory microbiota populations from baseline to three months rifaximin treatment with particular attention to *Shigella*, *Escherichia*, *Pseudomonas*, *Bacteroides*, *Eubacterium*, and *Faecalibacterium* species	Trial is ongoing

(*Continued*)

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Table 4
(*Continued*)

Disorder	Identifier/References	Study	Status/Results
Patients age ≥ 45 with diagnosis of probable AD according to NINDS/ADRDA guidelines and documented abnormal memory deficits through neuropsychological testing	NCT03998423 Sponsor: University of Wisconsin, Madison Collaborator: Wisconsin Partnership Program	Intervention: Phase I clinical trial of oral fecal microbiota transplant to AD patients (single dose is 30 capsules (22.5 g) of oral fecal microbiota) given once (at baseline), twice (baseline and 8 weeks), or three times (baseline, 8 weeks, and at 24 weeks) Total participants: 5 enrolled at study termination Feasibility and safety of oral fecal microbiota transplants Preliminary study to outline parameters for a future, larger study Stool samples collected at baseline, 8 weeks, 24 weeks, and 1 year and analyzed for composition by 16S rRNA sequencing for transmission of species present in donor sample but not in recipient stool at baseline	Study terminated to minimize risk to participants; fecal material tested positive for SARS-CoV-2 (COVID-19)
AD patients and their gender-matched cognitively normal sibling(s)	NCT04081285 Sponsor: The Chinese University of Hong Kong	Non-interventional Total participants: 250 (anticipated) Outcome assessments: Identification of epigenetic biomarkers for AD in blood (DNA methylation) and RNA-sequencing of gut microbiome	Trial is ongoing
AD patients with challenging behavior, without challenging behavior, and cognitively healthy age-matched elderly	NCT03593941 Sponsor: University of Aberdeen	Intervention: controlled diet in a home care facility Total participants: 60 Outcome assessments: Gut microbiota profile evaluation, fecal sample profile of SCFA and DNA profile in AD patients	Trial status is unknown
Longitudinal cohort of patients with or at risk for developing brain disease, including stroke and AD; as well as healthy individuals with no known risk for developing brain disease	NCT03592563 Sponsor: The Chinese University of Hong Kong	Intervention: Behavioral assessment via Neuro Quality of Life questionnaire, at 6-month intervals (proposed study period July 2019 through December 2048) Total participants: 5000 Outcome assessments: Identification of risk factors that contribute to neurological disorders over time. Blood sample collected for genome sequencing or PCR gene expression and stool samples for microbiome study	Trial is ongoing
Patients aged 65 to 75 years at risk of developing mild cognitive impairment	NCT03923712 Sponsor: University of Cádiz	Intervention: Behavioral assessment, supervised exercise program, including aerobics, strength-training, cognitive and coordination exercises over 5 months Total participants: 98 Outcome assessments: Neuroimaging markers for brain structures, suite of cognitive assessment measurements, untargeted metabolomics and proteomics profiling of blood serum, and telomere measurements by RT-qPCR; as well as vital signs and cognitive change from baseline	Trial is ongoing

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Patients over 40 years of age with mild cognitive impairment, AD (mild, moderate, and severe), or cognitively normal	NCT03136679 Sponsor: Baylor Research Institute	Non-interventional Total participants: 220 Outcome assessments: Correlation of metabolic biomarkers in plasma and urine samples with severity of cognitive decline	Trial is ongoing
Cognitively normal patients with impaired fasting glucose that have undergone genotyping of AD-related risk genes	NCT03117829 Sponsor: University of Wisconsin, Madison Collaborator: National Institute on Aging	Intervention: 12-week diet and exercise regimen Total participants: 30 Outcome assessments: Monitoring of cerebral blood flow, determination of altered metabolomic biomarkers and interaction of diet with microbiome assessed by metabolic profiling of fasting blood and urine	Completed, results pending
Patients aged 50 to 80 years with diagnosis of mild cognitive impairment	NCT04082611 Sponsor: John Wayne Cancer Institute Collaborator: Institute for Systems Biology, St. Joseph's Healthcare Foundation	Intervention: coached, data-driven, multi-modal lifestyle clinical recommendations Total participants: 60 Outcome assessments: Evaluation of neuroimaging, longitudinal multi-omics, including metabolomics, proteomics, genetics, and microbiome to discern relations to cognition and behavioral change assessments over 1 year	Trial is ongoing
APOE4 positive patients aged 60 to 80 years fulfilling criteria for subjective cognitive decline	NCT03978052 Sponsor: Parc de Salut Mar Collaborator: Fundacion IMIM, Barcelonabeta Brain Research Center, Pasqual Maragall Foundation	Intervention: Dietary supplement with EGCG and multi-modal lifestyle recommendations Total Participants: 200 Outcome assessments: Change from baseline of AD and inflammation biomarkers; changes in composition and metabolome of gut microbiome	Trial is ongoing
Patients aged 50 years or older, diagnosed with mild-moderate AD (ADAS-cog $\geq$ 12 and CDR-SOB $\leq$ 2)	NCT04044131 Sponsor: Istanbul Medipol University Hospital	Intervention: dietary supplement cocktail metabolic cofactors including serine, L-carnitine tartrate, N-acetylcysteine and nicotinamide riboside over 8 weeks Total participants: 120	Trial is ongoing
Patients diagnosed with PD > 18 years old (Hoehn Yahr 2–4)	Collaborator: ScandiBio Therapeutics AB, Alanya Alaadin Keykubat University, Sahlgrenska University Hospital, KTH Royal Institute of Technology	Outcome assessments: Change from baseline in serum omic profile to determine molecular network associations, biomarkers. Change from baseline in fecal and saliva microbiome utilizing shotgun metagenomics platform	

ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; *APOE4*, apolipoprotein E; AVLT, auditory learning verbal test; BBB, blood-brain barrier; CDR-FTLD, Clinical Dementia Rating frontotemporal lobar degeneration; CDR-SOB, Clinical Dementia Rating sum of boxes; CDR, Clinical Dementia Rating; CFU, colony forming units; EGCG, epigallocatechin gallate; IL, interleukin; MCI, mild cognitive impairment; MMSE, Mini-Mental State Exam; MNA, Mini-Nutritional Assessment; MoCA, Montreal Cognitive Assessment; MRI, magnetic resonance imaging; NfL, neurofilament light; NINDS/ADRDA, National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association; PCR, polymerase chain reaction; PD, Parkinson's disease; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SCFA, short chain fatty acid; TNF-α, tumor necrosis factor alpha.

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composition across people, we thus believe that cohort sizes of several hundreds of individuals are needed in order to unravel novel and robust associations.

Antibiotic resistance concerns with long-term antibiotic use

Because peripheral dysbiosis in the gut can influence microglial activation in the CNS and lead to neurodegenerative processes, chronic administration of low dose, specific antibiotics to titrate access and influence of proinflammatory bacteria or fungi may prove useful for slowing down or inhibiting progressive neurodegenerative disease without inducing further dysbiosis. Elucidating the inherent anti-inflammatory behavior of an antibiotic outside of its bacteriostatic or bactericidal qualities may prove beneficial as a dual-action treatment option for neurodegenerative disease (Table 5).

Antibiotics are primarily developed to control or eliminate pathogenic overgrowth of bacteria responsible for an acute infection. Two main concerns are associated with such an approach; over-prescription of antibiotics have contributed to the rise in antibiotic-resistant mutations in bacteria, making the treatment of infections more complex and heightening the demand for new antibiotics that are effective against drug-resistant bacteria [143]. Further, prolonged chronic administration of antibiotics is associated with major gut and peripheral side effects [144, 145]. Depending on the type of infection and specific antibiotic indicated, antibiotics are usually prescribed for treatment courses lasting 3–10 days at doses ranging from 300 mg/day to approximately 2,000 mg/day [146]. Standard antibiotic regimens can alter the host microbiome long-term. A one-year study of the administration of a weeklong course of ciprofloxacin or clindamycin in humans impacted species diversity in feces for one and four months, respectively; however, a weeklong course of minocycline resumed to normal diversity after one month and amoxicillin had no significant change from baseline over one year [147]. Recent studies have explored the ability of low-dose antibiotic administration for chronic infections. Low-dose antibiotic regimen (50 mg nitrofurantoin, 100 mg trimethoprim, 250 mg cefalexin) taken orally once daily for 12 months reduced the number of urinary tract infections (UTI) in individuals that use self-catheterization to empty their bladder [148]. However, there was also an overall increase in antibiotic resistance to commonly-prescribed antibiotics in bacterial urinary isolates [148]. The authors propose the observed antibiotic resistance and concurrent reduction in symptomatic UTIs could be due to changes in host response to chronic low-dose antibiotic treatment that promotes growth of colonizing bacteria and opposes virulent growth [148]. These are promising results for the pursual of chronic low-dose antibiotic treatments if populations of commensals can be maintained to discourage colonization of others but further study warrants exploring cumulative low-dose antibiotic regimens for use with specific microbiome profiles to expand effectiveness and lower drug resistance and toxicity from chronic administration.

Use of antibiotics for treatment of neurological disease

Antibiotic treatment is widely known to affect the populations of the gut microbiome and murine experiments have explored the effect that broad spectrum antibiotics can have in inducing gut dysbiosis and impact gut-brain signaling. A C57BL/6N mice study found through an 11-day oral gavage treatment of a high-dose antibiotic cocktail (including bacitracin, neomycin, ampicillin, meropenem, and vancomycin) was sufficient to impair object recognition memory associated with expression of tight junction proteins TJP1, claudin-5, and occludin in the hippocampus and amygdala [149]. Interestingly, even though serum concentrations of cytokines IL-1β, IL-6, IL-10, TNF-α, and IFN-γ did not differ between control and antibiotic-treated groups despite treatment with antibiotics substantially decreasing bacterial load, species diversity, and production of SFCAs; neural-signaling molecules were affected including BDNF reductions in medial prefrontal cortex, hypothalamus, and hippocampus, and increases of NMDA receptor GRIN2B and serotonin transporter SLC6A4 in amygdala [149].

One way gut dysbiosis is suspected to influence the CNS is through communication via the vagus nerve. Experiments with male, 8-week-old (adult) C57BL/6 mice subjected to chronic social defeat stress (CSDS) with CD-1 aggressor mice found that mice, after 14-day antibiotic treatment (ampicillin, neomycin sulfate, metronidazole) in ad libitum drinking water, given fecal transplants from CSDS-susceptible mice upregulated IL-6 in plasma, downregulated expression of synaptic proteins GluA1 and PSD-95 in prefrontal cortex, and *Lactobacillus intestinalis* and *Lactobacillus reuteri* were associated with depressive behavior in a vagus nerve-dependent manner [150].

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Table 5
Antibiotics with Anti-Inflammatory Effects

Antibiotic	Experiment vessel	Anti-inflammatory effect	References
Trovafloxacin	CCI injury to C57BL/6 mice	↓IL-1β, ↓IL-6, ↓TNF-α, ↓MPO, ↓GFAP, ↓Iba-1, ↓CD68	[204]
Ciprofloxacin	*in vitro* LPS-induced inflammation of SD rat primary microglia cells	Inhibition of NF-κB activation ↓TNF-α, ↓IL-1β	[205]
Clarithromycin, Erythromycin, Azithromycin, Roxithromycin	Systematic review of 45 human immunological biomarker studies	Decrease in number or function of immunological markers greatly outweighed increases Most frequent changes include decreases in IL-8, neutrophil count, TNF-α, IL-1β, ECP, MMP-9 Azithromycin was more associated with no change in immunological markers than Erythromycin, Clarithromycin, and Roxithromycin	[206]
Tobramycin-Copper complex	HLMVEC cells with fluorescent indicator DCFDA for reactive oxygen species	Complex taken up by endothelial cells prevented transendothelial neutrophil migration Significant anti-oxidant activity, potential SOD mimetic	[207]
Amphiphilic Tobramycin	Plastic-adherent macrophage-like THP-1 cells stimulated with bacterial LPS	Amphiphilic tobramycin inhibited LPS-induced TNF-α and IL-1β secretion relative to controls with simultaneous exposure to LPS. Exposure to tobramycin 30 minutes before LPS, IL-1β levels less affected; amphiphilic tobramycin may alter macrophage pattern-recognition receptors	[208]

CCI, controlled cortical impact; CD, cluster of differentiation; DCFDA, 2',7'–dichlorofluorescin diacetate; ECP, eosinophilic cationic protein; GFAP, glial fibrillary acidic protein; HLMVEC, human lung microvascular endothelial cells; Iba-1, Ionized calcium binding adaptor molecule 1; IL, interleukin; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SD, Sprague-Dawley; SOD, superoxide dismutase; THP-1, Tamm-Horsfall Protein 1 human monocyte cell line; TNF-α, tumor necrosis factor alpha.

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Altering the gut microbiome with high-dose antibiotics may deleteriously affect neurotrophic factors, but the targeting of a particular gut microbe may have benefits. Though limited by a small sample set, one study found by eliminating *Helicobacter pylori*, a bacterial species more abundant in AD, in the gut with a 1-week cocktail of clarithromycin (500 mg bid), amoxicillin (1,000 mg bid), and omeprazole (20 mg bid), followed by daily 20 mg dose of omeprazole for 1 month, gradually improved cognitive function two years following treatment [151]. Although the current way antibiotics are prescribed to treat acute infections and curb microbial dysbiosis may not have the same benefit in treating neurocognitive decline, there is justification to explore the possible concurrent beneficial effects of antibiotic treatment of neurodegenerative disorders.

But like with any drug, dosing matters. Reducing inflammation due to dysbiosis in the gut through the use of antibiotics may still be an alternative approach to treating neurodegenerative disorders. Pivotal experiments in APP/PS1 mice models of early-onset AD have shown that chronic, broad-spectrum antibiotic treatment regimens (from age 2 weeks to 6 months) are able to reduce amyloid deposition and increase soluble Aβ levels, alter microglial morphology surrounding plaques, and alter gut microbiota composition with marked increase in phyla *Firmicutes* (family *Lachnospiraceae*) and *Verrucomicrobia* (genus *Akkermansia*) [152]. *Akkermansia muciniphila* is a commensal gram-negative eubacterium that is positively correlated to gut barrier integrity and gut mucosal thickness, particularly in obesity and metabolic disorders [153]. Its abundance has been inversely related to histopathological inflammation in the human bowel [154]. Prolonged absence of complex polysaccharides and fiber derived from plants in the diet have been associated with decreased substrate for commensal, SCFA-producing microbiota and higher abundances of mucin-degrading microbes, including *Akkermansia mucinophila*; potentially leading to increased permeability of the gut mucosal layer, increased interaction of gut luminal contents with the epithelium, and subsequent increases in inflammation [155]. Further extensive experiments with APPPS1-21 mice exposed to a long-term antibiotic cocktail (including kanamycin, gentamicin, colistin, metronidazole, and vancomycin) observed a general decrease in Aβ pathology and morphological alterations in microglia that were present in only male mice [156].

The use of tetracycline class of antibiotics for potential treatment of neurodegenerative disorders has been investigated. *In vitro* experiments with low-dose doxycycline were shown to promote α-synuclein oligomerization into off-pathway, non-toxic oligomeric structures [157]. Though no effect on astrogliosis was observed, intraperitoneal doxycycline administration to Huntington's disease R6/2 model mice exhibited decreased microglial activation and enhanced neuronal survival [158]. Anti-amyloid properties of doxycycline are also seen with Aβ aggregates [159]. Molecular dynamics simulations show that doxycycline binds the hydrophobic C-terminal residues of $A\beta_{42}$ to prevent further fibril stabilization [160]. Low-dose doxycycline has also been shown to decrease LPS-induced inflammatory cytokine expression of TNF-α, IL-6, and IL-8 *in vitro* [161]. Long-term treatment of doxycycline (up to 200 mg/day) and/or rifampicin (up to 300 mg/day) over 1 year provided no significant improvement in cognitive decline in patients with mild to moderate AD, though the effect on the individuals' microbiome, or on Aβ or tau serum, or in CSF levels is unknown as the study only assessed changes in cognition, daily function, and behavior [162]. It is possible that issues with clinical treatment of AD with doxycycline reside within treating more-progressed stages of disease and that the potential anti-amyloid and anti-inflammatory benefits seen in *in vitro* experiments may translate better to prodromal patients if doxycycline were to be used as an AD prophylactic [159]. In a large, randomized Phase IIb clinical trial of two-year treatment with the tetracycline antibiotic minocycline with patients with mild AD (determined only by standardized Mini-Mental State Exam (sMMSE)$\geq$24), neither the 400 mg/day or the 200 mg/day treatment group slowed or improved cognitive decline over control as measured by the sMMSE or Bristol Activities of Daily Living Scale, with the higher dose of minocycline poorly tolerated long-term [163]. It is possible, because no biomarker analyses were performed to strengthen an AD diagnosis for inclusion in the trial, there may have been trial participants without AD diagnosed as such.

The cephalosporin antibiotic ceftriaxone is reported to exhibit neuroprotective effects by increasing expression of the glutamate transporter GLT-1 in various *in vivo* models of AD, PD, and cerebral ischemia; as well as reduction of pro-inflammatory mediators, such as TNF-α, IL-1β, IFN-γ, and NF-κB in animal models of PD, cerebral ischemia, and traumatic brain injury [164, 165]. Many analogues of sulfonamide

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antibiotics exhibit free radical scavenging activity *in vitro*, including some Aβ fibril and oligomer inhibition properties [176]. The 4-quinolone scaffold of quinolone antibiotics shows antioxidant activity against lipid peroxidation as well as hydroxyl radical production [166], although fluoroquinolone use has been associated with increased production of reactive oxygen species [167] and rare, but lasting, serious adverse effects including tendon rupture and nerve damage [168, 169].

It is unsurprising that diet has a large effect on the microbiome as its overall composition and proliferation is determined in no small part by what we eat, but the effect of diet on antibiotic efficiency in microbiota populations is emerging. In C57BL/6J mice, administration of amoxicillin decreased cecum glucose levels and significantly expanded the relative abundance of the phylum *Bacteroidetes*, specifically *B. thetaiotaomicron* and *B. vulgatus*, in harvested cecum compared to controls [170]. Interestingly, this change was attributed to amoxicillin, but not ciprofloxacin, treatment affording a metabolically-favorable environment for *Bacteroides* species. Further, by increasing the mice's dietary glucose, amoxicillin's ability to promote *B. thetaiotaomicron* amounts relative to control were diminished. In other experiments where C57BL/6J mice were fed a Western-style (high-fat, high-sugar) diet, large increases were found in phyla *Bacteroidetes* and *Verrucomicrobia* and a decrease in *Firmicutes* relative to vehicle [171]. Upon ciprofloxacin treatment an expansion of *Firmicutes* was only observed in the Western diet with no significant change with normal diet, potentially related to the diet-dependent ciprofloxacin-induced transcriptional changes in mucin substrate utilization and metabolic processes [171]. Collectively these studies suggest that diet and the metabolic environment in the gut may play a role in how effective some antibiotics are in modifying microbiome composition.

Indeed, administration of antibiotics to treat a neurodegenerative disorder may benefit with an accompanying dietary regimen prescription, of sorts, to stabilize aberrant populations to support healthy commensal growth and discourage opportunistic harmful growth. However, many more omics studies with large sample sets will be required to understand the complex interplay between antibiotics-induced host and microbial gene expression, sustained inflammatory processes and metabolite release, and the extent to which diet intervention will be efficacious in the long term. Unfortunately, there are few results for antibiotic treatment for neurodegenerative diseases in humans. This more than likely stems from the difficulty in containing and relating exogenous differentiating factors, such as diet, exercise, genetics, age, and medications, to the progression of disease. Future experiments will necessarily require targeted biomarker panels that would include direly-needed information on diagnosable ranges of pro-inflammatory mediators associated with AD progression, in concert with an analysis of gut microbiome dysbiosis present at early, potentially treatable stages of AD. The main approaches included in this personalized treatment plan are included in Fig. 2.

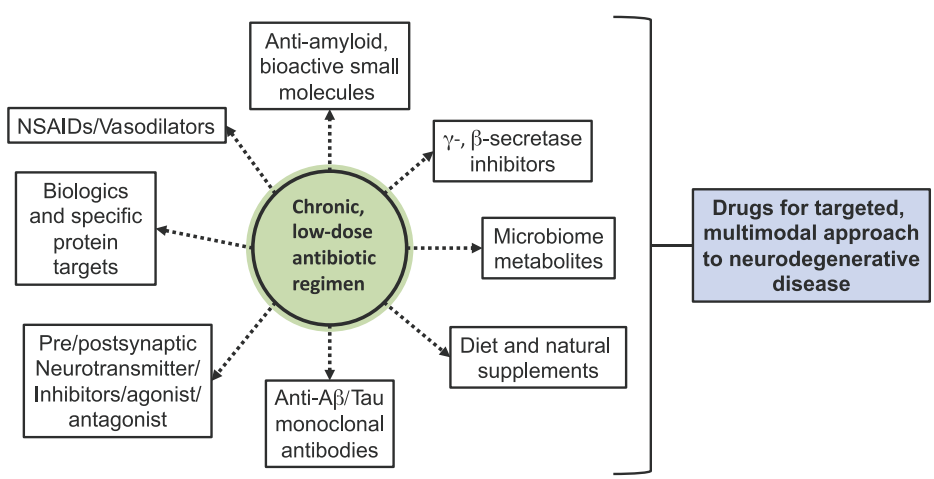


Fig. 2. Main approaches for personalized combination treatment of low-dose antibiotics with other neurodegenerative disease-modifying or -ameliorating drugs.

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CONCLUSION

The effect of the gut microbiome and other infections on CNS degeneration is supported by a large body of evidence of animal studies and relatively-limited substantive efficacy studies in humans. Many approaches for altering gut microbiota propose supplementing deficient gut microbiota with probiotic supplements, diet, metabolites, antibiotics, and fecal transfer from normal healthy subjects to sick subjects. Unfortunately, these proposed modes of treatment have demonstrated correlation to cognitive decline in a limited number of participants. However, a systematic profiling and staging of an AD profile coupled with a tailored treatment approach has yet to be demonstrated for efficacy and safety of chronic administration in controlled, randomized clinical trials. Here we emphasized the ability of slowing early AD by enhancing peripheral microglia recruitment to the CNS as a disease-modulating approach for profiling disease staging that intuits designing a specific treatment to titrate harmful bacteria and fungi access in early disease. We propose an expansive trial to establish the fecal microbial composition and serum metabolic profile of 500 patients diagnosed with mild to moderate AD to discover species and metabolic connections related to the severity of AD. The ambitious scope of this kind of study will be appropriately powered to assist with early diagnosis of AD as it relates to the microbiome. These efforts will contribute tremendously to the development of future probiotic or antibiotic treatments that have the potential to address the complex, multi-faceted nature of an individual's neuroinflammatory progression of AD, with the intent of eventually ameliorating its downstream cognitive effects by titrating the gut microbiome of harmful constituents and metabolic irregularities.

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Drosophila as a Model for Microbiota Studies of Neurodegeneration

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Abstract. Accumulating evidence show that the gut microbiota is deeply involved not only in host nutrient metabolism but also in immune function, endocrine regulation, and chronic disease. In neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis, the gut-brain axis, the bidirectional interaction between the brain and the gut, provides new route of pathological spread and potential therapeutic targets. Although studies of gut microbiota have been conducted mainly in mice, mammalian gut microbiota is highly diverse, complex, and sensitive to environmental changes. *Drosophila melanogaster,* a fruit fly, has many advantages as a laboratory animal: short life cycle, numerous and genetically homogenous offspring, less ethical concerns, availability of many genetic models, and low maintenance costs. *Drosophila* has a simpler gut microbiota than mammals and can be made to remain sterile or to have standardized gut microbiota by simple established methods. Research on the microbiota of *Drosophila* has revealed new molecules that regulate the brain-gut axis, and it has been shown that dysbiosis of the fly microbiota worsens lifespan, motor function, and neurodegeneration in AD and PD models. The results shown in fly studies represents a fundamental part of the immune and proteomic process involving gut-microbiota interactions that are highly conserved. Even though the fly's gut microbiota are not simple mimics of humans, flies are a valuable system to learn the molecular mechanisms of how the gut microbiota affect host health and behavior.

Keywords: Alzheimer's disease, cross-seeding, *Drosophila melanogaster*, gut-brain axis, gut microbiota, inflammation, neurodegeneration, Parkinson's disease

INTRODUCTION

Trillion of microbes such as bacteria, fungi, and viruses live inside our body and on all of our surfaces, with the largest concentration found in the gut [1]. Among such symbiotic microbes, the microorganisms on the surface of the gut are called gut microbiota. The gut microbiota has recently been shown to play an important role in the physiology and disease. Our symbiotic partners provide immune and metabolic functions by employing various molecular and genetic signaling pathways to protect the host from pathogens [2, 3]. Gut microbiota form biofilms, a layer of bacterial extracellular matrix, which protects them from attack [4]. The gut microbiota is affected by the host's genetic background, age, diet, comorbidities, and antibiotic use [5–7]. Disruption of gut microbiota is referred to dysbiosis

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and can contribute to various diseases. Recently, the involvement of gut microbiome has been reported in many areas such as aging, obesity, diabetes, fatty liver disease, cancer, and neurodegenerative diseases [5, 8–12]. Furthermore, accumulating evidence indicate that dysbiosis of the gut microbiota can cause low-grade inflammation which exacerbates chronic diseases such as atherosclerosis, asthma, and chronic kidney disease [13–16].

The brain and the gut strongly influence each other. This bidirectional relationship is called the "gut-brain axis" [17, 18]. Although much about the gut-brain axis is still unclear, it is well known that mental stress can cause diarrhea and constipation through changes in the gut microbiota. The relationship between gut microbiota and brain function has recently been analyzed in experiments using mice. Diaz Heijtz et al. compared the behavior of specific pathogen free mice with a normal gut microbiota with germ-free mice and showed that mice with microbiota gradually became accustomed to unfamiliar environments and reduce their amount of movement, whereas germ-free mice continued to move indefinitely in unfamiliar environments and tend to feel less fear. Furthermore, the gene expression of brain-derived neurotrophic factor (BDNF) was lower in germ-free mice than in mice with normal gut microbiota, suggesting that gut microbiota influence the development of BDNF-mediated neural circuits that control anxiety-related behavior [19]. Hsiao et al. reported using a mouse model of autism spectrum disorders that a metabolite called 4-ethylphenylsulfate produced by gut microbiota caused autism spectrum disorder-related behaviors, and that these phenotypes improved when *Bacteroides fragilis* was taken orally [20]. Furthermore, Sampson et al. reported that Parkinson's disease (PD) model alpha synuclein (α-syn) overexpressing mice that are germ free are protected from the disease [21]. Thus, evidence is accumulating that the gut microbiota is significantly involved in various neurologic disorders.

Drosophila melanogaster (henceforth *Drosophila*), one of many species of fruit fly, has classically been used as an optimal research model in the field of genetic engineering. In recent years it has been also attracting attention as an experimental animal for gut microbiota research in neurodegenerative diseases [22, 23]. *Drosophila* research has addressed the involvement of the microbiota on physical function, longevity, aging, and obesity. Furthermore, since 2018, a series of articles have been published analyzing gut microbiota in fly models of the neurodegenerative disease [24–27]. *Drosophila* has many advantages as a laboratory animal: short life cycle, numerous and genetically homogenous offspring, less ethical concerns, availability of many genetic models, and low maintenance costs. *Drosophila* has orthologs for about 75% of human disease-related genes and the fly's nervous system shares basic structure and functional features with humans [28]. Various neurodegenerative diseases such as Alzheimer's disease (AD), PD, amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth disease, and spinocerebellar atrophy adopt *Drosophila* disease model [29–33]. Moreover, *Drosophila* has a simpler gut microbiome than mice, which can be changed to any bacterial composition by experimental manipulation [34]. This makes *Drosophila* a suitable model for the study of the gut microbiome.

In this review, we discuss the history, physiology, anatomy of the gastrointestinal tract, and gut microbiota of *Drosophila*. We will then present research on *Drosophila* gut microbiota and neurodegenerative disease, and finally discuss how the findings from *Drosophila* gut microbiota can be adopted for therapeutic development of neurodegenerative diseases.

THE HISTORY, PHYSIOLOGY, AND ANATOMY OF *DROSOPHILA*

Drosophila has contributed to the Nobel Prize in Physiology or Medicine at least five times [35–37]. The first case was Thomas H. Morgan in 1933, who demonstrated the chromosome theory of inheritance, which states that chromosomes are carriers of genes. By collecting mutations in *Drosophila* and conducting mating experiments among them, Morgan produced the first chromosome map. He was able to locate the genes on the chromosomes by comparing a chromosome map with the pattern of salivary gland chromosomes of *Drosophila* and proved that the genes were on the chromosomes. The second case was Hermann J. Muller in 1946, who discovered that X-ray radiation could induce gene mutations in *Drosophila*. In the 1920 s he conducted an experiment in which male *Drosophila* was irradiated with X-rays, proving that the effects of radiation exposure can be transmitted to offspring and that radiation can artificially generate gene mutations. The third case was Edward B. Lewis, Christiane Nusslein-Volhard, and Eric F. Weischaus. They identified the genes involved in embryonic development using

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Drosophila with random mutations. Once embryonic development was delayed, the mutated gene was found to be involved, thus revealing genes necessary for *Drosophila* embryonic development. Many of identified genes were found in other species, indicating that similar developmental mechanisms in *Drosophila* apply to organisms other than flies. They were awarded the Nobel Prize in Physiology or Medicine in 1995. The fourth was Bruce A. Beutler, Jules A. Hoffmann, and Ralph M. Steinman. Hoffmann discovered the role of the *Drosophila* Toll gene in the immune system. Beutler demonstrated that one of the mammalian Toll-like receptors, TLR4, is a transmembrane component of the receptor complex of the cell wall of Gram-negative bacteria. Together with Steinman's discovery of dendritic cells, the mechanism by which the innate immune system initiates the acquired immune system was identified, and these three researchers were awarded the Nobel Prize in Physiology or Medicine in 2011. The fifth case was Jeffrey C. Hall, Michael Rosbash, and Michael W. Young. They cloned a *Drosophila* clock gene (named "*period*") and found that the mRNA and proteins encoded by *period* change in response to circadian oscillations. They were awarded the Nobel Prize in Physiology or Medicine in 2017 for their elucidation of the molecular mechanisms of circadian rhythms. Thus, many important biological discoveries have been made with *Drosophila*, and it continues to demonstrate its value as an attractive model animal not only in genetic engineering but also in other fields of biology.

The life cycle of *Drosophila* is about 10 days: the first instar larvae hatch in about one day at 25°C after spawning. The first instar larvae molt into the second instar larvae after one day at 25°C, and the third instar larvae after one more day. Third instar larvae live for two days and then pupate. They spend four to four and half days as pupae. Under eutrophic conditions, adult females are ready to lay eggs two to three days after emergence. The entire developmental period of *Drosophila* is easily affected by temperature, with a twofold delay in developmental time at 18°C. Development almost completely ceases at 12°C, and it dies at temperature below at 5°C. *Drosophila* is also not tolerant to high temperatures, and will die if reared continuously above 30°C. *Drosophila* survives for two to three months in captivity, and the maximum number of eggs laid by one female is about 300 to 500 [28].

One of the most useful experimental tools in *Drosophila* is the GAL4-UAS targeted expression system, in which GAL4 is a yeast-derived transcription factor that binds to UAS sequences and activates the expression of downstream genes. The main feature of this system is that the GAL4 strain which determines "where to express", and the UAS strain which determines "what to express", are generated independently. By analyzing the phenotypes obtained from the progeny of crosses between the targeted GAL4 and UAS strains, it is possible to efficiently perform overexpression in various combinations. Many strains that specifically express GAL4 in various tissues and cells and strains that have UAS upstream of many genes have been created and are available from stock centers around the world [38]. Furthermore, in *Drosophila*, the GAL4-UAS system can be combined with RNA interference (RNAi) to suppress specific gene expression in any tissue or cell. Transgenic lines expressing double-stranded RNAs for target mRNAs are being created and are available for most of the protein coding genes in *Drosophila* [39].

The digestive tract of *Drosophila* consists of three parts: foregut, midgut, and hindgut (Fig. 1). The foregut contains the oral cavity, esophagus, and crop (present only in adult fly), and is responsible for the physical preparation and salivary digestion of food. The midgut is the center of digestion and absorption, with over 300 digestive enzymes at work. The hindgut absorbs substances such as water and nutrients released from the Malpighian tubule, which is the equivalent of the mammalian kidney. The ingested food first enters a pouch-like structure called the crop. It then passes through the midgut, and is excreted through the hindgut. The midgut of the fly is composed of a single layer of epithelial cells, which contain the enterocytes, enteroendocrine cells, and intestinal stem cells, and is covered with muscle on the outside. On the luminal side of gut, there is a membrane composed of chitin fibrils and chitin-binding proteins called the peritrophic matrix [40, 41]. This membrane is known to allow only products degraded by digestive enzymes present on the membrane to pass through, and does not allow undigested food or bacteria to pass. Therefore, the peritrophic matrix is considered to be a physical barrier that protects the gut epithelium from pathogens. Although the role of the peritrophic matrix and its components in *Drosophila* is still unclear, the peritrophic matrix of many insects is thought to contain lysozyme and peptidoglycan degrading enzymes with antimicrobial activity, and the membrane may also serve as an essential site for gut immune responses [42].

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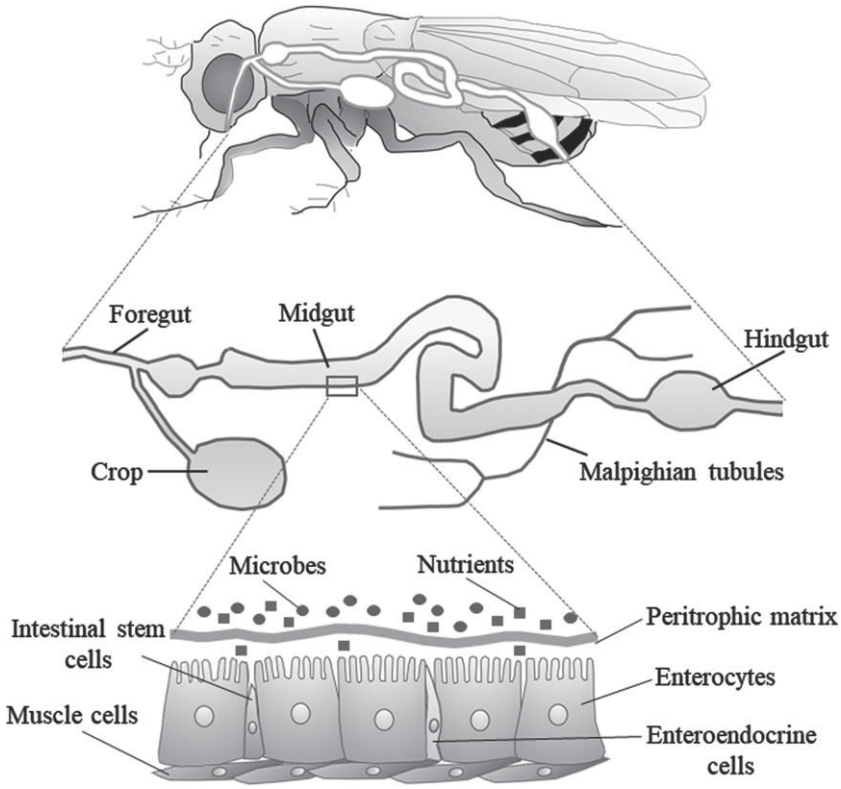


Fig. 1. Structure of the digestive tract in *Drosophila.* The upper panel shows a schematic diagram of the body and digestive tract of *Drosophila.* The middle panel shows the general parts of the digestive tract, which is divided into three parts: foregut, midgut, and hindgut. The lower panel shows the fine structure of a portion of the midgut. The layer consisting of enterocytes, enteroendocrine cells, and intestinal stem cells is covered with muscle cells. There is a peritrophic matrix between the enterocytes and the microbes, which physically separates the enterocytes from the microbes.

Flies have a much simpler gut microbiome consisting of only five to 20 microbial species, compared to humans which have about 500–1,000 microbial species in their gut [43]. Furthermore, relatively simple established methods can be used to generate germ-free flies or flies with standardized gut microbiota (such flies with standardized gut microbiota are called "gnotobiotic flies") [34]. In detail, we can obtain axenic flies by simply soaking the eggs in sodium hypochlorite and rearing them in sterile food. If we want to give them any bacteria, we can simply mix the bacteria into sterile food. Previous research showed that even human-derived *Lactobacillus* could be colonized in germ-free flies [44]. Compared to specific-pathogen-free mice, which can only be obtained by dissecting pregnant mice, the simplicity of sterile manipulation of *Drosophila* is one of the greatest advantages of using *Drosophila* in the study of gut microbiota.

The simple and easily regulated gut microbiome of *Drosophila* helps us to understand the impact of gut microbiota on host physiology as follows. Storelli et al. reported that rearing germ-free *Drosophila* larvae under nutrient deprivation significantly reduce the growth rate of the larvae. When *Lactobacillus plantarum* was reintroduced in the germ-free larvae, the decrease in growth rate was restored. Furthermore, they showed that *L. plantarum* genetically affected the TOR (Target of Rapamycin)-dependent nutrient sensing system [45]. Shin et al. found that rearing germ-free *Drosophila* monoassociated with pyrroloquinoline quinone-dependent alcohol dehydrogenase mutant bacteria delayed larval development and reduced body size. Such effect was restored by reintroduction of *Acetobacter pomorum*. Furthermore, they showed that this recovery effect was due to the acetic acid produced by *A. pomorum* modulating the larval insulin signaling pathway [46]. It is becoming clear that gut microbiome can influence a variety of *Drosophila* phenotypes, including aging and behavior [47–49]. Although there have been fewer studies of the gut microbiome in *Drosophila* than in mice,

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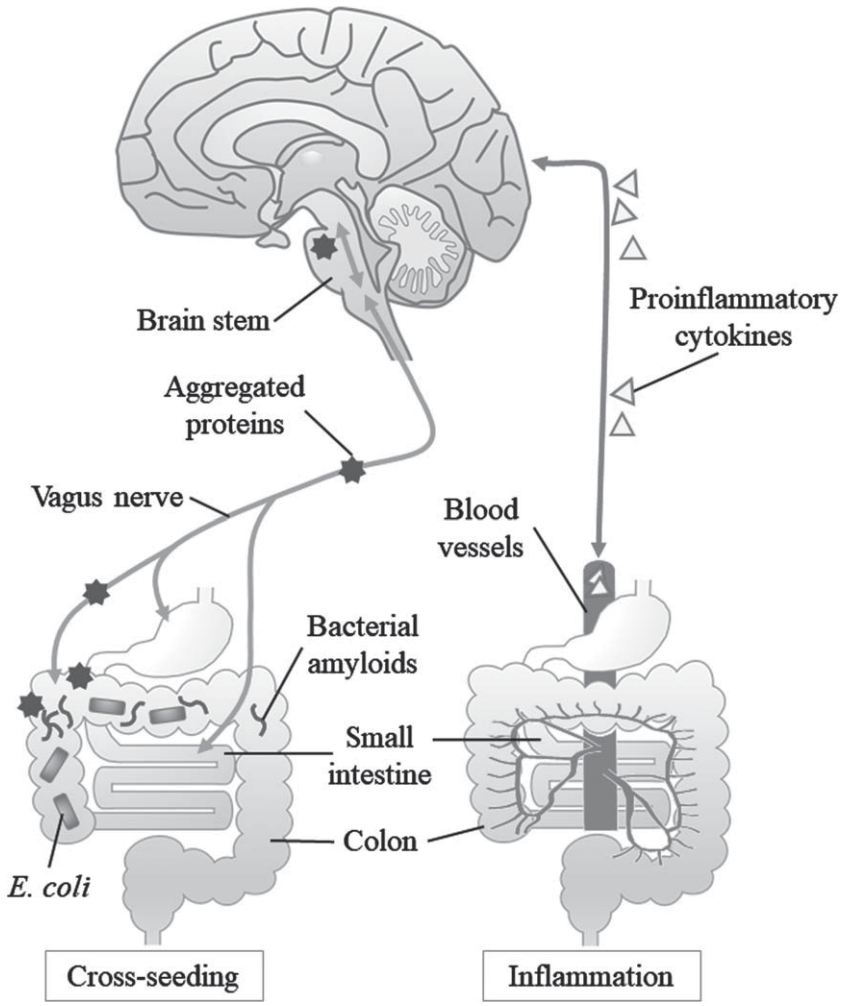


Fig. 2. Inflammation and cross-seeding: two hypotheses of abnormal aggregated protein ccumulation involving the gut. The left panel shows the "cross-seeding" hypothesis. Aggregated proteins (especially α-syn) produced in the gut propagate in a prion-like manner through the vagus nerve to the brainstem. Bacterial amyloid (e.g., curli) promote abnormal protein aggregation through cross-seeding. The right panel shows the "inflammation" hypothesis. Dysbiosis of gut microbiota activate the innate immune system and increase the production of proinflammatory cytokines and induce inflammation in the brain via systemic circulation.

physiological and anatomical simplicity in the use of *Drosophila* will aid the new discoveries, and help to find new pathways that will be applied to mammals.

THE NEURODEGENERATIVE DISEASES AND GUT MICROBIOTA

The gut and brain influence each other through four pathways: the endocrine, immunological, metabolic and neural pathways. In response to change in the gut environment, gut hormones such as 5-hydroxytriptamine (5-HT) and cytokines secreted by enteroendocrine cells and immune cells, respectively, reach the brain through the bloodstream. Bacterial metabolites and neurotransmitters such as fatty-acids, γ-aminobutyric acid (GABA), and precursor of 5-HT also go through the circulatory system and pass through the blood-brain barrier to signal the brain [50]. Afferent and efferent neurons of the vagus nerve connect the gut and brain stem. Recently, studies with rodents indicate that molecules such as GABA and α-syn can be delivered to the brain via the vagus nerve in the trans-synaptic way [51–55]. This work suggests that the gut-brain axis plays a more important role than we had imagined.

There are two keywords for the relationship between gut microbiota and neurodegenerative diseases: inflammation and cross-seeding (Fig. 2)

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[56–58]. A dysbiotic gut microbiome and damaged enteric epithelia can provoke systemic inflammation via activation of immune cells and release of cytokines [59, 60]. The hallmark of neurodegenerative diseases, such as AD and PD, is the misfolding and aggregated proteins in the brain, such as amyloid-β (Aβ) and α-syn, respectively. Minter et al. reported that Aβ deposition in a mouse model of familial AD was reduced by changes in gut microbial composition due to long-term use of antibiotics, whereas soluble Aβ was increased, and the same time, the expression of circulating cytokines was altered and the number of plaque-localized astrocytes and microglia was reduced [12]. Harach et al. found alteration in the gut microbiota of familial-AD model mice (amyloid-beta precursor protein, APP, transgenic mice) compared to wild-type mice. They also showed that germ-free APP transgenic mice had dramatically reduced Aβ deposition in the brain compared to mice with normal gut microbiota. Interestingly, while recolonization of gut microbiota in germ-free APP transgenic mice increased brain Aβ levels, microbes from standard (not germ-free) APP transgenic mice increased brain Aβ levels more effectively than that from wild-type mice [61]. AD patients and AD model mice showed different gut microbial composition from healthy controls and wild-type mice, and a stress response pathway such as the MAPK signaling pathway was enhanced [62, 63]. Zhan et al. reported that lipopolysaccharide, a bioactive substance produced by Gram-negative bacilli, and *Escherichia coli* (*E. coli*) fragments co-localized in Aβ plaques in the postmortem brains of AD patients, and that lipopolysaccharide was also deposited in perivascular areas of the brain, suggesting that microbial-mediated disruption of the blood-brain barrier might be involved in the neurodegeneration of AD [64]. These reports indicate that systemic inflammation and gut leakage play important roles in AD.

In the experimental setting, the abnormal assemblies of these proteins originate from the disease-specific misfolded proteins and finally adopt an amyloid polymer structure [65]. Many members of the human microbiome including *E. coli* produce extracellular fibers, which form amyloids. Curli, the first-described bacterial amyloid produced by *E. coli*, shares structural and pathological properties with human amyloids [66]. Besides, curli fibers induce high level releasing of proinflammatory cytokines such as tumor necrosis factor-alpha, interleukin-6, and interleukin-8 in human cells [67]. Moreover, the bacterial amyloid curli activates Toll-like receptor 2, which also recognizes and targets human amyloids such as Aβ and α-syn [68, 69]. Altogether, dysbiosis of the gut microbiota can cause inflammation and lead to neurodegeneration.

The other keyword, "cross-seeding", is related to direct abnormal proteinaceous changes in neurodegeneration. In many neurodegenerative diseases, experimental evidences support the concept that disease-specific proteins such as Aβ and α-syn acquire pathogenicity by a prion-like molecular process: the process of amyloid fibril formation proceeds after initiation by the "seed" that serves as the template [70, 71] These pathogenic protein assemblies are also known to propagate from the periphery to the brain in a prion-like manner [65, 72]. A key question is where does the process begin? In PD constipation precedes the onset of motor symptoms many PD persons, showing that PD is closely related to the gastrointestinal tract [73]. As Braak showed in PD, Lewy bodies, which are composed of α-syn, have a pathway from the dorsal nucleus of the vagus nerve upward to the substantia nigra [74]. Cersosimo showed that colonic biopsy in PD patients revealed accumulation of α-syn in the submucosal plexus in 72% of PD patients, while no accumulation was observed in the control group [75, 76]. Svensson et al. showed that the incidence of PD was significantly reduced in people who underwent total vagus nerve resection for the treatment of duodenal ulcer [77]. This report supports the hypothesis that pathological changes in PD begin first in the enteric plexus and propagate through the vagus nerve to the brain. Dysbiosis of the gut microbiota can weaken the barrier function of the gut epithelium and trigger the accumulation of α-syn in the enteric plexus. Thus, bacterial amyloids such as curli may initiate protein aggregation in enteric plexus. A pathway has also been proposed by Braak et al. and others [78] from the olfactory epithelium to the brain. In the roof of the nose the olfactory receptors AR processes of neurons that reside outside the central nervous system that are directly connected to the olfactory bulb as well as the entorhinal cortex. Prion-like protein misfolding may be initiated in olfactory neurons thorough exposure to bacterial products in the roof of the nose [79].

Is there a precedent for the gut factors to cause initiation of a protein folding disorder? This is precisely our current understanding of Creutzfeldt Jakob disease, bovine spongiform encephalopathy as well as kuru. In these conditions exposure to the prion protein in the gut thorough food initiates templated

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Table 1
Summary of *Drosophila* Microbiome Research on AD and PD.

Disease	Year	Flies	Summary	Ref
AD	2017	$A\beta_{42}$-transgenic flies	Gram-negative rod infection shortened the lifespan, impaired motor function, and increased cell death in the brain.	84
AD	2019	Flies expressing Human APP and BACE1	*Lactobacillus* administration improved phenotypes	85
AD	2020	$A\beta_{42}$-transgenic flies		86
AD	2020	$A\beta_{42}$-transgenic flies		87
PD	2019	α-syn transgenic flies	Phenolic acid metabolites were effective in delaying the deterioration of motor function	90
PD	2020	Endogenous PINK1 mutant flies	EGCG improved motor function and prolonged survival.	91

AD, Alzheimer's disease; PD, Parkinson's disease; APP, Amyloid-beta precursor protein; BASE1, beta-site amyloid precursor protein cleaving enzyme 1; $A\beta_{42}$, Amyloid beta 42; α-syn, alpha-synuclein; EGCG, epigallocatechin gallate.

misfolding of the cellular form of the prion protein in the neurons of the gut which is subsequently transmitted to the brain through the autonomic nervous system, as well as the blood. The ability of functional bacterial amyloid proteins such as curli and others to initiate prion-like misfolding of neuronal proteins α-synuclein end Aβ have been reported in *in vitro* studies [80, 81]. Although the enteric nervous and immune systems are much simpler than those of mammals, an innervated digestive tract and a primitive immune system (including specialized hemocytes involved in phagocytosis) of *Drosophila* can serve as an appropriate model for studying gut-brain communication [82, 83].

DROSOPHILA FOR MICROBIOTA STUDY OF ALZHEIMER'S DISEASE

Wu et al. used transgenic flies expressing human $A\beta_{42}$ as an AD model and showed in 2017 that the gut-brain axis is a fundamental factor in neurodegeneration of AD. Gram-negative rod (Ecc15) infection shortened the lifespan of $A\beta_{42}$-transgenic flies, impaired motor function, and increased cell death in the brain. In these flies, phagocytes were more easily recruited to the brain as a result of enhanced migration of brain phagocytes, and these cells were more likely to produce reactive oxygen species with the elevated JNK pathway. When phagocytes were genetically knocked out, the JNK pathway was suppressed and the flies' lifespan was extended. This paper clearly showed that in the AD model *Drosophila*, changes in gut bacteria can activate immune cells to produce inflammatory cytotoxicity of central nervous system [84]. In recent years, several papers have reported on the therapeutic effects of specific bacteria and substrates (called probiotics and prebiotics, respectively) or combination (called symbiotics) that are considered to be good for health promotion on *Drosophila* models of AD [85–87]. Among these, Tan et al. and Liu et al. used human $A\beta_{42}$ transgenic flies as an AD model, and Westfall et al. used transgenic flies expressing human APP and β-secretase 1 (BACE1) enzymes as an AD model. All reports used *Lactobacillus* as probiotics and showed that *Lactobacillus* administration improved phenotypes such as disrupted compound eye formation, shortened survival period, impaired motor function, and Aβ deposition. Although the number of papers is still limited, the combination of *Drosophila*'s advantages, such as the ease of regulation of gut microbiome and the homology of neural tissue with humans, will help us to study the role of gut microbiome in AD patients (Table 1).

DROSOPHILA FOR MICROBIOTA STUDY OF PARKINSON'S DISEASE AND OTHER NEURODEGENERATIVE DISEASES

It has been shown that the PD model *Drosophila* exhibits reduced locomotion, shortened lifespan, and neurodegeneration [88, 89]. There have been two reports on the gut microbiota in the *Drosophila* PD model. Ho et al. showed that phenolic acid metabolites were effective in delaying the deterioration of motor function in human α-syn transgenic flies, and that phenolic acid metabolites inhibited protofibril formation by binding α-syn *in vitro* [90]. The other report is by Xu et al. [91] (Table 1). When *Drosophila* mutants with endogenous PINK1 were fed Epigallocatechin-3-gallate (EGCG), a potent polyphenol, improved motor function and prolonged survival were observed. The abundance of gut microbial composition was increased in these ECGC-treated flies, and when such abundance was reduced by antibiotic administration, the treatment effect of EGCG disappeared. By transcriptomic profiling using PINK1 mutant flies, they showed that the

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expression of genes involved in fly-specific immune hormones was altered in PINK1 mutant flies, and that these gene expression changes were restored by ECGC consumption.

In ALS, another significant neurodegenerative disease, the relationship between the phenotype of ALS and gut microbiota has been investigated using mouse models. Blacher et al. showed that *Superoxide dismutase-1* (*SOD1*) transgenic mice exhibit abnormalities in gut bacteria and changes in the composition of metabolites by bacteria before the onset of ALS, and that the prognosis is worse when these mice are raised in sterile conditions or after treatment with antibiotics. The authors showed that the abundance of *Akkermansia muciniphila* continued to decrease in *SOD1* transgenic mice as the disease progressed. They also showed that colonization of antibiotic-treated *SOD1* transgenic mice with *A. muciniphila* improved motor symptoms, whereas colonization with *Ruminococcus torques* or *Parabacteroides distasonis* worsened symptoms. The authors also found that the symptoms worsened when colonized with *Ruminococcus torques* or *Parabacteroides distasonis*. The authors further showed that colonization with *A. muciniphila* increased the concentration of nicotinamide in the cerebrospinal fluid of *SOD1* transgenic mice. Similar improvement (vitamin B3) in motor symptoms was observed when nicotinamide was administered [92]. Burberry et al. showed that immune cells such as neutrophils infiltrate the spinal cord and microglia are activated in the *C9orf72* mouse model. They also found that the diversity of the gut microbiota of *C9orf72* transgenic mice was reduced in pro-inflammatory environments, compared to pro-survival environments. They further showed that reducing the microbial load with broad-spectrum antibiotics or transferring microbes from mice grown in a pro-survival environment can improve the inflammation-associated phenotype and extend the lifespan of *C9orf72* transgenic mice raised in a pro-inflammatory environment [93]. The results in mice suggest that the gut microbiota changes with the onset and progression of ALS, that microbial-derived metabolites such as nicotinamide alter the progression of the disease, and that the gut microbiota may alter the motor function and lifespan of ALS model mice through immune responses. However, since gut microbiota is very sensitive to environmental changes, the results of the mouse study should be validated using other models. For now, there are no studies on gut microbiota using *Drosophila* model of ALS, but it is likely that they will be used in the near future.

APPLICATION AND LIMITATION OF *DROSOPHILA* GUT-BRAIN AXIS RESEARCH

We have introduced the role of the gut microbiota in neurodegenerative diseases, and the characteristics of *Drosophila* as a laboratory animal, for the study of gut microbiota in neurodegenerative diseases. A significant paper on the gut-brain axis was published in 2021 using *Drosophila*. Kim et al. have identified an amino acid sensing mechanism in *Drosophila* that leads to behavioral changes and is partially driven by the gut microbiota. In flies deficient in essential amino acids derived from the diet or produced by the microbiota, elevated expression of the neuropeptide CNMamide (CNMa) in the anterior midgut gut cells occurs via the Gcn2 and Tor pathways, which in turn modulates feeding behavior via the activity of neurons expressing CNMa receptors. This was then shown to modulate feeding behavior via the activity of neurons expressing CNMa receptors [94]. It is interesting that one of the specific molecules that directly controls the brain-gut axis has been identified.

Drosophila is also a useful laboratory animal to test the effects of probiotics. Westfall et al. showed that new probiotics and symbiotics prolonged survival in *Drosophila* and improved gene expression related to insulin signaling, fat metabolism, immunity, and oxidative stress. They showed that probiotics and symbiotics prolonged *Drosophila* survival and improved gene expression related to insulin signaling, fat metabolism, immunity and oxidative stress [95].

The study of gut microbiota by *Drosophila* has some limitations. Firstly, due to physiological and developmental differences, it is difficult to directly apply the results of *Drosophila* gut microbiota studies to mammals. Secondly, most invertebrates, including *Drosophila*, do not have the acquired immune system found in mammals [82]. Therefore, the inflammatory response that can be reproduced in fly disease models is likely to different than the inflammatory response in humans. It is difficult to explain complex changes in gut bacteria in disease with only one experimental model. To decipher the complexity of gut microbiota, we need information from multiple models. The results shown in fly studies may represent a

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fundamental part of the whole picture of host and gut microbiota interactions that are conserved in multiple species. Even though the fly's gut microbiota are not simple mimics of humans, flies will be a wonderful system to learn the molecular mechanisms of how the gut microbiota affect the host's health and behavior.

CONCLUSION

Drosophila is a useful laboratory animal for the study of gut microbiota and neurodegenerative diseases. Studies of *Drosophila* will allow us to test the hypothesis that the gut microbiota is involved in human neurodegeneration. Disease modeling by *Drosophila* has just started using microbiota studies and is expected to be applied to a wider field in the future.

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Bacterial Amyloid Curli Associated Gut Epithelial Neuroendocrine Activation Predominantly Observed in Alzheimer's Disease Mice with Central Amyloid-β Pathology

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Abstract.
Background: Substantial evidence from recent research suggests an influential and underappreciated force in Alzheimer's disease (AD) pathogenesis: the pathological signals originate from outside the brain. Pathogenic bacteria produce amyloid-like proteins "curli" that form biofilms and show functional similarities to human amyloid-β (Aβ). These proteins may contribute to neurological disease progression via signaling cascade from the gut to the brain.
Objective: We propose that curli causes neuroendocrine activation from the gut to brain that promotes central Aβ pathology.
Methods: PGP9.5 and TLR2 levels in response to curli in the lumen of Tg2576 AD mice were analyzed by immunohistochemical and qRT-PCR analysis. Western blot and human 3D *in vitro* enteroids culture systems were also used. 16S rRNA gene sequencing was used to investigate bacterial dysbiosis.
Results: We found significant increase in bacterial-amyloid curli with elevated TLR2 at the mRNA level in the pre- and symptomatic Tg-AD gut compared to littermate WT controls. This data associates with increased gram-positive bacterial colonization in the ileum of the symptomatic AD mice. We found fundamental evidence for vagus nerve activation in response to bacterial curli. Neuroendocrine marker PGP9.5 was significantly elevated in the gut epithelium of symptomatic AD mice, and this was colocalized with increased TLR2 expression. Enteroids, 3D-human ileal mini-gut monolayer *in vitro* model system also revealed increase levels of TLR2 upon stimulation with purified bacterial curli fibrils.
Conclusion: These findings reveal the importance of pathological changes within the gut-vagus-brain signaling in response to luminal bacterial amyloid that might play a vital role in central Aβ pathogenesis seen in the AD brain.

Keywords: Alzheimer's disease, amyloid-β, bacterial amyloid, curli, dysbiosis, enteroendocrine cell, gut barrier, gut-brain axis, neuroendocrine, PGP9.5, TLR2, vagus nerve

INTRODUCTION

Our understanding of the "gut-brain axis" (GBA) and the mechanisms by which peripheral events can affect processes in the central nervous system (CNS) remains relatively unexplored [1]. Recent studies

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have provided robust evidence that gut impairment is connected to the progression of neurodegenerative diseases including Alzheimer's disease (AD) [1]. A hallmark of aging is the presence of chronic low-grade systemic inflammation (i.e., "inflammaging") [2]. We propose that a potential source of this inflammation come from the gut mucosa [1]. Age-related alterations in the gut microbiota composition have been documented, with decreased bacterial diversity and stability [3, 4]. This leads to gut barrier breakdown, a further increase in pro-inflammatory cytokines with the presence of bacteria-derived products into host and subsequent blood-brain barrier impairment and neuroinflammation [5, 6]. Others and our previous studies show that aging itself is associated with mild gut impairment and central inflammation, and this can be worsened in young or reversed in aged mice by modifying the microbiota [3, 4]. However, vagus communication within the gut-brain axis is still poorly understood. It establishes one of the major extrinsic connections between the brain and the gastrointestinal tract and sends information about the state of the inner organs to the brain via afferent fibers [7]. There is preliminary evidence that vagus nerve stimulation is a promising add-on treatment for gastrointestinal disorders [7] but is not fully understood and also it lacks information in terms of other disease types. Currently, all treatments that exist for AD patients are initiated after the emergence of clinical symptoms, when significant pathology is already present in the brain. Once the disease is symptomatic, the pathology is so severe that any treatment is likely to be futile. Investigation at pre-symptomatic stage, well before the disease has progressed in the brain, is crucial to understand the initiation and progression of the disease.

It is clear that late-onset AD is collectively modified by number of genetic factors that govern diverse cellular and molecular pathways, including many genes involved in the immune responses [8]. The role played by the immune system in AD pathogenesis is prominent but is by no means limited to the brain. Copious evidence from clinical and experimental research suggests an influential, yet largely underappreciated, force in AD pathogenesis: immune signals originating outside the brain. In the gut, there is a single layer of epithelial cells separating the lumen from the underlying tissue. Within the epithelial layer lies highly specialized cells called enterochromaffin cells, a subset of enteroendocrine cells, that are dispersed and can be excited electrically. These enterochromaffin cells (ECs) sense ingested nutrients and microbial products from the lumen [9]. The mechanism we propose is that part of intestinal bacteria can form biofilms by producing amyloid-like proteins that behaves similar to mammalian Aβ [10–12]. Gut inflammation in reaction to bacterial amyloid can alter gut integrity and allow the translocation of antigens [13–16] including bacterial biofilm associated amyloids "curli" into the gut mucosa [10–12]. This may trigger a specific immune response just not only in the systemic pathway but also in the vagal pathway that contributes to the central Aβ pathology [16] with age.

Therefore, we hypothesize that the gut physiology in response to its microbiota composition might play a vital role in AD progression. This is possible by toll-like receptor (TLR) activation in the gut. TLR2 activation has been recently shown to increase amyloid aggregation [17] and inhibition of TLR2 attenuates neuronal Aβ levels in AD mice [17, 18]. We propose that microbial amyloid-like proteins may cause TLR associated inflammatory cascade that promotes central Aβ pathology [19] via gut-brain axis interaction. This interaction might be stronger via afferent vagus signals from the gut to the brain. In support of our hypothesis, we found significantly increased luminal curli (bacterial-amyloid), associated with elevated gut TLR2 mRNA levels in the pre-symptomatic transgenic (Tg) 6-month-old and symptomatic Tg $\geq$ 15-month-old AD mice compared to littermate wild-type (WT) controls. In association with TLR2 elevation, we found increased gram-positive bacterial colonization in the ileum of the symptomatic AD mice compared to WT controls and the bacterial colonies appeared morphologically different with increased filamentous shape compared to cocci shape found in the lumen of aged WT mice gut. In addition, we found increased curli expression in pre-symptomatic Tg 6-month-old compared to WT littermate control mice gut. It is possible that TLR2 activation is due to increased translocation of bacterial products like curli (bacterial amyloids) for a prolonged period of time and that causes gut dysfunction. We found fundamental evidence for vagus nerve activation in response to bacterial curli. This was investigated by studying the expression levels of PGP9.5 (neuroendocrine marker) within the ileum. The data obtained clearly shows that TLR2 activation caused significant elevation of PGP9.5 expression within the ECs of AD mice ileum compared to WT mice. TLR2 + PGP9.5 + signals were also elevated in the sub-mucosa where vagus nerve fibers are enriched [20]. In addition, enteroids 3D human ileal mini-gut

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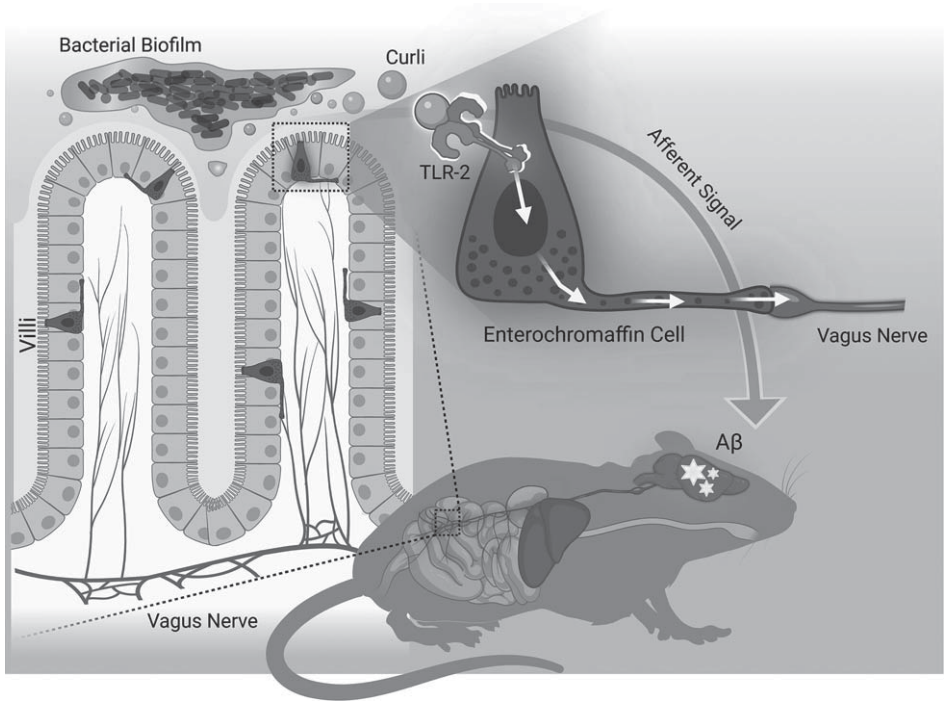


Fig. 1. Schematic representation of the gut neuroepithelial circuit activation (the neuropod cells) in response to bacterial curli breach in the presence of central Aβ pathology observed in AD mice. TLR2, Toll-Like Receptor 2; Aβ, amyloid-beta. (Designed using Bio Render).

monolayer *in vitro* model also revealed increase levels of TLR2 upon stimulation with purified bacterial curli fibrils, adding supportive evidence to the *in vivo* study. Overall, our study reveals the first set of evidence on the importance of immune changes within the gut vagal pathway in response to luminal bacterial amyloid that might play a vital role in central Aβ pathogenesis seen in the AD brain (Fig. 1).

METHODS

Mice

The Tg2576 transgenic mouse model of AD was used in this study. These mice overexpress the 695-amino acid isoform of human Alzheimer amyloid precursor protein (APP_{695}) containing a double mutation of Lys670Asn, Met671Leu (i.e., Swedish mutation) under the control of the hamster prion protein promoter [21], resulting in elevated levels of Aβ aggregates, extensive cerebral amyloid pathology, and cognitive deficits [22]. These animals develop parenchymal plaques beginning at around 9–10 months of age with some vascular amyloid which eventually becomes widespread in the cortical and limbic structures by around 15 to 16 months of age [23].

The study was conducted under protocols approved by the Center for Laboratory Animal Medicine and Care (CLAMC)/Institutional Animal Care and Use Committee (IACUC) at the University of Texas McGovern Medical School. We formed the breeding pairs with young (2 months old) Tg/WT males (129S6.Cg-Tg(APPSWE)2576Kha N20+?) mice (model 1349) and young WT/WT (129S6.Cg-Tg(APPSWE)2576Kha N20+?) females mice, that were purchased from Taconic. Prior to breeding, all the animals purchased were housed for one month to normalize their microbiota and adapt to the new environment. The breeding pairs were used to generate F1 generation followed by genotyping to confirm the presence of inherited mutation. Tg2576 and WT littermate mice obtained from F1 generation were used in the study. All the experiments shown here were performed with animals obtained from F1 generation. The pups were weaned 21 days after birth and both sexes were used in the study. Mice were housed up to 5 per cage (individually ventilated, changed weekly or bi-weekly under HEPA-filtered workstations) in standard facilities with a 12-h light/dark schedule

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(lights on at 7AM) in a temperature- (21.7–22.8 C) and humidity-controlled (40–60 RH) controlled vivarium, with ad libitum access to food (LabDiet 5053 and 5058, pelleted, irradiated at manufacturer, stored at room temperature for up to 6 months) and water (filtered tap water, pH 6–8, not acidified nor chlorinated). The animal used in the study were pre-symptomatic Tg mice that were 6 months of age and symptomatic Tg mice that were15 to 16 months of age with their respective age-matched littermate controls.

Immunohistochemistry (IHC) and microscopy

IHC was performed as previously described in [1]. Formalin-fixed, paraffin-embedded 5 μm-thick intestinal sections were used for the following staining protocols. For lectin staining, terminal mucin glycans were examined using a panel of FITC-conjugated lectins: *Ulex europaeus* agglutinin-1 (UEA-1) for terminal fucose. De-paraffinized sections were rinse in PBS-Tween and block with PBS containing 10% FBS for 1 h at room temperature (RT). Sections were then stained with labeled lectin (10 μg/ml) for 1 h at RT. Sections were washed with ice-cold 1X PBS and counterstained with DAPI for 10 min at RT and mounting using fluoroshield (F6182-20 ml, Sigma).

For all other gut staining protocols, after dewaxing and rehydration steps, a heat-inducing antigen retrieval procedure using 1 mM EDTA buffer at pH 8.0 was performed, with subsequent washing in PBS. After a blocking step, sections were incubated with primary antibodies (Table 1) overnight followed by incubation with fluorescent secondary or HRP immunoglobulins (1 : 1000, multiple routinely used vendors) for 60 min.

Immunoreactions were developed with diaminobenzidine (DAB) diluted in DAB Substrate Buffer (Peroxidase chromogen or Substrate solution from N-Histofine DAB-2V, Tokyo, Japan). The sections were counterstained with hematoxylin for IHC or DAPI (ThermoFischer) for f-IHC prior to visualization. Images were obtained on a Keyence BZ-X810 all in one confocal microscope.

Brain sections fixed in tissue freezing media were cut with 12 μm thickness. Thioflavin S (Millipore Sigma, Mfr. No. T1892-25G) staining for detection of Aβ plaques in the brain sections was performed by incubating the sections with 1% thioflavin-S in 0.01 mol/L PBS in the dark for 8 min at 22°C, followed by washing in 70% ethanol for 3 min.

CsgA expression was quantified by our newly established scoring protocol. Briefly, Curli expression per 10 μm of lumen was quantified using scores from the 20X images obtained. Five images per section and 3 slides per animal were used. In total n of 6 animals per group for symptomatic timepoints and 3 animals per group for pre-symptomatic timepoints were used. The scores were performed as, score: 4 (dark brown IHC stain with disrupted mucus barrier), score: 3.5 (dark brown IHC stain with intact mucus barrier), score: 3 (medium brown IHC stain with disrupted mucus barrier), score: 2.5 (medium brown stain with intact mucus barrier), score: 2 (light brown IHC stain with reduced intact mucus barrier), score: 1 (light brown IHC stain with intact mucus barrier) and score: 0 (little to no brown stain).

Fluorescent in situ hybridization (FISH)

Formalin-fixed paraffin-embedded intestinal tissue sections (5 μm) were initially treated with lysis buffer for 1 h at 37°C and then hybridized at 51°C with a 20 bp bacteria-specific probe (EUB 338: GCTGCCTCCCGTAGGAGT) to visualize and quantify the proximity of luminal bacterial colonies and the corresponding antigen load on the epithelium. Following the overnight hybridization, the intestinal sections were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, Dihydrochloride, Cat #: D1306 by Invitrogen) for visualization of cell nuclei. Images were obtained and analyzed using a Keyence BZ-X810 all in one fluorescence microscope.

Table 1
The information of primary antibodies

Primary antibody	Host	Dilution ratio	Mono/Polyclonal	Source	Catalog number
CsgA	Rabbit	1 : 10000	Monoclonal	Obtained from Dr. Chapman lab	
Muc2	Rabbit	1 : 100	Polyclonal	Novus Biologicals	NBP1-31231
CD282 (TLR2)	Rat	1 : 100	Monoclonal	Invitrogen	14-9021-82
TLR2	Rabbit	1 : 100	Monoclonal	Invitrogen	MA5-32787
PGP9.5	Rabbit	1 : 200	Polyclonal	GeneTex	GTX109637

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Curli bacterial amyloid protein purification

CsgA protein from *E. coli* was purified as described previously [24]. Briefly, *E. coli* CsgA was overexpressed in NEB 3016 cells. Induction performed at OD 0.9 with 0.5 mM IPTG for 1 h and cells were pelleted down. Pellet was resuspended in 25 mL 8 M guanidine chloride in 50 mM KPi buffer pH 7.4 and incubated at room temperature for 1 h with shaking. Following this, the cell lysate was centrifuged at 10000 g for 20 min at 4°C. The supernatant was then sonicated with a probe sonicator at level 3 for three 20-s bursts. Then 800 μL of Sigma Ni-NTA resins were added and incubated at room temperature for 1 h with continuous slow shaking. The supernatant was then loaded on to 5 ml polypropylene columns and washed with 10 mL of 50 mM KPi buffer pH 7.4, followed by 3 mL of wash buffer (12.5 mM Imidazole in 50 mM KPi buffer pH 7.4) and finally CsgA protein was eluted out with 3 mL of elution buffer (125 mM Imidazole in 50 mM KPi buffer pH 7.4. Fraction 1 was 500 μL and fraction 2 was 1000 μL (only two fractions were collected). Fraction 2 contained CsgA. This fraction was then first buffer exchanged to 50 mM KPi buffer pH 7.4 and then passed through a 30 kD cut off column and the filtrate was collected (CsgA). The CsgA protein concentration was estimated using BCA assay prior to use (this protein purification is performed in Chapman lab).

Human ileal enteroids (HIE) cultures

HIE cultures were generated from crypts isolated from the ileal tissues of adult patients undergoing bariatric surgery as previously described [25]. These established cultures were obtained at Baylor College of Medicine through the Texas Medical Center Digestive Diseases Center Gastrointestinal Experimental Model Systems (GEMS) Core. Three-dimensional HIE cultures were prepared from the tissue samples and maintained in culture as described previously [25]. For these studies, ileal HIEs from patient IL103 were used. Complete medium without growth factors (CMGF-) and CMGF+ were prepared as previously described [25]. Briefly, CMGF- consisted of advanced Dulbecco's modified Eagle medium (DMEM)/F-12 medium supplemented with 100 U/mL penicillin-streptomycin, 10 mmol/L HEPES buffer, and 1× GlutaMAX (all Invitrogen, Carlsbad, CA). CMGF+consisted of CMGF- medium with 50% (vol/vol) Wnt3A-conditioned medium, 20% (vol/vol) R-spondin–conditioned medium, 10% (vol/vol) Noggin-conditioned medium, 1× B-27 supplement (Invitrogen), 1×N-2 supplement (Invitrogen), 1 mmol/L N-acetylcysteine (Sigma-Aldrich, St. Louis, MO), 50 ng/mL mouse epidermal growth factor (Invitrogen), 10 mmol/L nicotinamide (Sigma-Aldrich), 10 nmol/L Leu-Gastrin I (Sigma-Aldrich), 500 nmol/L A-83-01 (Tocris Bioscience, Bristol, UK), and 10 nmol/L SB202190 (Sigma-Aldrich).

Differentiation medium consisted of the same components as CMGF + without Wnt3A-conditioned medium, R-spondin–conditioned medium, SB202190, and nicotinamide, and only 5% (vol/vol) Noggin-conditioned medium. hW-CMGF+, for creating and maintaining lentivirus-transduced HIEs, consisted of CMGF+mixed with an additional 50% (vol/vol) Wnt3a-conditioned medium. All HIEs were passaged in phenol red-free, growth factor–reduced Matrigel (Corning, NY).

HIE monolayer preparation

HIE monolayers were prepared from 3D cultures and seeded into flat 96-well plates. In brief, 96-wells (Corning) were pretreated with Matrigel diluted in 1×PBS (1 : 40) and incubated at 37°C. 3D HIEs were lifted from Matrigel and washed with an ice-cold solution of 0.5 mmol/L EDTA in 1×PBS and dissociated with 0.05% trypsin/0.5 mmol/L EDTA for 4 min at 37°C. Trypsin was inactivated with CMGF- +10% fetal bovine serum and the cell solution was pipetted vigorously and filtered using a 40-μm nylon cell strainer (Corning) to dissociate into single cells. Then cells were centrifuged for 5 min at 400×g, resuspended with CMGF+ and 10 μmol/L Y-27632 Rock inhibitor, and plated into prepared wells. After 48 h in CMGF+ and 10 μmol/L Y-27632 Rock inhibitor, the medium was changed to differentiation media with the addition of 10 μmol/L Y-27632 Rock inhibitor and indicated concentrations of doxycycline (Thermo Fisher Scientific, Waltham, MA). Differentiation medium with Y-27632 and doxycycline was changed every day for 4–5 days to differentiate cells.

Bacterial curli incubation with HIEs

HIE monolayers were differentiated for 4 to 5 days. Upon differentiation, the monolayers were treated with bacterial curli at three different concentrations. HPLC purified bacterial curli of 40 μM was used. We used saline control, 0.025 nM, 0.05 nM, and 0.5 nM curli concentrations to treat bacterial enteroids.

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The enteroids were incubated for 12 h at 37°C. After incubation, supernatants were removed and enteroids were washed in 1x Pbs. Enteroids were treated with RIPA lysis buffer for western blot protein analysis. These were reproduced five times. Lysed enteroids were stored at −80°C until analyzed.

mRNA gene expression

To quantify relative mRNA expression levels of TLR2, TLR4, and GAPDH mRNA was extracted from intestinal mucosa samples using the miRNeasy® mini kit (QIAGEN). 1.0 μg of RNA was reverse-transcribed to single-stranded cDNA using the RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Fischer, USA). Reverse transcriptase real-time (RT) PCR was performed using the Quant Studio 3 Real-Time PCR system (Applied Biosystems, USA). The RT-PCR reaction mix (adjusted with H_2O to a total volume of 20 μl) contained 2 μl template DNA, 10 μl Sybr green master mix (Thermo Fischer, USA), Relative mRNA target gene expression levels ($\text{Ratio} = [(E_{\text{target}})^{\text{dCPtarget(control}-\text{sample)}}] / [(E_{\text{ref.}})^{\text{dCPref.(control}-\text{sample)}}]$) were normalized to the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and used as a reference. Subsequently, intestinal mucosal gene of interest of the WT littermate control group at 16 months (Yg-WT) was set to 1.0 and used as the calibrator to identify the relative mRNA fold difference between the WT littermate controls and Tg2576 mice at 6 and 15 months. Similar methodology was used for mRNA extraction from enteroids treated with bacterial curli and saline treated control group.

Immunoblotting

Cell pellets were collected from enteroids monolayers and solubilized in RIPA lysis buffer containing protease and phosphatases inhibitor (Roche), followed by homogenization by sonication. After centrifugation at 5000 g for 10 min, the supernatant was collected as the protein lysate. Protein concentrations were determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Twenty-five micrograms of protein from each sample were resolved onto 4 to 15% SDS-PAGE gradient gels (Bio-Rad). Following electrophoresis, proteins were transferred to nitrocellulose membrane. After blocking with 5% non-fat milk in TBST, blots were probed with 1 : 500 dilution of primary antibody (TLR2, cat# MA5-32787, Invitrogen) overnight at 4°C, followed by a 1 : 2000 dilution of anti-rabbit HRP secondary antibody (Cat# 7074, Cell Signaling Technology) for 1 h at room temperature. SuperSignalTM West Femto Maximum Sensitivity Substrate (Thermo Fisher) was applied and the blot was imaged on a chemiluminescent imaging system. The blot was then stripped and probed with HRP-conjugated mouse anti-beta actin diluted 1 : 3000 (Cell Signaling Technology) before substrate addition and imaging. Beta actin was used as the loading control. Image J is used to analyze the band intensity.

Statistics

Data were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed data are presented as means with standard error while the medians with their range are given for non-normally distributed data. Significance of differences between Yg-WT (age-matched littermate controls for pre-symptomatic Tg2576), Yg-Tg (pre-symptomatic, 6-month-old Tg2576), Ag-WT (age-matched littermate controls for symptomatic Tg2576), and Ag-Tg (symptomatic,≥15-month-old Tg2576) mice were analyzed using the One-way of variance test for normally distributed data (or) the Kruskal-Wallis test for non-normally distributed data, followed by either Bonferroni /Tukey or Dunn's comparison *post-hoc* tests. Differences between Yg-WT and Yg-Tg group was tested using students *t*-test followed by the Mann-Whitney test for non-normally distributed data. All the experiments are performed blinded. Differences between the groups were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. SPSS 16.0 (IBM, USA) for Windows 7 was used for data analysis. Prism 5.0 software (Graph Pad Software, Inc., La Jolla, CA, USA) for Windows, was used for data presentation and also for data analysis. All investigators were blinded to genotype.

RESULTS

Dysbiosis was significantly found in the small intestinal microbial colonization of symptomatic AD mice compared to WT littermate controls

First and foremost, we confirmed the Aβ pathology in symptomatic AD mice brain showing the presence of Aβ plaques in the cortex, hippocampus, and thalamus regions (Fig. 2A). The composition of gut microbiota can be influenced by intestinal epithelial dysfunction associated immune activation [1].

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We have previously reported the presence of large intestinal gut dysfunction in pre-symptomatic and symptomatic Tg mice compared to their respective WT littermate controls [1]. Similarly, we performed 16S rRNA sequencing to examine compositional differences in the gut microbiota of Tg2576 mice at 15 to 16 months of age with the littermate controls. We examined the bacterial diversity of gut microbiota in the small intestinal (SI) luminal content (ileal) samples. After visualization and analysis of alpha-diversity, or within-sample diversity, we observed no differences in OTUs between the symptomatic AD compared to WT littermate controls groups ($p = 0.24$, Fig. 2B). Upon visualization of beta-diversity, or between-samples diversity, with Unweighted UniFrac distances by principal coordinate analysis (PCoA), a significant clustering effect ($p = 0.003$) emerged along the PC1 axis (23.9% variation explained) at the symptomatic timepoint in Tg2576, when compared to age-matched WT littermates (Fig. 2C). Examination of 16S data at the family level showed a significant increase in Lactobacillaceae abundance in the symptomatic Tg2576 mice when compared to age-matched WT littermates. Our 16S data show that significant bacterial compositional differences termed as dysbiosis (imbalanced in beneficial versus detrimental bacterial composition), exist in the SI of symptomatic Tg2576 mice gut microbiota when compared to WT littermate controls and that shift denoted significant increase in gram positives within the SI lumen.

Bacterial amyloid "Curli" breach observed in symptomatic AD mice gut when compared to littermate WT controls

Dysbiotic bacteria within the gut lumen may form biofilms by producing bacterial amyloid-like proteins known as curli [10–12]. To investigate if dysbiosis found in Tg2576 AD mice show increased curli burden in the gut lumen, we used immunohistochemistry technique against bacterial curli biofilm in the cecal gut tissue and found significant increase in curli abundance in both pre-symptomatic (6-months of age) and symptomatic AD mice (≥15 months of age) compared to age-matched WT littermate controls. We found significant increase in bacterial curli at both pre- and symptomatic timepoints showing the presence of biofilm bloom within the gut earlier than Aβ pathology occurs in the brain which persists into symptomatic age (Fig. 3A (i)-(iv), B). In addition, ileum also showed increased curli abundance in

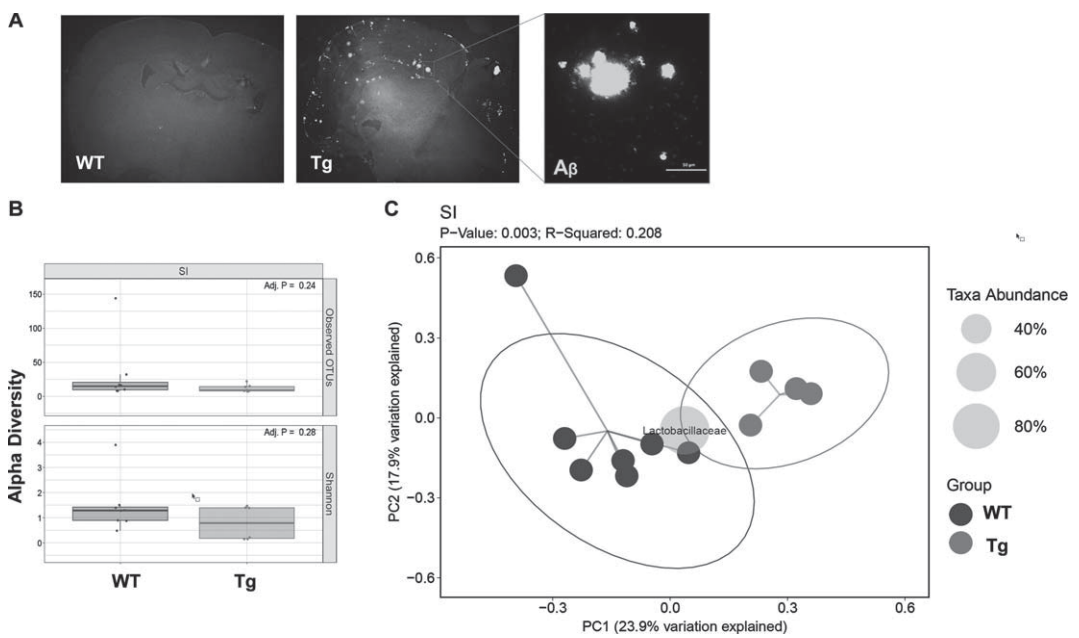


Fig. 2. Compositional differences in gut microbiota by 16S rRNA sequencing of small intestinal luminal content. A) Presence of Aβ plaques (green signal) in the symptomatic Tg2576 AD mice brain compared to age-matched WT controls using thioflavin-S staining. Magnification-2X. B) Visualization of alpha diversity shows changed with-in the sample diversity. They show no significant changes with-in the bacterial population of animals from the same groups. C) Beta-diversity, or between-samples diversity, with Unweighted UniFrac distances by principal coordinate analysis (PCoA) shows a clustering effect by strain between Tg2576 and WT littermate controls at symptomatic timepoint of 15 months, within the small intestine. $n = 5$-7 per group. p-values were calculated using ALTIMA sequencing software platform

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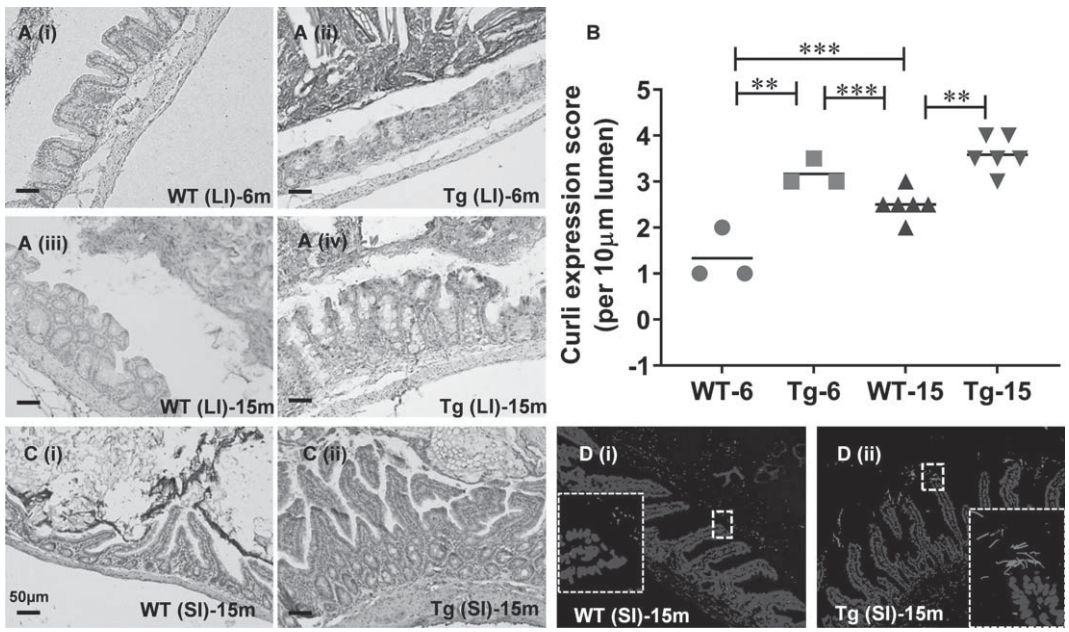


Fig. 3. Bacterial amyloid curli protein expression by IHC in the gut lumen. A-C) Bacterial amyloid Curli protein (CsgA) staining within the large intestinal (A) and small intestinal (C) gut lumen (bacterial mass), showing a significant increase expression in pre-symptomatic and symptomatic Tg2576 AD mice when compared to age-matched WT controls (B) quantification of curli expression. A(i) and A(ii) are from pre-symptomatic timepoints of 6 months of age; A(iii), A(iv), C (i), and C(ii) are from symptomatic time-points of 15 months of age. $n = 3,6$ per group. D) Bacterial breach through the mucosal barrier onto the intestinal epithelium detected by FISH in the small intestine of Tg2576 mice and their WT littermate controls at 15 months. Red - bacterial biofilm. Blue - host epithelial nuclei. $n = 6$ per group. A, C, D) Magnification 20x. $n = 3$, 6 per group. Data are expressed as mean ± s.d., as well as individual values, and are obtained from > three independent experiments at various times. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. p-values were calculated using One-Way analysis of variance with Bonferroni correction.

symptomatic-AD mice compared to WT littermate control. Bacterial curli was proportionately increased in aged WT-littermate controls (Fig. 3C) compared to young WT controls. It is known that aging induces dysbiosis [3, 4], and we found increased curli expression in aged gut compared to young gut in WT littermate controls (Fig. 3A (i), 3A (iii)). Together with curli analysis, bacterial biofilm using FISH quantification in the SI tissue revealed that bacterial colonies appeared morphologically different with filamentous shape in the ileum of symptomatic AD mice compared to cocci shaped found in WT littermate controls and gut bacterial breach was proportionately increased in AD mice as previously observed [1] (Fig. 3D).

Significant increase in the gut mRNA and protein TLR2 levels of AD mice and this was associated with increased amyloid plaque formation in the brain

TLRs are the first line of defense in recognizing pathogens and activate innate immune responses. They are expressed in the gut epithelial cells, lamina mucosa especially dendritic cells, intraepithelial lymphocytes, and vagus nerve cells within the submucosa. TLR2 is activated by enrichment of materials from gram-positive and TLR4 is activated due to gram-negative bacteria [26]. We set to understand the host-microbiota link due to bacterial dysbiosis and its associated increase in bacterial amyloid-curli seen in symptomatic AD mice compared to WT littermate controls. TLRs sensing might play a major role in immune activation against bacterial curli in these animals. We found significant increase in the mRNA levels of TLR2 (Fig. 4A) and TLR4 (Supplementary Figure 1) in the ileum of symptomatic AD mice compared to the WT littermate controls. Interestingly this difference was strikingly elevated in the SI where vagus nerve and immune activation is dominant. In addition, TLR2 activation was significantly stronger than TLR4 activation which provides us the clue that the pathogenic molecule activates TLR2 associated pathway. Importantly, we found TLR2 activation was ≥5-fold elevated in the ileum of the pre-symptomatic AD mice at 6 months of

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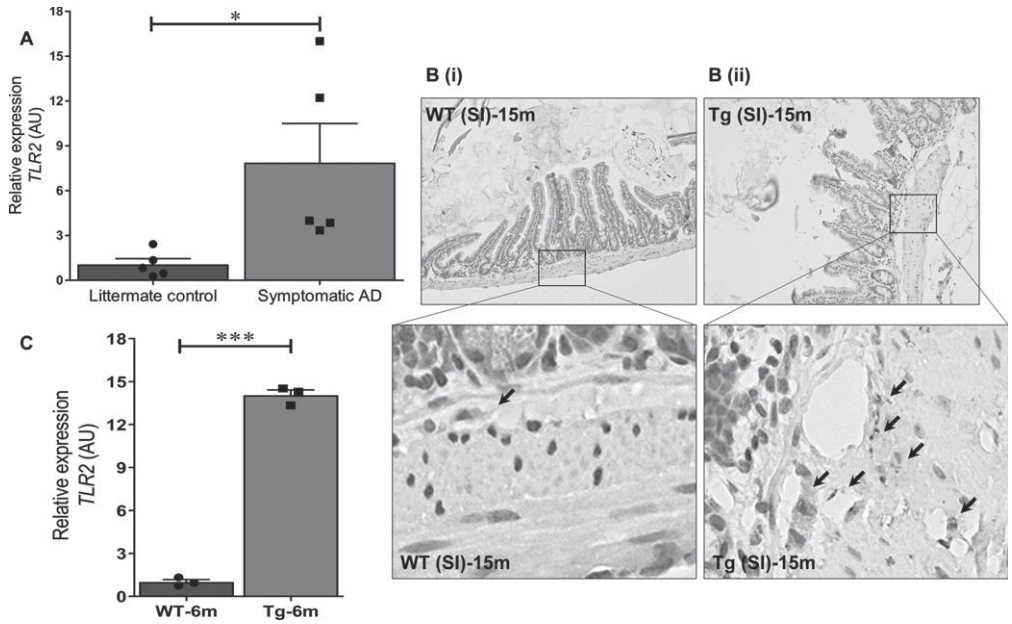


Fig. 4. TLR2 is elevated in the gut tissue of AD mice. A) TLR2 mRNA expression is significantly increased in symptomatic Tg2576 measured by qRT-PCR. B) Immunohistochemical staining of small intestine shows a significant increase in TLR2 expression in Tg2576 mice (B(ii)) when compared to WT littermate controls (B(i)) at 15 months. Brown-TLR2, blue-nuclei. C) The transcriptomic analysis targeting TLR2 in the small intestine of Tg2576 mice at pre-symptomatic timepoint of 6 months of age shows significant increased TLR2 to the WT controls. $n = 3$ or 5 per group. B) Magnification 20x. $n = 6$ per group. A, C) Data are expressed as mean ± s.d., as well as individual values, and are obtained from three independent experiments at various times. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. p-values were calculated using students t-test.

age compared to littermate WT control (Fig. 4C). At 6 months of age the Tg2576 animals show no pathology in the brain [1]. In addition, we found 2-fold increase in TLR2 mRNA levels of symptomatic AD mice compared to WT-littermate controls. Immunohistochemical protein analysis (Fig. 4B) also supported the mRNA data that TLR2 was found elevated in the gut sub-mucosa and in the intestinal epithelium of AD mice compared to WT mice.

Increased PGP9.5 neuroendocrine marker and TLR2 activation seen in the ileal epithelial enterochromaffin cells of the symptomatic AD mice

Because we were interested in both the systemic and vagus nerve role in the gut-brain axis (GBA) communication of the central Aβ pathogenesis and to understand the TLR2 activation associated signaling mechanism within the GBA, we performed fluorescence-IHC on the ileal gut tissues of AD and control WT mice. Intriguing changes were recorded at the TLR2 protein levels. We additionally co-stained these gut sections with PGP9.5 (stains neuroendocrine cells). We found increased vagal afferent activation within the ileal tissue observed in symptomatic AD mice compared to littermate controls. Interestingly, PGP9.5 neuroendocrine marker was colocalized with TLR2 and this was present in ECs of the ileal epithelium (Fig. 5). TLR2 expression was also found sparsely in age-matched WT littermate controls but was significantly lower than AD mice. However, the number of $TLR2^{+}$ $PGP9.5^{+}$ cells were significantly increased in AD mice compared to the control mice (Fig. 5m). Additionally, these $TLR2^{+}$ $PGP9.5^{+}$ cells were also strongly expressed at the sub-mucosa that defines vagus nerve activation from the lumen to the mucosa (Figs. 4B, 5k, 5l) and potentially to the CNS.

Central TLR2 levels increased with Aβ plaque formation in the symptomatic AD mouse brain

Since we found increased TLR2 signals together with afferent vagal activation at the sub-mucosa in the AD mouse gut (Figs. 4 and 5), we set out to understand the TLR2 activation in the brain. Interestingly, we found significantly elevated levels of TLR2 activation in the cortex of AD mouse brain (Fig. 6) by immunohistochemical analysis and this was

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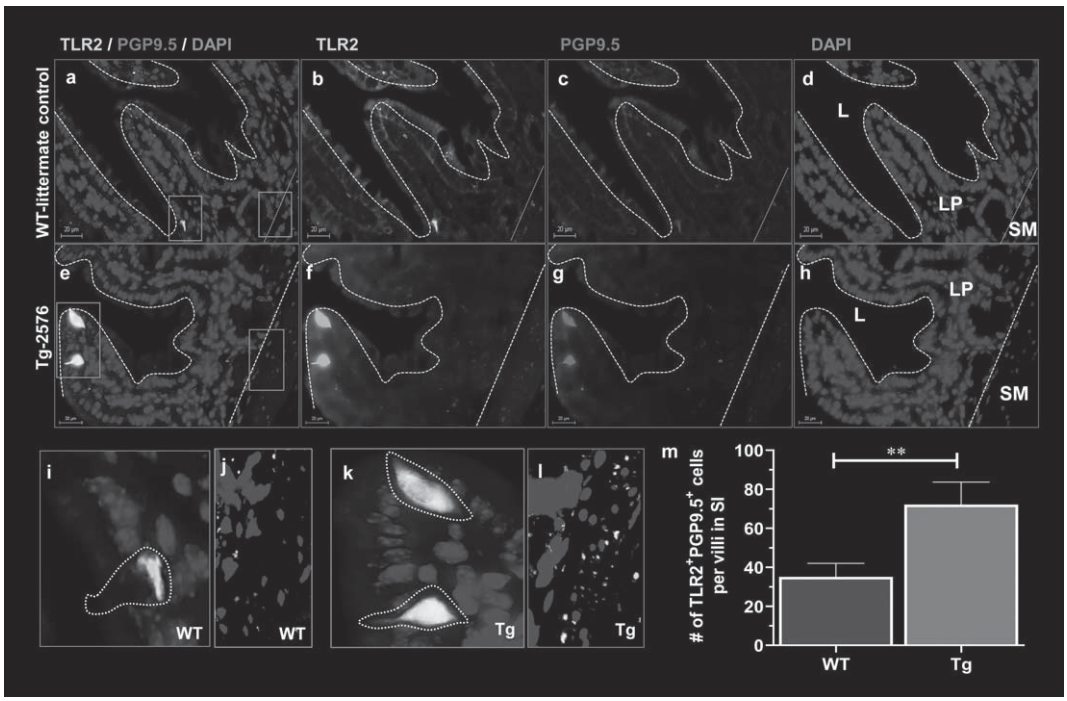


Fig. 5. TLR2 colocalizes with neuroendocrine marker PGP9.5 in the enterochromaffin cells within the epithelium and sub-mucosa of AD mice ileum. Immunofluorescent microscopy image of small intestinal villus showing TLR2 (green color) in ECs (subset of enteroendocrine cells) co-localized with PGP 9.5 neuroendocrine marker (red color) of vagus nervous system. Expression of TLR2 and PGP 9.5 in Symptomatic Tg2576 (e-h) are higher than WT (a-d) in the small intestine. The merged cells with overlapping expression are highlighted in the enlarged view, WT ileum epithelium (i) and sub-mucosa (j), whereas Tg AD mice ileum epithelium (k) and sub-mucosa (l). Images are taken at 60x magnification and exposures are set using staining of WT mouse tissues. All scale bars indicate 20 μm. (m) The percentage of $TLR2^+$ & $PGP9.5^+$ cells per villi in small intestine of symptomatic Tg2576 is higher than WT-littermate controls. Data are represented the mean ± s.d. of 5 mice in each group. TLR2 positive cells were calculated per villi for 15 villi per animal tissue. The TLR2 expression was also associated with PGP9.5 expression (low in WT animals). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *p*-values were calculated using students *t*-test. LP, lamina propria; SM, sub-mucosa; L, lumen.

associated with significant presence of Aβ plaque deposits in the brain (Fig. 2A). This was observed significantly in the cortex and hippocampus regions of the AD brain compared to age-matched WT littermate control mice.

Elevated TLR2 levels after bacterial curli treatment in the human ileal enteroids

To validate if bacterial curli activates TLR2 specific responses in the gut epithelium containing enterochromaffin cells, we used an *in vivo* specialized method of gut 3D enteroids cultures from human ileum. This was then prepared as monolayer. These enteroids monolayer where then treated with saline control and purified bacterial curli at varying concentrations (high-0.5 nM, medium-0.05 nM, and low-0.025 nM). We found increased TLR2 protein levels after 12 h of incubation with bacterial curli (Fig. 7A). Enteroids showed 2-fold increased levels of TLR2 with as low as 0.025 nM of curli protein incubation for 12-hours (Fig. 7B).

DISCUSSION

A growing body of evidence suggests that intestinal pathobiontic bacteria produce amyloid-like proteins that forms biofilms [10–12]. These proteins may contribute to neurological disease progression from the gut to the brain [13–16]. Amyloids are a class of protein with unique self-aggregation properties [27], and their aberrant accumulation can lead to cellular dysfunction associated with neurodegenerative diseases [27, 28]. While genetic and environmental factors can influence amyloid formation, molecular triggers are not well defined. Growing evidence suggests that non-identical amyloid proteins may accelerate reciprocal amyloid aggregation in a prion-like fashion [29]. While humans encode ∼30 amyloidogenic proteins, the gut microbiome

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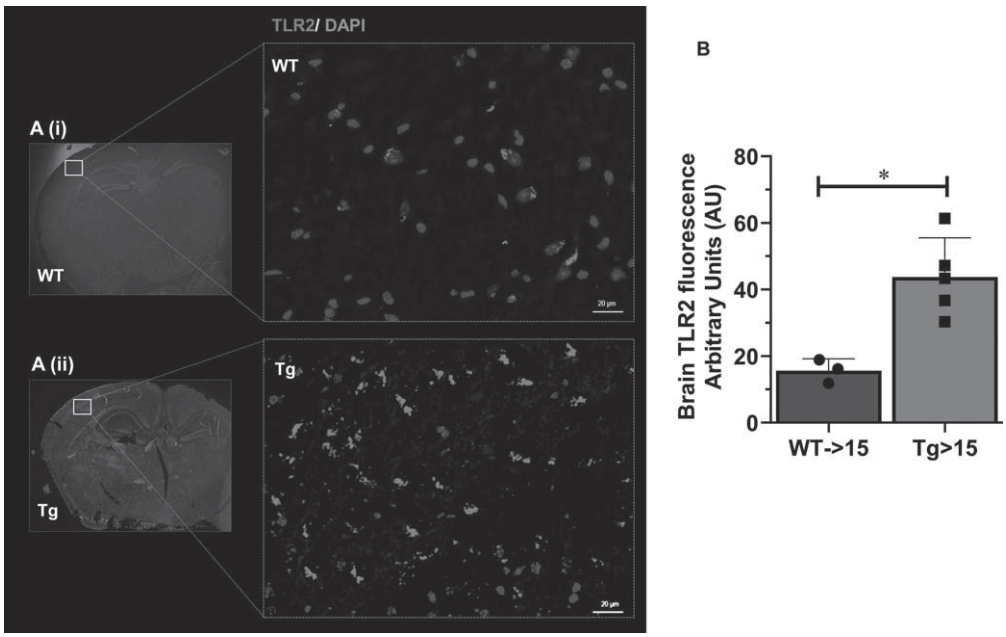


Fig. 6. Expression of TLR2 is markedly increased in symptomatic Tg2576-mice brain (Cortex). A) Immunofluorescence staining of TLR2 in the brain indicates significant increase of TLR2 expression in the cortex of Tg2576 AD mice (Tg) compared to the wild-type littermate controls (WT). Images are taken at 2x and 60x magnification and exposures are set using staining of WT mouse tissues. Images are representative of at least 3 independent experiments, each including 5 mice of each group. Scale bars indicate 20 μm. B) Fluorescence intensities of TLR2 expression in cortex sections from Tg and WT mice. Data are expressed as fluorescence intensity in arbitrary unit (a.u.) and represent the mean ± s.d. of 5 mice. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *p*-values were calculated using students *t*-test

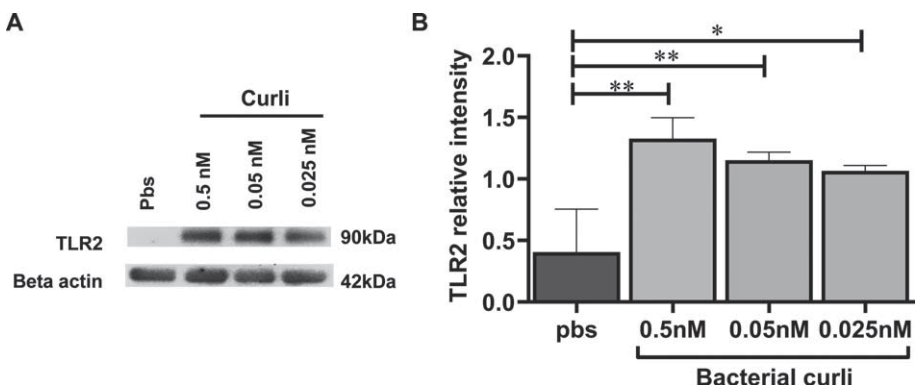


Fig. 7. Bacterial Curli accelerate TLR2 protein expression in human ileal enteroids (HIE). A, B) Immunoblotting analysis of TLR2 expression in HIE. The assay is performed in triplicate and analyzed by NIH ImageJ Software. Samples are compared to β-actin as a housekeeping protein. Data are presented as the mean ± s.d. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *p*-values were calculated using One-Way analysis of variance with Bonferroni correction.

also produces functional amyloids to build the biofilm matrix [30] that lines the intestinal mucus layer. Gut inflammation can alter gut integrity, gut bacterial composition as well and allow the translocation of antigens including biofilm associated amyloids into the gut mucosa [10–12]. Bacterial amyloid may trigger those local inflammation [31]. Bacterial amyloid translocation [13–15] may trigger [31] and accelerate amyloid aggregation in the brain [32–35]. Viral, bacterial, and fungal infections might be causative factors for the inflammatory responses found in AD [36]. We have already documented in our previous study that gut dysfunction occurs prior to Aβ accumulation in the brain using the same Tg2576 AD mouse model [1]. Most of the previous analysis was from large intestine, however, to understand gut-brain axis communication, the SI is of utmost importance due to its primary role in vagal activation [37]. Similar to

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the large intestinal gut dysbiosis found earlier [1], in our current study using 16S rRNA gene sequencing, data show significant bacterial compositional differences termed as dysbiosis (imbalance in normal gut microbiota compared to WT controls), found in the SI of symptomatic Tg2576 mice (15 to 16 months of age) gut microbiota when compared to WT littermate controls, and that shift denoted significant increase in gram positives within the SI lumen. We also found increased proportion of family Lactobacillaceae that again relates to our previous observation [1]. We sort to understand the main role of increase in Lactobacillaceae family. We will in our future studies understand the strain type of Lactobacillaceae bloom in these AD gut. However, since we found significant dysbiosis occur in AD mice SI, we were interested to look at immune activation from the gut. Therefore, we explored the gut integrity by looking at bacterial biofilm using FISH and found that the bacterial type differs in AD mice compared to WT controls. They were found more of rod shape and filamentous in the AD mice gut compared to cocci shaped bacteria found in WT mice. As a consequence, we will focus our future analysis based on these morphological changes. Most importantly, our control animals were age matched WT littermates and they live in the same cage. Since mice are coprophagic, the bacterial cross seeding might be an existing problem. Therefore, our future experiments will have age-matched non-littermate WT controls in addition. We mainly focused this study in SI because, microbial diversity in the SI is of utmost importance due to its specialized nature of containing ECs that are known to activate not just the systemic but primarily vagus nerve signaling pathways [37].

Loss of gut barrier integrity may cause pathogenic bacterial amyloid [10–12] translocation [13–15] and peripheral inflammation [38–40], and may influence toll-like receptor 2 induced innate immune responses [31]. We hypothesize that gut TLR2 activation may accelerate central Aβ pathology [19]. Interestingly, we found increased levels of bacterial amyloid-curli in the Tg AD mouse large intestine at pre-symptomatic timepoint before the Aβ pathology was found in the brain [1] compared to WT controls. However, we observed bacterial-curli presence in both aged WT and symptomatic AD mice large intestine. Similarly, we also found curli proteins in SI gut tissue of both WT and symptomatic AD mice. The increased bacterial amyloid-curli biofilm in WT animals' gut lumen might be due to age-associated changes in the gut microbiota that are known to have detrimental effects [3, 4]. However, gut barrier integrity is impaired significantly in AD mice than in WT mice with age. Therefore, curli translocation and TLR2 activation would be profound in aged AD mice. We found TLR2 activation was 4- to 5-fold increase in pre-symptomatic AD mice compared to WT controls in the ileum. In addition, symptomatic aged AD mice also showed significantly increased TLR2 compared to WT controls but was not as high as pre-symptomatic timepoint. The increased levels of gut TLR2 was associated with loss of gut barrier integrity [1] and luminal bacterial-curli biofilm abundance found in pre-symptomatic AD mice compared to young WT control mice. Interestingly, we found TLR2 activation to be significantly stronger at the sub-mucosa regions of the ileal tissue in the symptomatic AD mice analyzed with IHC. It is previously known that vagal nerve plexus is present abundantly in the sub-mucosal regions and lamina propria within the gut tissue [20] and they extend the nodes to the epithelium and directly communicate with gut epithelial ECs [9]. ECs serve as bidirectional signal transducers between the gut lumen and nervous system [41, 42]. Vagal, afferent innervation of ECs provides a direct pathway for enterochromaffin-cell signaling to neuronal circuits [42, 43]. Since we found increased protein levels of TLR2 at the sub-mucosa, the data guided us to look at the intestinal epithelium more closely. PGP9.5 is a neuroendocrine/enteroendocrine marker that is known to be activated during vagus nerve stimulation [44]. Using fluorescence IHC, we found that ileal gut ECs significantly express TLR2 and this expression was co-localized with PGP9.5 (neuronal endocrine marker) [9, 45].

Dysbiotic pathogenic bacteria in the gut lumen form biofilms and they might produce amyloid-like proteins [10–12]. Specifically, *Salmonella* derived bacterial amyloid curli fibrils contribute to Nos2 expression in animal models by stimulating TLR2 signaling [31, 46]. Bacterial amyloids can activate TLR 2/9 receptors [31] and exacerbate memory deficits [10–15]. TLR2 activation also worsens white matter damage in AD [19]. TLR2 activation has been shown to increase amyloid aggregation and inhibition of TLR2 attenuates neuronal Aβ levels in AD mice [17, 18]. It is possible that TLR2 activation is due to increased translocation of bacterial products like curli (bacterial amyloids) [31] due to gut dysfunction in AD [1]. On that note, we also found increased TLR2 levels in the symptomatic AD brain compared to age-matched WT controls. We

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believe that TLR2 activation mainly progress from the gut in response to bacterial amyloid-curli. We have shown that gut TLR2 elevation colocalizes with neuroendocrine marker within the ECs. Our data provided the first evidences that gut TLR2 activation causes afferent vagus nerve signaling in response to bacterial-curli that travel to the brain in an AD mouse model. More research on this line of thought is necessary and we are doing further experiments to understand this pathway more in detail. However, to verify that bacterial curli activates TLR2 within the gut epithelium, we used 3D enteroids culture of human ileum which also has intact ECs apart from other specialized epithelial cells and where treated with purified bacterial curli protein fibrils. This experiment revealed that bacterial-curli induces TLR2 activation.

In conclusion, our data clearly shows that bacterial amyloid burden is present in AD mouse gut even before Aβ pathology occurs in the brain. Even though WT aged mice show curli presence, an intact gut barrier is significantly reduced in AD mice gut [1] which might cause increased curli translocation in AD mice. Bacterial curli activates TLR2 specific signals within the epithelium and in the sub-mucosa of the gut. Importantly, TLR2 activation co-localized with significantly elevated neuroendocrine marker PGP9.5 within the gut of AD mice that provides evidence for vagus nerve activation in response to bacterial curli [44]. We also found increased levels of TLR2 activation within the brain of AD mice as seen by others [17, 18]. Therefore, it provides preliminary evidence that TLR2 activation from the gut to brain in response to bacterial curli might play a major role in central Aβ pathology of AD. However, we need more evidence to prove whether this signaling pathway is clinically important in order to treat the pre-symptomatic AD patients before pathology is seen in the brain when treatments are likely to be ineffective. Our future studies will primarily focus on unraveling the gut-vagus-brain connection with clinical implications in AD.

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SUPPLEMENTARY MATERIAL

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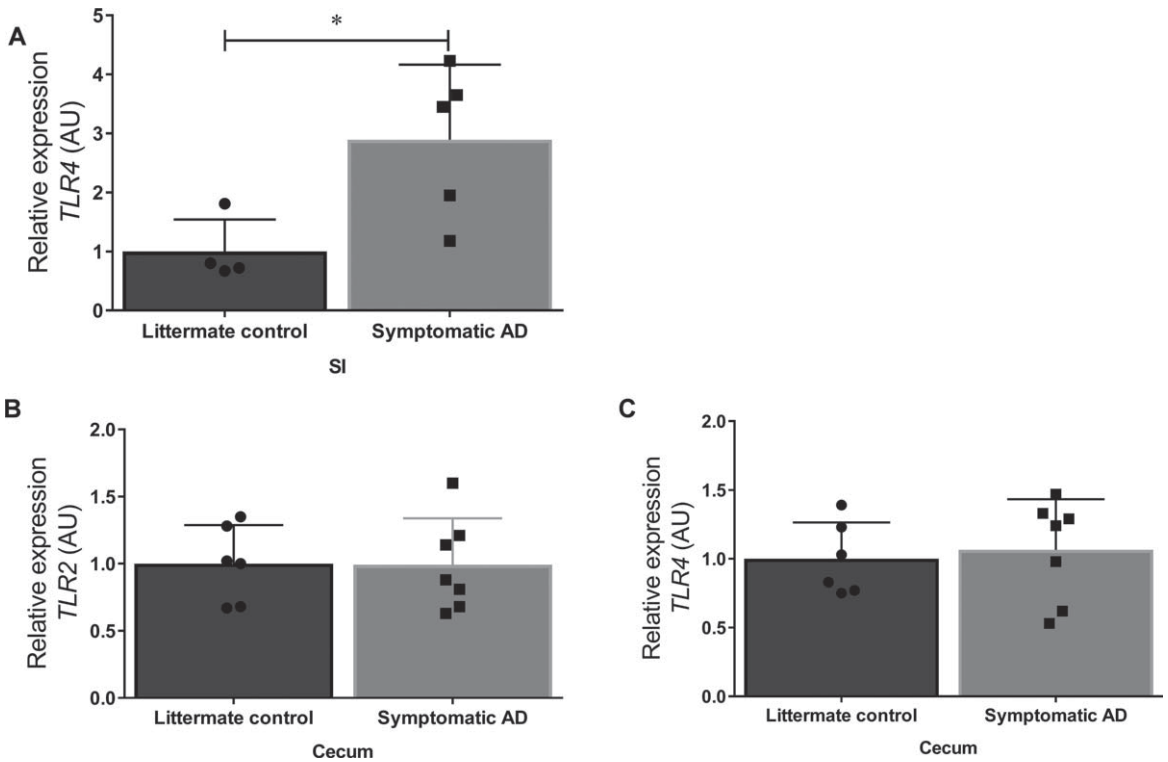
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SUPPLEMENTARY MATERIAL

Bacterial Amyloid Curli Associated Gut Epithelial Neuroendocrine Activation Predominantly Observed in Alzheimer's Disease Mice with Central Amyloid-β Pathology



Supplementary Figure 1. The transcriptomic analysis targeting TLR4 in the small intestine (A) and cecum (C) of Tg2576 mice at 15 months shows a significant increase of the expression of the TLR4 gene compare to the WT controls only in the small intestine tissue. B) The TLR2 gene expression in cecum of Tg2576 mice at 15 months does not show any significant difference with their WT controls.

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Diet Patterns, the Gut Microbiome, and Alzheimer's Disease

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Abstract. Given the complex bidirectional communication system that exists between the gut microbiome and the brain, there is growing interest in the gut microbiome as a novel and potentially modifiable risk factor for Alzheimer's disease (AD). Gut dysbiosis has been implicated in the pathogenesis and progression of AD by initiating and prolonging neuroinflammatory processes. The metabolites of gut microbiota appear to be critical in the mechanism of the gut-brain axis. Gut microbiota metabolites, such as trimethylamine-n-oxide, lipopolysaccharide, and short chain fatty acids, are suggested to mediate systemic inflammation and intracerebral amyloidosis via endothelial dysfunction. Emerging data suggest that the fungal microbiota (mycobiome) may also influence AD pathology. Importantly, 60% of variation in the gut microbiome is attributable to diet, therefore modulating the gut microbiome through dietary means could be an effective approach to reduce AD risk. Given that people do not eat isolated nutrients and instead consume a diverse range of foods and combinations of nutrients that are likely to be interactive, studying the effects of whole diets provides the opportunity to account for the interactions between different nutrients. Thus, dietary patterns may be more predictive of a real-life effect on gut microbiome and AD risk than foods or nutrients in isolation. Accumulating evidence from experimental and animal studies also show potential effects of gut microbiome on AD pathogenesis. However, data from human dietary interventions are lacking. Well-designed intervention studies are needed in diverse populations to determine the influence of diet on gut microbiome and inform the development of effective dietary strategies for prevention of AD.

Keywords: Gut microbiome, gut metabolites, dietary patterns, Alzheimer's disease

GUT MICROBIOME AND ALZHEIMER'S DISEASE

The human microbiome represents an ecosystem of trillions of microorganisms (i.e., bacteria, fungi, and viruses) which are primarily found within the gastrointestinal tract [1]. While some bacteria and viruses can be attributed to disease, gut microbes have important roles in human health, including influencing immune and metabolic functions [2]. Labelling specific bacteria as 'good' or 'bad' for the host is challenging, and not always achievable above strain-level due to the high intra-species functional variation. The gut microbiota provides mediums for the fermentation of non-digestible substrates, like dietary fibers and resistant starches. This fermentation supports the growth of specialist microbes that produce metabolites such as short chain fatty acids (SCFAs). The

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major SCFAs produced are acetate, propionate, and butyrate [2] and have many effects on host physiology including regulation of the gut barrier and influencing inflammatory responses [3]. The ecosystem of the human microbiome is a diverse, complex entity and identification of the diversity of organisms present in microbial communities is possible by utilising sequencing technology [4]. The richness of this ecosystem is often measured by assessing changes in the alpha diversity of a sample (i.e., the variation of microbes in a single sample), or the beta diversity of microbial communities between samples [5]. By doing so, we can understand the number, abundance and types of microbes present within or between samples, and how these might change in response to stimuli or disease [6].

During the adult aging process, the composition of the gut microbiome changes, the diversity of the ecosystem is reduced, and potential pathogens can be promoted (e.g., pro-inflammatory microbes) [7]. Alzheimer's disease (AD) is a progressive, debilitating disease of cognitive decline, presenting as a current global public health challenge [8]. The pathophysiology of AD has been strongly linked to inflammatory pathways, both in the central nervous system and the periphery, with microglial macrophages in the brain becoming chronically activated, promoting sustained production of pro-inflammatory cytokines that contribute to a cycle of neuroinflammatory processes [9]. There is evidence to suggest that communication within the brain-gut-microbiome axis is bi-directional, whereby gut microbes communicate to the central nervous system through nervous (vagal nerve), endocrine, and immune (cytokines) signaling pathways [10]. Given this complex bidirectional communication system between the gut and brain, there is growing interest in the gut microbiome as a novel and potentially modifiable risk factor for AD.

Gut dysbiosis is commonly referred to as an imbalance in the gut microbial community that can be associated with disease [11]. The concept of dysbiosis is complex, with the term being broadly used in the literature to represent a difference between healthy and diseased patients, or within a patient through the disease process [12]. This broad concept, in the absence of a defined healthy or normal microbiome, makes the nuance of dysbiosis difficult to define. Nonetheless, mechanistic studies have demonstrated how an imbalance of the gut microbiome ecosystem can be associated with the pathogenesis of AD by initiating and exacerbating these neuroinflammatory processes [13]. There are consistent data to indicate that the microbiome of AD patients has reduced diversity in comparison to sex and age-matched individuals [14]. Furthermore, alterations of the microbiome ecosystem may affect the synthesis and secretion of several brain-derived neurotrophic factors and pathophysiological changes consistent with AD [15, 16].

Considerations for microbiome research

Improved technology and ability to profile the microbes in clinical samples has resulted in a massive research interest in the microbiome. There are important considerations for designing and executing a microbiome study, detailed extensively elsewhere [17]. Of particular note is accounting for potential confounders between groups of interest to mitigate false positives (see [18]). There are two principal sequencing approaches, 16S rRNA gene sequencing and metagenomic sequencing. The former is an amplicon-based approach and as such is limited to taxonomic classification typically to genus level. The latter approach sequences DNA without amplifying a specific universal gene and, as such, allows identification to species and strain level, as well as information on genetic capacity. This metagenomic approach is more costly but the extra detail can provide important insight into diet-cognition correlations. Notably, both techniques generate proportional data and resulting analyses and interpretation must consider taxonomic changes are in 'relative abundance'. In other words, if something goes up, something else must go down. To complete such qualitative approaches, investigators can perform follow-up experiments (e.g., quantitative PCR) of species of interest to determine the true microbial load or copy numbers of the target organism.

DIETARY PATTERNS, GUT MICROBIOME, AND AD

Diet is an important modifiable factor influencing the composition of the gut microbiome, indicating the potential for dietary interventions to modulate microbial diversity, composition, and stability [19]. Several studies have shown that modulations of the gut microbiome community can result in biological changes, potentially contributing to chronic disease risk [20]. In relation to the influence of diet on AD, mechanistic studies have shown how the synergistic effects of nutrients/foods when consumed as part of a usual dietary pattern are likely to exert greater effects than

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single nutrients on inflammatory processes and neurodegeneration [21]. As a result, there has been much interest in examining the role of dietary patterns such as the Mediterranean diet (MD), as a potential strategy for AD prevention. Although current evidence is inconclusive [22–24], there is a body of evidence mainly from population based studies, with some trial evidence, demonstrating that greater adherence to healthy dietary patterns, like a MD, is associated with slower cognitive decline and reduced AD risk [24–27].

Only a small number of intervention studies have to date examined the interrelationship between the MD, gut microbiome, and cognitive function. The NU-AGE study was a one-year, randomised, parallel trial to investigate whether a tailored Mediterranean-like dietary pattern could counteract or slow down the inflammatory processes during aging [28]. The MD is characterised by high intake of fruits, vegetables, whole grains, nuts, and legumes; moderate intake of fish, poultry, and alcohol (particularly red wine, with meals); and low intake of red and processed meats with olive oil used as the main fat source [29]. Participants were adults aged 65–80 years across five European countries and were randomly assigned to either a NU-AGE diet group (MD) or control group (national dietary guidelines for relevant country). In terms of adherence, after one year, the diet group improved mean intake of 13 out of 16 NU-AGE dietary components ($p < 0.05$), with a significant increase in total NU-AGE index (difference in mean change = 21.3 ± 15.9 points, $p < 0.01$) [30]. Furthermore, among NU-AGE participants, the MD was associated with microbiome alterations. Adoption of the MD increased abundance of specific beneficial taxa [*Faecalibacterium prausnitzii, Eubacterium, and Roseburia*] that were positively associated with improved cognitive function, and negatively associated with inflammatory markers including C-reactive protein and interleukin-17 [31]. Furthermore, a decline in microbiome diversity was observed among those allocated the low MD adherence group.

Brain glucose utilisation can be impaired during aging, with accelerated decline in glucose uptake and insulin resistance observed in cognitive impairment and AD [32]. However, ketones can provide an alternate energy source for the hypometabolic brain in AD, sharing protein-mediated uptake mechanisms similar to that of glucose [33]. The ketogenic diet (KD) consists of high fats, moderate proteins, and very low carbohydrates (around 5% to 10% of total caloric intake, or below 50 g per day) and stimulates ketone production [34]. There are suggestions that increased uptake of ketones via a KD, particularly in those with cognitive impairment could provide a therapeutic target against neurodegeneration and AD [35]. Experimental data show potentially beneficial effects of the KD on neurotransmission, neuroinflammation, insulin sensitivity, amyloid accumulation, and oxidative stress [36]. However, to date there is no consensus on the effects of KD on the intestinal microbiota. Murine studies have demonstrated an increase in the relative abundance of beneficial gut microbiota (*Akkermansia* and *Lactobacillus*) and reduction of potentially pro-inflammatory taxa (*Desulfovibrio* and *Turicibacter*) in response to KD [37]. In contrast, another study in rats found that the KD over 8 weeks induced gut dysbiosis and lowered gut microbiome diversity. In the same study, rats fed a higher carbohydrate diet (68% energy from carbohydrates, 19% energy from protein, and 13% energy from fats) showed increased microbiome diversity and higher relative abundance of Bacteriodetes [38]. Furthermore, a higher carbohydrate diet potentiated insulin signaling and reduced neuroinflammation in this study. In AD mice models, a high fat diet (HFD, containing 60% energy from lard-based fat, 20% from protein, 20% from carbohydrate), caused increased beneficial changes in the gut microbiome composition [specifically in the phyla *Firmicutes, Bacteroidetes, and Actinobacteria*] [39]. Hence, it is not clear how KD per se affects the gut microbiome, but the proportion and quality of both carbohydrate and fat in the diet appear to be important modulators of gut microbiota diversity and taxonomic composition. Furthermore, variations in the characteristics of the study populations investigated, e.g., in terms of gender, age, and presence of chronic disease, may have an influence on the impact of a KD intervention.

The Modified Mediterranean KD (MMKD) allows slightly higher carbohydrate consumption to permit increased intake of vegetables and fruits, while promoting fats and proteins derived from sources such as olive oil and fish [40]. Nagpal et al. [40] conducted a pilot study among 17 participants (11 with mild cognitive impairment (MCI), and 6 cognitively normal (CN)) to investigate the effects of a MMKD versus control diet (dietary guidelines from the American Heart Association, namely high intakes of fruit and vegetables, whole grains, and healthy protein sources, with lower intakes of processed foods, added sugar and salt) on markers of

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AD and gut microbiome over 6 weeks. The intervention resulted in no significant impact on the overall gut microbiome in terms of the alpha diversity and beta diversity indices. The optimum timeframe for observing changes in the gut microbiome in response to diet intervention is not known, but 6 weeks may not be long enough to detect any impact on bacterial diversity. However, there were several healthy gut bacterial phyla, families, and genera differentially altered after the short MMKD intervention in MCI versus CN participants. For example, phylum *Actinobacteria*, family *Bifidobacteriaceae*, and genus *Bifidobacterium* were significantly reduced among the MCI group after MMKD compared to CN participants [40]. Furthermore, MMKD was also associated with fungal-bacterial networks that correlated with AD markers in MCI patients suggesting that diet regulation of gut-brain axis involves interactions of the broader gut microbiome ecosystem in a relatively short timeframe [41].

In a further small study, using advanced metagenomic sequencing, participants who switched to a lacto-ovo-vegetarian diet (from habitual omnivorous diet) for 3 months demonstrated some significant changes in the gut microbiome, compared to those following an omnivorous control diet [42]. After 3 months of the lacto-ovo-vegetarian diet, the relative abundance of *Alistipes* was reduced, coinciding with increased relative abundance of *Roseburia inunilivorans, Ruminococcus lactis, Lactobacillus plantarum*, and *Streptococcus thermophiles*. However, there was no detected difference in alpha-diversity in response to the vegetarian intervention. An additional comparison of both control groups (long-term omnivores and vegetarians) revealed compositional differences at genus and species levels, supporting the idea that long-term dietary patterns are a major driver of gut microbiota assembly. In conclusion, a switch to the vegetarian diet had an impact on gut microbiota composition, but its functional relevance on gut microbial co-metabolism remains to be elucidated [42].

Wan et al. [43] performed a study to investigate the effect of different proportions of dietary fat intake on gut microbiota. In this 6-month randomised controlled-feeding trial, 217 healthy young adults were allocated to three diets with varying the amounts of fat, containing lower-fat (20% energy), moderate-fat (30% energy), and higher-fat (40% energy) and underwent fecal metabolomic analysis using 16S rRNA sequencing. At the phylum level, the moderate and higher fat diets decreased the ratio of Firmicutes to Bacteriodetes after intervention. At the genus level, the higher fat diet decreased the relative abundance of *Faecalibacterium* and increased the relative abundance of *Alistipes* and *Bacteroides*, while the lower fat diet increased *Faecalibacterium* and *Blautia* relative abundance after the intervention. The changes in relative abundance of *Blautia* was negatively associated with the changes in serum total cholesterol, low-density lipoprotein cholesterol, and non-high-density lipoprotein cholesterol, whereas the change in *Bacteroides* relative abundance was positively correlated with the changes in these blood lipid markers [43]. In this study, intake of dietary fiber on all the three diets was maintained at the baseline level of consumption, approximately 14 g per day. The total amount of carbohydrate was highest in the lower-fat diet group, mainly from white rice and wheat flour (bread). Given that dietary fiber intake was similar across the groups, it is plausible that the potentially beneficial effects of the lower-fat diet could be due to the increased amount of resistant starch found in these food products, which can in turn promote beneficial abundance of gut microbiota [43].

Nuts are a key component of the MD and may have independent benefits for the gut microbiome in part due to their high dietary fiber and unsaturated fatty acid content. In a randomised controlled trial (cross-over design) of 96 healthy participants, a walnut-enriched diet was administered for 8 weeks followed by a switch to nut-free diet [44]. Fecal samples were collected for 16S rRNA gene sequencing analysis. No difference was found in alpha-diversity, but beta-diversity of bacterial profiles demonstrated a distinct clustering of the walnut and control groups. In the walnut group, a significantly increased relative abundance of Ruminococcaceae and bifidobacteria coincided with a decrease in *Clostridium* sp. Cluster XIVa species *Blautia; Anaerostipes* compared to control diet. Thus, walnut intake may promote compositional shifts of the gut microbiota to potentially probiotic and SCFA-producing species that may account for potential health benefits associated with walnut consumption. However, links to health-promoting, walnut-dependent metabolites remain speculative, since this study reported compositional differences and did not quantify actual microbial metabolites [44].

In summary, the potential for dietary interventions to modulate microbial diversity is mechanistically plausible; however, as the current evidence in relation to cognition stems from either animal studies, or smaller, pilot human intervention studies measuring the effect of a narrow range of dietary patterns,

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it is not yet known if gut dysbiosis contributes to the development of AD and/or progression of cognitive impairment.

POTENTIAL MECHANISTIC PATHWAYS LINKING DIET, GUT MICROBIOME, AND AD

Bacterial metabolites and toxins appear to be critical in the gut-brain axis and could provide important insight into mechanistic pathways linking diet, gut microbiome, and AD. Bacterial endotoxins such as lipopolysaccharide (LPS) and microbiota metabolites, such as trimethylamine-n-oxide (TMAO), and SCFAs, are suggested to mediate systemic inflammation and cerebral amyloidosis; processes that are suggested to be the principal pathogenic pathways in AD [45]. These may be amenable to diet manipulation as discussed in more detail below.

Lipopolysaccharides

LPS are components of the outer membrane of gram-negative proinflammatory bacteria and have been implicated in the pathogenesis of AD. Plasma LPS levels are reported to be 3-times higher in AD patients than in healthy controls [46]. LPS have been found to be significantly increased in gray matter as well as the vulnerable neocortex and hippocampus regions of the AD brain, compared to age-matched controls [47]. Furthermore, mice injected with LPS accumulated amyloid-β in the hippocampus and exhibited severe cognitive impairment [48]. Furthermore, LPS is thought to promote amyloidosis via the activation of inflammatory signaling pathways. LPS bind to microglial receptors (Toll-Like Receptor (TLR) TLR2, TLR4, and CD14) in the brain and activate nuclear factor kappa B (NF-kB) transcription factor to increase oxidative stress and neuroinflammation and promote the accumulation of amyloid-β proteins and neurofibrillary tangles [49]. In older adults, higher plasma LPS, pro-inflammatory cytokine levels, and markers of endothelial function were associated with a higher risk of amyloidosis, suggesting that LPS could be a key pathophysiologic factor linking between the gut microbiome and AD pathology [50].

Relatively little is known about how diet can influence LPS. Diet-induced damage to the gut endothelium can cause metabolic endotoxemia or increased plasma LPS from the gut. Chronic high-fat diets are associated with endotoxemia and can damage the blood–brain barrier and allow LPS to enter into the brain [49]. Higher adherence to a MD has been associated with lower endoxemia [51], mainly driven by increased fruit and vegetable intake. In older adults, greater adherence to both the MD and a healthy 'prudent' diet (rich in vegetables and fruits and low in cookies) were associated with lower circulating 3-hydroxy fatty acids (3-OH FAs), a proxy measure of LPS burden (β [95% CI] for each additional point of score: –0.12 [–0.22, –0.01] and –0.27 [–0.48, –0.07], respectively) [52]. In contrast, greater adherence to a 'traditional' high meat diet was associated with higher concentration of 3-OH FAs (β [95% CI] 0.22 [0.001, 0.46]) [52]. Further research is warranted to determine whether plant-based diets are effective in reducing metabolic endotoxemia as a means to potentially modulate neuroinflammation and AD pathology.

Trimethylamine N-oxide

TMAO is a pro-inflammatory toxin derived from the gut metabolism of choline, betaine, and carnitine in animal foods, e.g., meat, fish, eggs, and dairy. These nutrients are metabolised by gut microbes, particularly Firmicutes and Proteobacteria, to produce trimethylamine (TMA), which is subsequently oxidised to TMAO in the liver by flavin monooxygenases 3 (FMO3) [53]. TMAO can alter cholesterol homeostasis [53] and potentially increase cardiovascular disease risk [54]. Emerging evidence suggests that TMAO may contribute to accelerated neurodegeneration and AD. Mice treated with TMAO show increased oxidative stress and neurotoxicity, impaired mitochondrial function, inhibition of mammalian target of rapamycin (mTOR) signaling, neuroinflammation, and impaired cognitive performance [55]. Findings from computational analysis reported that TMAO was one of the metabolites highly associated with AD [56]. Furthermore, TMAO is observed at higher concentrations in cerebrospinal fluid from AD patients compared to cognitively healthy individuals [57]. Hence, there is growing interest in dietary manipulation of TMAO as a potential target for AD prevention and treatment.

High protein, high fat western diets are associated with increased TMAO while plant-based dietary patterns are associated with decreased TMAO levels [58–60]. High protein intake at twice the recommended Dietary Allowance significantly increased plasma TMAO levels in healthy men [58]. High fat diets may also increase TMAO [60] as well as the

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abundance of *Firmicutes* and *Proteobacteria*, which promote TMA production [53]. In contrast, a recent crossover trial demonstrated significant reductions in TMAO in response to a plant-based diet possibly by increasing the prevalence of bacterial genus such as *Prevotella* that inhibit TMA synthesis [59]. It is not clear how MD impacts TMAO. In the PREDIMED study, higher adherence to MD was associated with lower TMAO levels [61]. However, in adults at increased colon cancer risk, the MD did not affect TMAO and TMA levels, possibly due to a higher fish consumption which can act as a TMA precursor [62]. Further research is needed to understand the potential modulation effects of dietary patterns and food constituents on TMAO and AD biomarkers in humans.

Short chain fatty acids

Acetate, propionate, and butyrate are among the SCFAs derived mainly from gut microbial fermentation of dietary fiber [63]. SCFAs activate G-protein-coupled receptors to modulate neurohumoral gut signaling and exert antimicrobial and anti-inflammatory effects for maintenance of gut integrity and intestinal health [64]. Interestingly, SCFAs have direct effects in the brain and may play a role in modulating AD pathogenesis. Experimentally, SCFAs are shown to reduce amyloidosis and inhibit neurotoxic amyloid-β aggregations linked to cognitive impairment [65, 66], although data have not been consistent [67]. The underlying mechanisms by which SCFAs might influence neuropathological processes are not understood but may involve inflammatory and gene regulation pathways. In AD mice, butyrate treatment improved memory function and increased expression of genes implicated in associative learning through enhanced hippocampal histone acetylation [66]. Furthermore, high fiber diets or butyrate administration had potential beneficial effects on neuroinflammation, regulation of neurotrophic factors involved in neuronal integrity and cognitive performance in AD mouse models [65, 67, 68]. Collectively, preclinical studies lend support to a neuroprotective effect of SCFAs; however, the optimal dose and composition of SCFAs have not been comprehensively investigated. While increased blood levels of butyrate have been linked to lower amyloid burden in older adults [50], it is not clear whether diet manipulation to improve SCFAs has neuroprotective effects in humans. Predictive modelling from the NU-AGE study [31] suggested that greater adherence to the MD-style diet increased SCFA producing taxa, suggesting a mediating beneficial role for SCFAs in systemic inflammation and cognitive function. These preliminary findings warrant further investigation to characterise both the gut microbiome and measured SCFA metabolite response to diet intervention.

CONCLUSIONS

Accumulating data support the gut microbiome as a viable modifiable risk factor for AD. Altered gut microbiome toward a more pro-inflammatory state has been reported in patients with AD and even in early-stage MCI. Experimental and animal data also show effects of gut microbiome on AD pathogenesis, although is it not yet known if gut dysbiosis contributes to the initiation of AD and/or progression of disease. Diet is an important modulator of both gut microbiota and microbial metabolites and therefore may be an effective approach to reduce AD risk. However, data from human dietary interventions are lacking. Well-designed intervention studies are needed in diverse populations to determine the influence of diet on gut microbiome and inform the development of effective dietary strategies for prevention and treatment of AD. The few available studies have focused primarily on MD or variations of this dietary pattern, and there is a considerable lack of robust studies consistently testing similar interventions using advanced gut microbiome measurement techniques. Larger intervention studies, of sufficient power and duration ($\geq$12 months as indicated by the NU-AGE study [31]), are needed to test these hypotheses. Furthermore, factors such as gender, ethnicity, and genetics can influence both gut microbiome and AD risk but have not yet been comprehensively investigated in nutrition studies.

Future studies should also focus on the dietary mechanisms in relation to gut toxins and metabolites that can modulate complex gut-brain communications and AD pathology. In addition to LPS, TMAO, and SCFAs discussed above, the influence of diet on other gut metabolites, notably branched-chain amino acids and bile acids as potential candidates for AD biomarkers should be investigated. Furthermore, emerging evidence suggest that commensal gut fungi can influence systemic inflammation, intestinal disease [69], and potentially AD [70]; however the impact of diet on gut mycobiome remains largely understudied. To advance this field, shotgun metage-

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nomic sequencing should be utilized, as unlike 16S rRNA sequencing, it can read all genomic DNA in a sample, rather than just one specific region of DNA. For microbiome studies, this means that shotgun sequencing can identify and profile bacteria, fungi, viruses, and many other types of microorganisms at the same time [71]. This would allow for a more inclusive and detailed mechanistic approach to diet and microbiome studies.

The integration of advanced metagenomics, metabolomics, and informatics approaches in future studies will undoubtedly help to better characterise the role of specific microbiomes and advance our understanding of diet-regulated associations at the strain and species level that could be helpful for developing personlised dietary approaches to reduce the risk of neurodegeneration.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/22-0205r2).

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Section 2

Gut Microbiota and Alzheimer’s Disease

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The Gut Microbiota and Alzheimer's Disease

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Abstract. The gut microbiota comprises a complex community of microorganism species that resides in our gastrointestinal ecosystem and whose alterations influence not only various gut disorders but also central nervous system disorders such as Alzheimer's disease (AD). AD, the most common form of dementia, is a neurodegenerative disorder associated with impaired cognition and cerebral accumulation of amyloid-β peptides (Aβ). Most notably, the microbiota-gut-brain axis is a bidirectional communication system that is not fully understood, but includes neural, immune, endocrine, and metabolic pathways. Studies in germ-free animals and in animals exposed to pathogenic microbial infections, antibiotics, probiotics, or fecal microbiota transplantation suggest a role for the gut microbiota in host cognition or AD-related pathogenesis. The increased permeability of the gut and blood-brain barrier induced by microbiota dysbiosis may mediate or affect AD pathogenesis and other neurodegenerative disorders, especially those associated with aging. In addition, bacteria populating the gut microbiota can secrete large amounts of amyloids and lipopolysaccharides, which might contribute to the modulation of signaling pathways and the production of proinflammatory cytokines associated with the pathogenesis of AD. Moreover, imbalances in the gut microbiota can induce inflammation that is associated with the pathogenesis of obesity, type 2 diabetes mellitus, and AD. The purpose of this review is to summarize and discuss the current findings that may elucidate the role of the gut microbiota in the development of AD. Understanding the underlying mechanisms may provide new insights into novel therapeutic strategies for AD.

Keywords: Aging, Alzheimer's disease, amyloid, amyloid beta-peptides, blood-brain barrier, dysbiosis, gut microbiota, lipopolysaccharides, obesity, type 2 diabetes mellitus

INTRODUCTION

The microbiome of the human gastrointestinal (GI) tract is the largest reservoir of microbes in humans, containing approximately 10^{14} microorganisms from at least 1000 distinct microbial species that vary in diversity and stability between individuals and outnumber human host cells by about one hundred to one. The bacterial density of human gut microbiota is up to 10^{12} per mL, which is the highest density in any known microbial ecosystem (for review, see [1, 2]). The human microbiome has been estimated to encode approximately 4×10^6 genes, which collectively outnumbers the 26,600 human genes by about 150 to 1 [3–5] (for review, see [4, 5]). Due to the current limitations of culturing microorganisms from intestinal samples, the exact composition of the gut microbiota is not fully known. However, Bacteroidetes and

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Firmicutes are the two major bacterial phyla in the gut with others, such as Proteobacteria, Verrucomicrobia, Actinobacteria, Cyanobacteria, Fusobacteria, Spirochetes, and other microorganisms, comprising the remainder (for review, see [2, 6]). The intestinal microbiota is required for distinct functions that are known to be beneficial for host health, including maintenance of the intestinal barrier integrity, inhibition of pathogen adhesion to intestinal surfaces, vitamin K synthesis, energy salvaging from unabsorbed food by short-chain fatty acid (SCFA) production, and regulation and proper maturation of the immune system (for review, see [6, 7]).

The microbiota-gut-brain axis is a bidirectional communication system that is connected via neural, immune, endocrine, and metabolic pathways (for review, see [8–11]). Recent studies suggest that the gut microbiota plays a pivotal role in the modulation of gut and brain functions (for review, see [10, 12–14]). Dysbiosis and alterations of the intestinal microbiota composition contribute to the development and progression of many diseases in humans, including inflammatory bowel disease (for review, see [15, 16]), type 2 diabetes mellitus (T2DM) [17, 18], obesity (for review, see [19, 20]), cardiovascular disease events [21, 22], autism [23, 24] (for review, see [24]), depression (for review, see [25, 26]), Parkinson's disease [27–29] (for review, see [28]), and multiple sclerosis [30, 31]. Accumulating evidence also supports a possible connection between the gut microbiota and Alzheimer's disease (AD). In this review, we discuss the role of the gut microbiome in AD and summarize the studies from both animals and humans demonstrating the contribution of intestinal bacteria on cognitive dysfunction, specifically the development of AD.

BACKGROUND ON ALZHEIMER'S DISEASE

AD is the most common cause of dementia in the elderly and is clinically characterized by progressive cognitive impairment, which commences with a deterioration in memory (for review, see [32]). In 2010, it was estimated that approximately 36 million people were living with dementia worldwide, with numbers predicted to double every 20 years. The numbers were speculated to increase up to 66 million in 2030 and 115 million in 2050 (for review, see [33]). As life expectancy is increasing worldwide, the incidence of AD continues to rise significantly, which causes a heavy burden on family and society. Pathologically, AD is characterized by the extracellular deposition of amyloid-β peptides (Aβ) in senile plaques and the intracellular accumulation of hyperphosphorylated tau that forms neurofibrillary tangles (for review, see [32]). In addition, neuroinflammation is regarded as a factor involved in the pathogenesis of AD (for review, see [34]). Though the etiology of AD remains elusive, it is recognized as an interaction between genetic and environmental factors [35]. Apolipoprotein E (ApoE) is one of the most common susceptibility genes, composed of ε2, ε3, and ε4 alleles, in which ε2 is a protective allele, ε3 is a neutral allele, and ε4 is a high-risk allele (for review, see [36, 37]). In addition to the APOE locus, recent genome-wide association studies have identified some genetic risk factors for late-onset AD, among which is a rare susceptibility variant in TREM2 (triggering receptor expressed on microglia/myeloid cells-2) [38, 39] (for review, see [39]). Though the principal risk factor for AD is advanced age, there are other conventionally acknowledged factors that play a role, including family history, susceptibility genes, and cardiovascular risk vascular factors [39, 40]. Furthermore, a growing body of evidence suggests that the human gut commensal microbiota modulates brain function and behavior via the microbiota-gut-brain axis. Based on the following current studies, we have reason to speculate that the gut microbiota plays a potential role in AD-related mechanisms.

EXPERIMENTAL EVIDENCE THAT GUT MICROBIOTA MODULATION IMPACTS AD

Recent studies have investigated the impact of the gut microbiota on brain function, which indicates strong evidence for a link between the gut microbiota and cognition. As AD is characterized by impaired cognitive behavior, we have reason to speculate about the potential role of the intestinal microbiota in the development of AD. Approaches that have been used to parse the role of the gut microbiota on cognition or AD include the use of germ-free animals, antibiotic or probiotic interventions, microbial infections, and fecal microbiota transplantation (FMT).

Germ-free studies

Germ-free (GF) animals are reared in a sterile environment in gnotobiotic units, which eliminates

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the opportunity for postnatal colonization of their GI tract. Compared with conventionally reared mice that have an intact intestinal microbiota, GF mice demonstrated deficits in non-spatial and working memory tasks (e.g., novel object recognition test and spontaneous alternation assessed in the T-maze) and reduced expression of the brain-derived neurotrophic factor (BDNF) in the hippocampus [41]. BDNF is a neurotrophin that is crucial for synaptic plasticity and cognitive function and has been found to be decreased in the brains and sera of AD patients and to correlate with Aβ burden [42–44] (for review, see [42, 44]). However, Neufeld and colleagues found upregulated mRNA expression of BDNF in the hippocampus of female GF mice [45]. A significant decrease in hippocampal BDNF mRNA expression was observed in male GF animals, while a qualitative increase was observed in female GF mice, indicating that BDNF expression was modulated in a sex-dependent manner [46]. Moreover, GF mice exhibit an increase in adult hippocampal neurogenesis [47]. Adult hippocampal neurogenesis plays an important role in cognitive processes (for review, see [48]). GF mice displayed microglial immaturity and defects in microglial proportions, leading to impaired innate immune responses that can contribute to the pathogenesis of neurological diseases, including AD [49]. Previously, Sudo et al. found decreased expression of N-methyl-D-aspartate (NMDA) receptor 2A (NR2A) mRNA in the cortex and hippocampus of GF mice compared with specific pathogen-free mice [50]. More recently, Neufeld found a downregulation of the NMDA receptor NR2B subunit mRNA in the central amygdala of GF mice [45]. The NMDA receptor also plays a key role in synaptic plasticity and cognitive function (for review, see [51]). More specifically, increased activation of the NMDA receptor may be vital to the Aβ-dependent synaptic dysfunction seen in AD (for review, see [52]). Taken together, germ-free studies show utility in elucidating the underlying mechanisms of the microbiota-gut-brain axis.

Antibiotics

The administration of broad-spectrum antibiotics is one of the most commonly adopted artificial methods to cause intestinal dysbiosis, resulting in reduced abundance and diversity of the gut microbiota in experimental animals. Wang et al. treated weaned male Sprague-Dawley rats with ampicillin, which induced dysbiosis of the microbiota, spatial memory impairments, increased anxiety-like behavior, and decreased NMDA receptor levels in the hippocampus [53]. From weaning onwards, disruption of the gut microbiota after antibiotic treatment reduced anxiety-like behavior and induced cognitive impairment accompanied by significantly decreased BDNF levels in the adult brain [54]. This result agrees with another finding that shows prolonged antibiotic treatment of adult mice decreases hippocampal neurogenesis and cognitive function, which is partially mediated by $Ly6C^{hi}$ monocytes [55]. Erny et al. treated specific pathogen-free mice orally with broad-spectrum antibiotics for 1 month, resulting in an immature phenotype and unchanged number of microglia [49]. A newly published study reported that long-term broad-spectrum antibiotics treatment induced perturbations in gut microbial diversity, decreased Aβ plaque deposition, attenuated plaque-localized glial reactivity and markedly altered microglial morphology in a murine model of AD [56]. In summary, these studies highlight the utility of antimicrobial-based strategies in assessing the role of microbiota in cognition or AD-related changes. Future studies with antibiotics in humans is warranted to further explore the role of gut microbiota on brain function.

Probiotics

Probiotics are living microorganisms that, when ingested in adequate quantities, may offer a health benefits to the host. *Lactobacillus* and *Bifidobacteria* are the two main genera of probiotics used to investigate the impact on the host health. γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the human central nervous system (CNS) produced by *Lactobacillus brevis* and *Bifidobacterium dentium* via glutamate metabolism [57]. Postmortem studies of the cortical areas of AD patients have shown reduced frontal, temporal, and parietal GABA concentrations (for review, see [58]). There are an increasing number of studies in animal models assessing the impact of probiotics on cognitive behavior (see Table 1); however, not all probiotics produce positive effects. Infection with *Citrobacter rodentium* changes the microbiota and causes stress-induced memory impairment in mice, which can be restored by administering a daily treatment of probiotics (*L. rhamnosus* (R0011) + *L. helveticus* (R0052)) [41]. A study by Savignac et al. investigated the role of two *Bifidobacteria* strains in cognitive processes, showing that *B. longum 1714* could improve learning and memory as displayed

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Table 1
Impact of probiotics on cognitive function

Probiotics	Possible mechanisms	Effects on cognition
L. rhamnosus (R0011) + *L. helveticus* (R0052) [41]	prevent the drop in hippocampal BDNF expression	prevent stress-induced memory deficits in *C. rodentium*-infected mice
B. longum 1714 [59]	need further investigation	improve the ability of learning and memory in an anxious mouse
B. breve 1205 [59]	need further investigation	have little or no positive impacts on memory
Lactobacillus fermentum NS9 [53]	maintain NMDA receptor at the normal level	alleviate an ampicillin-induced spatial memory impairment
Lactobacillus helveticus NS8 [60]	restore BDNF levels in hippocampus	improve spatial memory of chronic restraint stress group
VSL#3 [55]	restore neurogenesis and increase $Ly6C^{hi}$ monocyte propotions in the brain	rescue the cognitive deficits of antibiotic-treated mice
Lactobacillus acidophilus+ *Lactobacillus casei*+ *Bifidobacterium bifidum*+ *Lactobacillus fermentum* [61]	need further investigation	have a positive effect on cognitive function in the AD patients

BDNF, brain-derived neurotrophic factor; NMDA receptor, N-methyl-D-aspartate receptor.

in the object recognition test, Barnes maze, and fear conditioning, while *B. breve 1205* had little or no positive impacts on cognition [59]. Treatment with *Lactobacillus fermentum* NS9 alleviated an ampicillin-induced spatial memory impairment and inhibited the ampicillin-induced reductions in NMDA receptor expression [53]. Liang et al. also observed that the probiotic *Lactobacillus helveticus* NS8 could significantly ameliorate cognitive impairments caused by chronic restraint stress and restore BDNF content in the hippocampus [60]. Möhle et al. showed that probiotic treatment could rescue the cognitive deficits of antibiotic-treated mice and fully restore neurogenesis [55]. More importantly, the latest randomized, double-blind, and controlled clinical trial demonstrated that a mixture of probiotics (*Lactobacillus acidophilus*+*Lactobacillus casei*+*Bifidobacterium bifidum*+*Lactobacillus fermentum*) consumption for 12 weeks had a positive effect on cognitive function and some metabolic statuses in the AD patients [61]. To summarize, certain probiotic strains show efficacy in modulating cognitive behavior or AD-related pathogenesis. Consequently, there is a need for future studies focusing on the best combinations of probiotic strains, the timing of administration, and the identification of the mechanism by which each strain exerts its effects. Moreover, clinical validation is also warranted to fully investigate the translatability to humans of the promising results seen in animal studies.

Microbial infection

Many researchers have begun to investigate the impact of microbial infections on the etiology of AD. *Citrobacter rodentium* is a gram-negative pathogenic bacterium, which could alter the intestinal microbiota. *C. rodentium*-infected mice displayed memory dysfunction only when exposed to acute stress, concomitant with a reduction in hippocampal BDNF expression and an increase in colonic proinflammatory cytokine levels [41]. Amyloid deposition formed in mice brains after intranasal infection with *Chlamydia pneumoniae* isolated from an AD brain, indicating that infection could be involved in the initial pathogenesis of AD [62]. Moreover, immunohistochemical analyses of the AD brain revealed that astrocytes, microglia, and neurons may be infected by *C. pneumoniae* and that these bacteria were found close to senile plaques and neurofibrillary tangles [63]. The correlation between *Helicobacter pylori* (HP) infection and AD has been reported in recent case-control studies. More specifically, the HP-infected AD patients tended to have more serious cognitive impairments, as shown by lower scores on the Mini-Mental State Examination [64]. HP infection might have an impact on the pathophysiology of AD through the release of proinflammatory cytokines and induction of oxidative stress (for review, see [65]). HP eradication can prolong the survival of AD patients, potentially indicating that HP infections may alter AD pathophysiology [66].

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Furthermore, other infectious agents such as herpes simplex virus, human immunodeficiency virus, and human cytomegalovirus are related to the pathogenesis of AD [67–69] (for review, see [69]). Interestingly, a new published study reported that a physiologic role of the Aβ, known to form pathogenic plaques in AD, is as an antimicrobial peptide to protect against microbial infection in the brain [70]. Although such studies with pathogens do not directly address the protective roles of the gut microbiota, it is possible that microbiota dysbiosis may increase the host susceptibility to infections, which could promote the onset of AD.

Fecal microbiota transplantation

FMT is an extreme intervention that replaces or replenishes the gut microbiota of an unhealthy individual by transplanting the microbiota from a healthy donor. An arguably effective therapy for treating patients with *Clostridium difficile* infection [71, 72] (for review, see [72]), FMT has also shown favorable outcomes in extra-intestinal diseases associated with intestinal dysbiosis (for review, see [73, 74]). Bruce-Keller et al. found that fecal transplantation from high-fat fed mice whose microbiota was depleted with antibiotics induced significant and selective disruptions in exploratory, cognitive, and stereotypical behavior in conventionally housed, control-diet mice, independent of body weight [75]. Harach et al. revealed that remarkable alterations were observed in the gut microbiota composition of conventionally raised 8-month-old APP/PS1 mice compared to wild-type mice, including reduced levels of Firmicutes, Verrucomicrobia, Proteobacteria, Actinobacteria, and a concurrent increase in Bacteroidetes and Tenericutes [76]. Furthermore, they generated a germ-free APP/PS1 mouse model and discovered a sharp reduction of Aβ pathology in the brain when compared to the conventionally raised APP/PS1 mice. Colonization of germ-free APP/PS1 mice with the microbiota from aged, conventionally raised APP/PS1 mice via oral gavage increased cerebral Aβ levels. In contrast, colonization with the microbiota from control wild-type mice was ineffective in increasing cerebral Aβ levels. Interestingly, our ongoing research suggests that colonization of 5-month-old APP/PS1 mice with the microbiota from young control wild-type mice via oral gavage for 4 months can improve the learning and memory abilities in the Morris water maze task, and these results have yet to be further verified. Although FMT seems to be a drastic treatment, the change in the composition of the gut microbiota may positively affect the cognition or pathology of AD. Many unanswered questions need to be addressed for this intervention, including the optimal procedure, safety issues, and long-term consequences (for review, see [77]).

GUT MICROBIOTA AND AGING

As advanced age is the predominant risk factor for AD, age-related changes may play a potential role in the onset of AD. It has been reported that the composition of the gut microbiota changes in the elderly, with a decrease in certain bacteria considered beneficial, such as Bacteroidetes, Lactobacillus, and Bifidobacteria [78–80] (for review, see [80]). However, Mariat et al. showed that the Firmicutes/Bacteroidetes ratio undergoes an increase from 0.4 in infancy to 10.9 in adulthood (25 to 45 years) and a decrease back to 0.6 in the elderly (70 to 90 years) [81]. More recently, Biagi et al. found that a core gut microbiome, comprising species in three families: *Bacteroidaceae, Lachnospiraceae*, and *Ruminococcaceae,* is associated with the human host throughout life, with decreasing cumulative relative abundance along with age. In particular, *Coprococcus, Roseburia, and Faecalibacterium* were found to be negatively correlated with age. In contrast, *Oscillospira* was positively correlated with age, as well as two subdominant members of the *Bacteroidales* order (*Odoribacter* and *Butyricimonas*) [82]. Although these findings are not consistent, alterations in the composition of the gut microbiota with reduced diversity of organisms is recognized, which can be influenced by different dietetic regimens and living environments (i.e., day-hospital, community, rehabilitation, or long-term residential care) [83–85] (for review, see [84, 85]). Aging is associated with an overstimulation of both innate and adaptive immune systems, resulting in a low-grade, chronic state of inflammation defined as inflammaging (for review, see [86, 87]), which may increase gut permeability ("leaky gut") and bacterial translocation (for review, see [88, 89]). Other studies also reported that some specific bacterial groups such as Bifidobacterium, Lactobacillus, or Faecalibacterium might modulate inflammation at the level of the gut epithelium [90–93]. Biagi et al. found that high plasma levels of pro-inflammatory cytokines (interleukin (IL)-6 and IL-8) in centenarians correlated with an enrichment in *Proteobacteria* and a decrease in several butyrate producing bacteria [94]. Moreover, the abundance of Bifidobacterium

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in the microbiota of elderly was negatively correlated with serum levels of tumor necrosis factor (TNF)-α and IL-10 [95]. Consequently, the microbiota composition might modulate the inflammaging process. Additionally, during aging, the blood-brain barrier (BBB) is compromised [96–99] (for review, see [96, 99]), which can not only influence the clearance of Aβ from the brain but also the secretome and receptor-mediated signaling involved in the neuroinflammation that is observed in AD (for review, see [100]). In conclusion, it is possible that age-related alterations in the gut microbiota facilitate inflammatory processes that may contribute to the neuroinflammation in AD.

THE POTENTIAL ROLE OF BACTERIALLY DERIVED LIPOPOLYSACCHARIDES AND AMYLOIDS IN THE PATHOGENESIS OF AD

Bacteria populating the gut microbiome are capable of excreting enormous quantities of lipopolysaccharides (LPSs) and amyloids, which may contribute to the pathogenesis of AD, especially during aging when both the GI tract epithelium and BBB become more permeable. It has been proposed that LPS and amyloids may directly pass through a compromised GI tract or BBB and/or indirectly pass through these protective physiological barriers via LPS/amyloid-triggered cytokines or other small proinflammatory molecules that are normally transited (for review, see [101–103]).

LPS, also called endotoxin, is a major component of the outer membrane of gram-negative bacteria. In addition, LPS is often used to induce inflammation in experimental animals. It has been observed that the chronic infusion of LPS into the fourth ventricle of rats can reproduce numerous inflammatory and pathological features seen in AD [104]. Moreover, a recen*t in vitro* study suggests that bacterial LPS potentiates the fibrillogenesis of Aβ peptides [105]. Mice treated with multiple intraperitoneal injections of LPS displayed significantly higher levels of $A\beta_{42}$ in the hippocampus in addition to cognitive deficits [106]. Jaeger et al. reported that intraperitoneal injections of LPS into mice increased the blood-to-brain influx and decreased the brain-to-blood efflux, resulting in Aβ accumulation [107]. A recent study reported that Bacteroides fragilis Lipopolysaccharide (BF-LPS) exposure to human primary brain cells is extremely potent inducer of the pro-inflammatory transcription factor NFκB (p50/p65) complex, a known trigger involved in inflammatory neurodegeneration in the AD brain [108]. Toll-like receptor (TLR) 4 expressed on microglia is capable of recognizing LPS, and its functions depend on the co-receptor CD14 [109, 110] (for review, see [110]). Liu Yang et al. also demonstrated that CD14 plays an important role in the phagocytosis of $A\beta_{42}$. Moreover, prominent CD14 immunoreactivity spatially related to the characteristic AD lesion sites was found in microglia in brain sections of AD patients, but not in those of the control subjects [111]. Therefore, the LPS-receptor TLR4/CD14 may play a role in AD physiopathology.

In addition to LPS, a significant quantity of functional amyloid can be generated by many bacterial strains, including *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *Salmonella enterica*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*, and may contribute to the pathology of AD through the accumulation of proteinaceous misfolded Aβ oligomers and fibrils (for review, see [112, 113]). A number of recent studies suggest that clumping of proteins with prion-like behavior might be a phenomenon shared by several common neurodegenerative diseases, including Parkinson's disease and AD. It has been hypothesized that functional amyloids produced by gut bacteria may be the source of misfolding of neuronal proteins such as alpha-synuclein, Aβ via cross-seeding, priming of the innate immune system, and activation of neuroinflammation [114, 115] (for review, see [114]). According to this hypothesis, AD should be considered a prion-like disease, and AD-related Aβ as prion-like proteinaceous nucleating particles (for review, see [116, 117]). When prions accumulate and exceed a certain threshold, they may induce a self-propagating process, leading to CNS dysfunction (for review, see [116]). It has been reported that after oral administration, prions can withstand the process of digestion and be incorporated by microfold cells or villous columnar epithelial cells in the gut. Prions might accumulate in follicular dendritic cells within Peyer's patches and other lymphoid follicles via an interaction with dendritic cells or macrophages. Then, prion-like proteins might move to the enteric nervous system by interacting with follicular dendritic cells, finally spreading to the CNS (for review, see [118]). Alternatively, murine senile amyloid proteins in Peyer's patches have been identified in a senescence-accelerated (SAM-P/1) mouse model, as

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detected by immunohistochemical staining and electron microscopy [119]. Furthermore, Galloway et al. showed that Aβ was found in epithelial cells of the small intestine by an immunohistochemical approach [120, 121]. Consequently, we have reason to speculate that the GI tract might also play a role in Aβ formation and propagation, possibly contributing to the initiation of AD.

The extracellular bacterial amyloids known as "curli fibers" that are produced by *Escherichia coli* K12 have been shown to facilitate biofilm development, adhesion, and protection against host defenses (for review, see [112, 122]). The major structural curli subunit gA amyloid precursor contains a pathogen-associated molecular pattern that, like the $A\beta_{42}$ peptide, is recognized by the human TLR2 (for review, see [112, 122] in a process that involves CD14 [123]. In addition, it has been hypothesized that bacterial amyloids might leak from the GI tract, which may contribute to the systemic and CNS amyloid burden. These bacterial amyloids might activate the NFκB signaling, which induces upregulation of proinflammatory microRNA-34a (miRNA-34a). Consequently, miRNA-34a would downregulate TREM2, which may impair the ability of the microglia to phagocytose and facilitate the accumulation of $A\beta_{42}$ peptide [101, 124–127] (for review, see [101, 127]). Moreover, microbially generated amyloids induce proinflammatory IL-17A, a driver of NFκB signaling, and IL-22, both of which are directly associated with AD [128, 129]. These cytokines might pass through both the GI tract and the BBB, reaching the brain and further triggering immunoreactivity, reactive oxygen species release, and signaling of TLR 2/1, CD14, and NFκB, which are known to play a role in neurodegeneration (for review, see [101, 103, 114]).

THE INTRICATE ASSOCIATION BETWEEN THE GUT MICROBIOTA AND THE DEVELOPMENT OF OBESITY, T2DM, AND AD

Obesity is a state of chronic, low-grade inflammation and is now considered a vital risk factor for development of T2DM. Hyperinsulinemia, hyperglycemia, glucose intolerance, hypertension, and atherosclerosis are also risk factors for T2DM (for review, see [130]). Interestingly, it has been estimated that up to 81% of AD patients have T2DM or impairment of glucose metabolism [131]. Insulin resistance and hyperinsulinemia trigger inflammation and are associated with the increased expression of inflammatory agents that contribute to the onset of AD [132, 133] (for review, see [132]). More specifically, it has been suggested that brain insulin resistance may characterize AD and promote cognitive decline [134]. Moreover, glucagon-like peptide 1 receptor agonists can protect the brain from defective insulin signaling and improve cognition in a transgenic AD mouse model [135]. Insulin may facilitate inflammation in the CNS through effects on Aβ through the following mechanism. First, insulin promotes the secretion of Aβ from intracellular neuronal compartments and inhibits its degradation with insulin-degrading enzyme [136]. Second, Aβ aggregates interfere with insulin receptor signaling, resulting in increased glycogen synthase kinase-3β activation, which in turn leads to an increase in Aβ synthesis and tau hypophosphorylation (for review, see [137]). Finally, recent evidence demonstrated that the receptor for advanced glycation end products-mediated inflammation is involved in inflammation-based mechanisms shared in T2DM and AD (for review, see [138]).

The association of a proportional increase in Firmicutes and reduction in the abundance of Bacteroidetes with obesity was concluded by various studies using animal models and human subjects [19, 139]. It was shown that the obesity trait could be transmitted from obese mice to GF mice by fecal transplants [140]. Recent studies have found that a high-fat diet can alter the composition of the gut microbiota with a dramatic reduction in various bacterial groups such as *Bifidobacterium spp.*, *Eubacterium rectal*, the *Clostridium coccoides* group and Bacteroides-related bacteria [141, 142]. It has also been observed that high-fat diet chronically increases plasma LPS levels 2- to 3-fold, which is termed "metabolic endotoxemia", and can lead to low-grade chronic inflammation, insulin resistance, and T2DM [141–143]. Studies also found that the plasma levels of LPS in patients with AD were three times higher than healthy controls [144]. Furthermore, the plasma LPS concentration is negatively correlated with the number of gut *Bifidobacterium spp*., which are known to maintain the gut barrier integrity and prevent bacterial/endotoxin translocation [145, 146]. Therefore, gut microbiota may provide a link from obesity and endotoxemia to AD pathogenesis.

The gut microbiota participates in the digestion of plant polysaccharides to SCFAs, predominantly acetate, propionate, and butyrate, which account for

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up to 15% of caloric intake, thereby contributing to the development of obesity (for review, see [147]). Apart from being a direct energy source, SCFAs also play a pivotal role in regulating host gene expression, metabolism and maintenance of the gut mucosal barrier integrity (for review, see [148]). SCFAs can serve as a ligand for two free fatty acid (FFA) receptors (FFA2 and FFA3) on intestinal enteroendocrine cells. Bjursell et al. reported that *Ffar2*-deficient mice (*Ffar2*-KO) did not develop obesity despite being fed a high-fat diet [149]. Furthermore, despite divergent metagenomic markers for T2DM between European and Chinese cohorts, two studies place emphasis on the role of butyrate-producing bacteria in regulating human glucose and lipid metabolism [17, 150]. Although a detailed analysis of the microbiota in patients with AD is lacking, given the alteration in the gut microbiota related to SCFAs in T2DM, which is a risk factor for AD, we have reason to speculate that SCFAs such as butyrate may provide another mechanism for the development of AD. Alternatively, SCFAs also serve as important modulators in immunity (for review, see [151]) via NFκB signaling, which could regulate the responsiveness of microglial cells to LPS. Different studies have demonstrated that butyrate can downregulate NFκB signaling triggered by LPS or cytokines on the different cells [152–154]. Furthermore, it has been shown that butyrate can also downregulate TLR4 expression and inhibit TLR4-mediated signaling transduction activation of NFκB (for review, see [155]). As a consequence, decreased levels of SCFAs might facilitate microglial activation induced by increased CNS levels of LPS or bacterial amyloids, which may be involved in the development of AD.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we showed that microbiota dysbiosis may lead to increased gut permeability and bacterial translocation, which are associated with risk factors for AD, such as aging, T2DM, and obesity. Studies conducted in animals have demonstrated that antibiotics, microbial infections, probiotics, and FMT could not only affect gut microbiota composition but also influence cognitive behavior or AD-related pathogenesis. To date, there are a paucity of studies directly focused on the gut microbiota and AD. We can further investigate the effects of antibiotics, probiotics and FMT in animal models of AD, while correlating them to the simultaneous assessment of the gut microbiota composition, the metabolites of the gut microbiota, the translocation of these gut-expressed signaling molecules to the CNS via systemic circulation or other pathway, the neuroinflammatory mechanisms, and the changes in the deposition of Aβ pathology. A recent study reported that gut microbiota from PD patients promote enhanced motor dysfunction compared to microbiota from healthy controls when transplanted into a mouse model of PD [29]. Given the recent finding suggesting that distinct microbes associated with PD, future work must analyze the structural differences of the gut microbial content between AD patients and healthy individuals to identify some AD-associated specific microbiota or microbial metabolites. Then we can transplant specific bacterial strains or the fecal microbiota of AD patients into GF animals or gut microbiota-depleted animals to observe whether they can induce AD-related pathological hallmarks. We can use these preclinical findings as one of several approaches to indicate the development of AD. However, we must use caution in extrapolating the exciting data that have largely emerged from animal work to humans especially given the different diets and microbiota composition between species. Further investigation through well-designed controlled human trials is needed to explore new treatments aimed at the direct manipulation of the gut microbiota, including probiotics to provide beneficial microbes, novel antibiotics to remove harmful bacteria, prebiotics to promote the growth of desired organisms, and even FMT to replace patients' gut microbiota with those of healthy donors. Additionally, dietary intake seems to be a main short-term and long-term regulator of the structure and function of gut microbiota (for review, see [156]). Moreover, dietary patterns characterized by higher intake of low-fat plant foods and specific plant-derived compounds may lower the risk of T2DM, metabolic syndrome and even AD (for review, see [157]). Thus, future randomized, clinically controlled dietary interventions targeting the gut microbiota studies in humans are required to determine the underlying mechanisms of diet-induced gut dysbiosis and the development of AD, which may open up dietary-based intervention strategies in AD. Interestingly, recent data have suggested that gut microbiota can activate the vagus nerve and that vagal signals from the gut can induce an anti-inflammatory reflex with afferent signals informing the CNS of the presense of inflammation to activate

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its efferents, releasing mediators including acetylcholine (ACh) via an interaction with immune cells to reduce inflammation (for review, see [158, 159]). Vagus nerve stimulation has been used successfully in the treatment of epilepsy and depression, and is under investigation as a therapy for AD [160–162] (for review, see [162]). Epilepsy, depression, and AD are all closely connected with neuroinflammation. Although the exact mechanism of the effect of vagus nerve stimulation is still unkown, it may activate the cholinergic anti-inflammatory pathway releasing ACh which binds to α-7-nicotinic ACh receptors (α7 nAChRs) of macrophages to inhibit the release of proinflammatory cytokines (i.e., TNFα) (for review, see [163, 164]). Moreover, stimulation of α7 nAChRs expressed on microglia cells can promote phagocytosis of Aβ in AD [165]. Taken together, understanding the modulation of vagal signaling pathway induced by the manipulation of gut microbiota, which targeted to stimulate anti-inflammatory regulation in AD may have important implications for the development of microbial-based therapeutic strategies.

The recognition of the potential contribution of microbiome-generated LPS and amyloids to the regulation of signaling pathways implicated in neuroinflammation, brain Aβ deposition, and AD pathogenesis are relatively recent discoveries. Furthermore, it has been hypothesized that the trigger for Aβ misfolding may originate in the gut. Therefore, therapeutic strategies to alter the amyloids produced by bacteria or reduce their production may deserve to be explored. Recent advances in metagenomics, RNA sequencing, metabolomics, metatranscriptomics, and metaproteomics may provide a better understanding of the cellular and molecular mechanisms underlying microbiota dysbiosis and the pathogenesis of AD. Consequently, these advances might enable the discovery of biomarkers suitable for the early diagnosis of AD as well as novel therapeutics and preventive strategies.

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The Role of the Gut Microbiota in the Metabolism of Polyphenols as Characterized by Gnotobiotic Mice

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Abstract. A growing body of experimental data suggests that microbes in the gut influence behavior and can alter brain physiology and neurochemistry. Although promising, researchers are only starting to understand the potential of the gut microbiota for use in neurological disease. Recent evidence demonstrated that gastrointestinal activities are linked to mood disorders such as anxiety, depression, and most recently, cognitive functions in age-related neurodegenerative disorders. Studies from our group and others are uncovering new evidence suggesting that the gut microbiota plays a crucial role in the metabolism and bioavailability of certain dietary compounds and synthetic drugs. Based on this evidence, this review article will discuss the implications of the gut microbiota in mechanisms of bioavailability and biotransformation with an emphasis on dietary polyphenol compounds. This will be followed by a survey of ongoing innovative research identifying the ability of individual gut bacteria to enhance the bioavailability of gut-derived, brain-penetrating, bioactive polyphenol metabolites that ultimately influence mechanisms associated with the promotion of resilience against psychological and cognitive impairment in response to stress. Lastly, current research initiatives aimed at promoting the generation of brain bioactive polyphenol metabolites by specialized gut microbes will be discussed, specifically the use of gnotobiotic mice to develop bioengineered second generation probiotics. We propose that leveraging the gut microbial ecosystem to generate brain targeted bioactive metabolites from dietary polyphenols can attenuate lifestyle risk factors and promote resilience against age-related cognitive decline.

Keywords: Gnotobiotic mice, inflammation, microbiota, oxidative stress, polyphenol metabolism

INTRODUCTION

In recent years, the scientific community has begun to understand the intricate and dynamic interactions between the symbiotic bacteria in the gastrointestinal (GI) tract, the gut microbiota, and neurological function [1–8]. The bacteria of the gut have been show to affect high order cognition and physiological functions, and they are crucial to physiological homeostasis [9–14]. Ongoing studies support the hypothesis that the gut microbiota's influence on brain function is mediated through metabolic processing of certain dietary compounds such as polyphenols [15–18] and synthetic drugs [19–22] by

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the resident bacteria. Studies from our group and others suggest that the polyphenol metabolites produced by the microbiota modulate immune-inflammatory cascades [23–29], synaptic maladaptation [30–32], mood disorders [33, 34] as well as age-related neurodegenerative disorders [18, 31, 32, 35]. Given this ability of the microbiota to generate unique bioactive compounds, efforts have been made to manipulate the gut microbiota to improve the delivery and efficacy of orally consumed compounds, including phytodrugs [36–38]. However, the role gut microbiota has on polyphenol metabolism remains elusive. Further efforts should explore how specific members of the gut microbiota promote polyphenol biotransformation in order to develop novel therapeutic strategies through probiotic bacteria.

WHAT IS THE GUT MICROBIOTA?

The gut microbiota is a community of diverse bacteria and microorganisms that form symbiotic relationships with their hosts to maintain host health [39, 40]. The influence of the gut microbiota on the host is so complex that the gut microbiota is now considered to be an independent endocrine organ [41] with vast metabolic activities and the capacity to modulate several physiological states including inflammation, oxidative stress [42], and as this review will focus on, neurological processes [43]. The significant impact of the gut microbiota on host metabolism was amply illustrated when transplantation of gut bacteria from twins discordant for obesity into mice resulted in identical metabolic phenotypes of the microbiota donor [44]. The composition of the gut microbiota varies significantly between healthy individuals due to diet, antibiotic usage, exposure to pharmaceutical agents, interactions with the environment, physiological and psychological stress [45, 46]. Although hundreds of various microbe families have been found in the GI tract [47], each individual harbors a unique combination of these microbes [48, 49]. Despite this diversity, a standard healthy gut microbiota profile has emerged as the composition of the microbiota is now recognized as an important factor in understanding individual nutritional needs [50, 51]. Recent research has shown that a healthy gut microbiota is composed of a high proportion of metabolically-active butyrate-producing bacteria including the *Ruminococcus* spp. and *Eubacterium* spp., *Bifidobacterium* spp., which degrade long chain dietary fibers, a low ratio of the principle phyla Firmicutes to Bacteriodetes, and a reduced proportion of inflammatory pathogens including the Proteobacteria [52–54]. As a more accurate profile of the gut microbiota develops, efforts will look at the metabolic activity of specific bacteria and their role in generating biologically active compounds, such as compounds derived from dietary polyphenols.

THE GUT MICROBIOTA AND ITS ROLE IN THE GENERATION OF BIOACTIVE POLYPHENOLIC METABOLITES

Research to date on the relationship between polyphenols and bacteria in the GI tract indicate that gut bacteria possess innate mechanisms that generate de novo and potentially bioactive compounds when provided with dietary polyphenols (Fig. 1). The gut microbiota is critical to enabling the bioavailability of ingested polyphenols as most parent polyphenols are not well absorbed by the small intestine [55]. Likewise, 90% of ingested polyphenols reach the large intestine and within the context of the resident microbiota, are transformed into bioavailable products. Following the ingestion of polyphenol compounds, usually in their glycosylated form, bacteria in the GI tract convert dietary polyphenols to low-molecular-weight phenolic compounds, such as phenolic acids [56, 57], that can be more efficiently absorbed by intestinal epithelial cells [58, 59]. In some cases, biotransformation by the gut microbiota is essential to providing bioavailable polyphenolic acids. Polyphenols have been shown to undergo a variety of enzymatic processes by bacterial populations in the GI tract. These include the hydrolysis of glycosylated flavonoids, acylation of flavanol-3-ols, and esterification of hydroxycinnamic acids [60]. Subsequent to enzymatic processing by bacteria, the polyphenol derivatives, usually the aglycons or hydroxyphenylacetic acids, are absorbed by epithelial cells of the small intestine. Once the polyphenol derivatives are in a form capable of being absorbed by the small intestine or colon, they undergo enterocyte phase II modifications, in which they are often converted to their O-Glucuronide or O-Sulfonate forms through methylation, sulfation, hydroxylation, and glucoronidation. Multimeric polyphenols that are not broken down in the small intestine move through the GI tract where their conjugating moieties are processed by colon specific bacteria and absorbed into the epithelial tissue for phase II metabolism [61]. The modified polyphenols, from the enterocytes, then enter the

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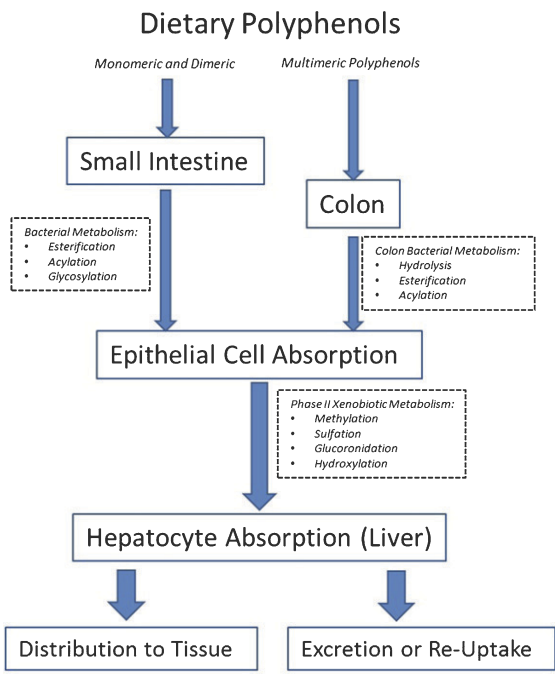


Fig. 1. The diversity of interpersonal bacteria differentially metabolizes polyphenols. After oral consumption, monomeric polyphenols undergo a variety of covalent modifications, such as acylation or esterification, catalyzed by the bacterial enzymes unique to the upper gastrointestinal tract. The modified polyphenols then transit into the epithethial cells of the upper GI tract where they undergo enterocyte modifications by phase II metabolism that involves the conjugation of glucuronide, methyl or hydroxyl groups. Multimeric polyphenol compounds pass through the small intestine because the resident bacteria do no transcribe hydrolytic enzymes required for the production of monomeric polyphenols, and their molecular weight prevents their uptake into upper GI epithethial cells. They pass into the colon where resident bacteria hydrolyze conjugating moieties of multimeric polyphenols allowing for their absorption and metabolic processing into simple phenols by colon epithelial cells. Another set of covalent modifications can occur when polyphenol metabolites pass through the portal vein and reach liver hepatocytes, whereupon they undergo additional phase II modifications. The polyphenol derivatives are then either distributed to the target tissue by circulation, or are excreted as bile for enterohepatic circulation or excretion. The interpersonal diversity of microbiota, represented in the scheme by the red bacteria, can substantially alter the potential bioavailability and bioactivity of a polyphenol's derivatives, and subsequently their therapeutic efficacy in models of induced cognitive dysfunction.

blood stream via the portal vein, are delivered to the liver where they can be further modified via conjugations, and finally are either secreted as bile components back into the intestine for enterohepatic recirculation, or into the blood stream to be delivered to the peripheral tissues including the brain [60].

One recent study demonstrated how a polyphenol-rich potato extract containing cholorogenic, caffeic, and ferulic acids was broken down in a colonic simulator containing human gut microbiota into several biotransformation products. A small proportion of the metabolites were transduced across a Caco-2/HepG2 co-culture; however, the absorption of the secondary metabolites following hepatic transformation resulted in significant increases of ferulic, dihydrocaffeic, 2-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, and coumaric acid [62]. In another study, a simulated gastrointestinal environment composed of rat gut microbiota metabolized three main anthocyanins from mulberry including cyanidin-3-glucoside, cyanidin-3-rutin, and delphinidin-3-rutinoside. Cyanidin-3-glucoside and cyaniding-3-ruitin resulted in protocatechuic, vanillic, and p-coumeric acids while delphinidin-3-rutinoside into gallic acid, syringic acid, and 2,4,6-trihydroxybenzaldehyde: processes that were dependent on the presence of the gut microbiota [63]. In a cohort of elderly Japanese individuals, the composition of the gut microbiota was highly correlated with the rate of quercetin metabolism. The abundance of Surreellaceae and Oscillospiraceae were negatively correlated with quercetin metabolism, while Fusobacteria and Enterobacteriaceae showed a positive correlation [64]. Furthermore, using 20 healthy volunteers who were administered green tea, one study found that colonic microbiota produced polyhydroxyphenyl-γ-valerolactones, which was the urinary catabolite produced in the greatest concentrations; it was shown to have a 10 times greater concentration than the other flavan-3-ol conjugates [65]. Recently phenyl-γ-valerolactones have been purported to provide protection to brown adipocytes against oxidative damage, which may provide an important observation for research into obesity [66].

Our group has shown that mice administered with grape seed polyphenol extract (GSPE) resulted in the production of 11 unique polyphenol metabolites, as measured in urine, and 4 as measured in the plasma, while only of the 2 metabolites, 3-HBA and 3-HPP, were detected in the brain following perfusion (Fig. 2, adapted from Wang et al. [35]). Both 3-HBA and 3-HPP are likely derivatives of the flavonol quercetin and are produced following ring scission by *Enterobacteria* spp. in the gut and subsequent enterocyte phase II modification such as dehydration or reduction [67]. Another study using dietary supplementation with a red wine polyphenolic extract found that the addition of *Lactobacillus plantarum* increased the conversion of monomeric flavanols, such as those found in GSPE, and their intermediate

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metabolites into phenylpropionic acids, in particular 3-(3´-hydroxyphenyl) propionic acid (3-HPPA) [68]. *In vivo* studies using germ-free or antibiotic-treated mice also support the necessity of gut microbes for the conversion of polyphenol compounds into bioactive and bioavailable compounds whose concentration without bacteria metabolism would be subclinical [18, 68–72].

THE IDENTIFICATION OF BACTERIA CAPABLE OF CONVERTING POLYPHENOLS INTO BIOACTIVE COMPOUNDS

To best understand the role of gut bacteria in the breakdown of polyphenols into bioactive metabolites, it is critical to determine specific bacterial strains responsible for the enzymatic processing of polyphenols. Understanding the catalytic abilities of each bacterial species will provide an opportunity to bio-engineer next-generation probiotics that most effectively transform polyphenols into the desired bioactive metabolites.

Several enzymes necessary for the biotransformation of dietary polyphenols have been identified. *Eubacterium ramulus* induces ring fission of the polyphenolic core generating several metabolites with altered health benefits [73]. For example, degradation of naringen by calchone isomerase and phloretin hydrolase converts the complex polyphenol into usable aromatic products [74]. From the green tea polyphenols catechins, epicatechin, epicatechin gallate, and epigallocatechin gallate, 4-hydrobeenzoic acid and vanillic acid are produced [75]. For example, ferulic acid, a hydroxycinnamic acid found in plant stems and various herbs, is broken down by the esterase activity of Lactobacillus spp. into 4-vinylguaiacol and hydroferulic acid [76]. Further breakdown converts these products into the caffeic and vanillic acid, which have potent therapeutic effects especially in Alzheimer's disease (AD) [77]. Soya-isoflavone daidzein is converted exclusively in equol only by *Adlercreutzia equolifaciens,* giving it a greater pro-estrogenic effect [78]. Furthermore, the conversion of ellagic acid into urolithin is conducted by *Gordonibacter urolithinfaciens*; however the accumulation of the variety of biotransformation products was dependent on the presence of the complex microbiota [79]. Another important biotransformation elicited by the gut microbiota, notably *Flavonifractor plautii*, is the conversion proanthocyanidins into 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) [60, 80]. This is an important conversion as the anti-inflammatory action of DHPV is greater than its parent metabolites, notably the TNF-α-mediated induction of proinflammatory factors from monocytes. In addition to these examples, several other metabolites of gut bacterial biotransformation in the GI tract are outlined in a comprehensive review by Rowland et al. [81].

Despite the large body of evidence supporting the role of the gut bacteria and their specific enzymes in the biotransformation of polyphenols, understanding the breadth of the microbiota's effect on polyphenol metabolism remains in its infancy. Likewise, novel approaches are necessary in order to elucidate the specific activities of select bacteria and their role in polyphenolic biotransformation. A recent approach to characterize gut bacterial populations leveraged a method of metagenomic binning of bacterial DNA methylation to create bacterial barcodes for efficient identification of unique bacterial strains [82]. Other groups, including ours, have used gnotobiotic and germ-free mice to manipulate bacterial populations to better understand host metabolism. With these new methods, we will be able to clarify the unique metabolism of gut microbiota, which differs between individuals, and how this may determine the bioavailability and bioactivities of polyphenol metabolites [83, 84].

HUMANIZED GNOTOBIOTIC MICE AS A MODEL SYSTEM TO EXPLORE THE RELATIONSHIP BETWEEN THE HUMAN GUT MICROBIOTA AND THE BIOAVAILABILITY OF DIETARY POLYPHENOL PREPARATIONS

Mice raised in germ-free environments provide an excellent system for understanding the role of gut bacteria in various aspects of host physiology. Germ-free mice can be colonized at specific life stages with a complex or defined consortia of microbes to understand the role of the gut microbiota in host physiology using a highly controlled and easily manipulated system. One germ free mouse model found that consistent with *in vitro* evidence supporting the relevance of gut microbiota in the bioconversion and bioavailability of phenolic metabolites from botanical polyphenol components [85–87], germ-free mice or antibiotic-treated mice had decreased bioconver-

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Metabolite	Urine *(nmol/day)*	Plasma *(μM)*	Brain *(μM)*
3-HBA	9.2	(-)	1.75
3-HPA	1	(-)	(-)
3-HPP	627.3	0.92	2.53
3-HHA	54.8	(-)	(-)
34-HBA	366.2	(-)	(-)
4-HHA	241.5	(-)	(-)
5-HPV	78.7	0.48	(-)
3,4-diHPA	2.5	(-)	(-)
3,4-diHPP	590.5	1.25	(-)
HA	22.2	4.72	(-)
FA	128.2	(-)	(-)

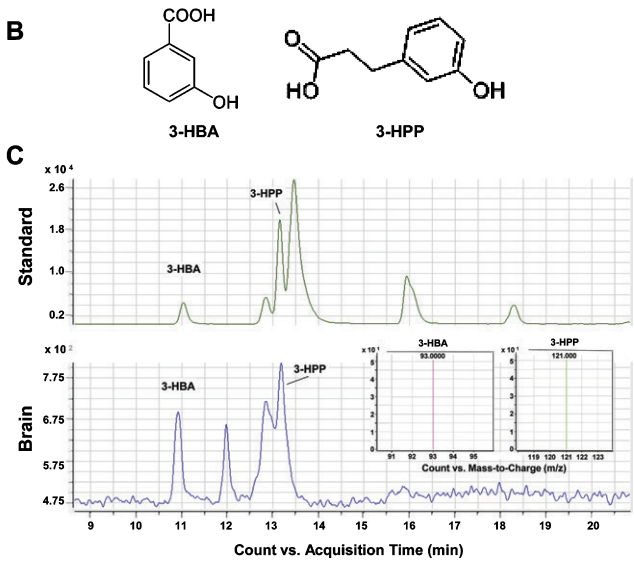


Fig. 2. Polyphenol metabolites measured in urine, plasma and in the brain following the administration of GSPE. A) The administration of GSPE, at 250 mg/BW, to Sprague Dawley rats by gavage for 10 days resulted in the identification of eleven unique phenolic acids in the urine, four of which were also found in the plasma, and two in the brain. B) Of the two quantified phenolic acids in the brain, 3-(3´-hydroxyphenyl) propionic acid (3-HPP) is a known derivative of the flavonol quercetin, the flavone apigenin, and of the flavan-3-ols catechin or epicatechin, which are processed by respective bacteria *Enterococcus casseliflavus, Clostridium coccoides,* and *C. orbiscindens* [60]. Meanwhile, the second brain quantified phenolic acid, 3-hydroxybenzoic acid (3-HBA), is known to be a derivative of the anthocyanin, cyanidin, which undergoes enzymatic processing by either *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, or Bifidobacterium lactis* [60]. C) The concentration of each phenolic acid was determined via LC-MS/MS, using analytical grade molecular standards (top) and a sample brain specimen (top).

sion of orally consumed polyphenol compounds to phenolic acid within their intestinal tract [88]. We recently developed and validated other methods for the transplantation of the human gut microbiota into gnotobiotic animals [89]. These humanized gnotobiotic mice have a high engraftment rate with 88% of genus-level taxa from the donor detected in the mice, while the genera that did not engraft were at very low abundance in the donor sample (0.008% on average). The applicability of these models to study human disease was evident when mice humanized with the microbiota of twins discordant for obesity developed phenotypes seen in the human host. Specifically, mice transplanted with gut microbiota of an obese twin had a higher increase in fat mass than mice transplanted with the corresponding lean twin microbiota [44]. The implications of these inter-individual variabilities on gut microbes and how they may influence the bioconversion of polyphenols in bioactive metabolites are further discussed below.

To the author's knowledge, there are currently no published studies that directly explore the relationship between the human gut microbiota and the diversity and abundance of bioactive phenolic metabolites *in vivo*. Therefore, we currently are pursuing studies to characterize germ-free mice models to understand polyphenol metabolism. The microbiota of different human donors can be transplanted into separate groups of gnotobiotic mice to estimate variation in bioavailability of bioactive botanical polyphenols as a function of donor microbiota (Fig. 3A). High-throughput bacterial isolation can then be used to isolate a large proportion of the gut microbes from two donors of interest into personalized arrayed culture collections (one donor is shown) (Fig. 3B). These personal culture collections can be screened using a combinatorial gnotobiotic approach combined with statistical modeling to identify specific microbial strains that modulate the bioactive compounds of interest (target metabolites will be based on our prior research) (Fig. 3C). We believe our studies complement transplantation of human microbiota to gnotobiotic mice [84, 90]. With advancing genomic technologies allowing researchers to isolate, archive, and retrieve a large number of gut bacteria [48, 91], the necessary tools to identify and use a combination of gnotobiotic mice to possibly identify the microbial strains that modulate the metabolism of bioactive polyphenols in the serum and the brain are now available [92].

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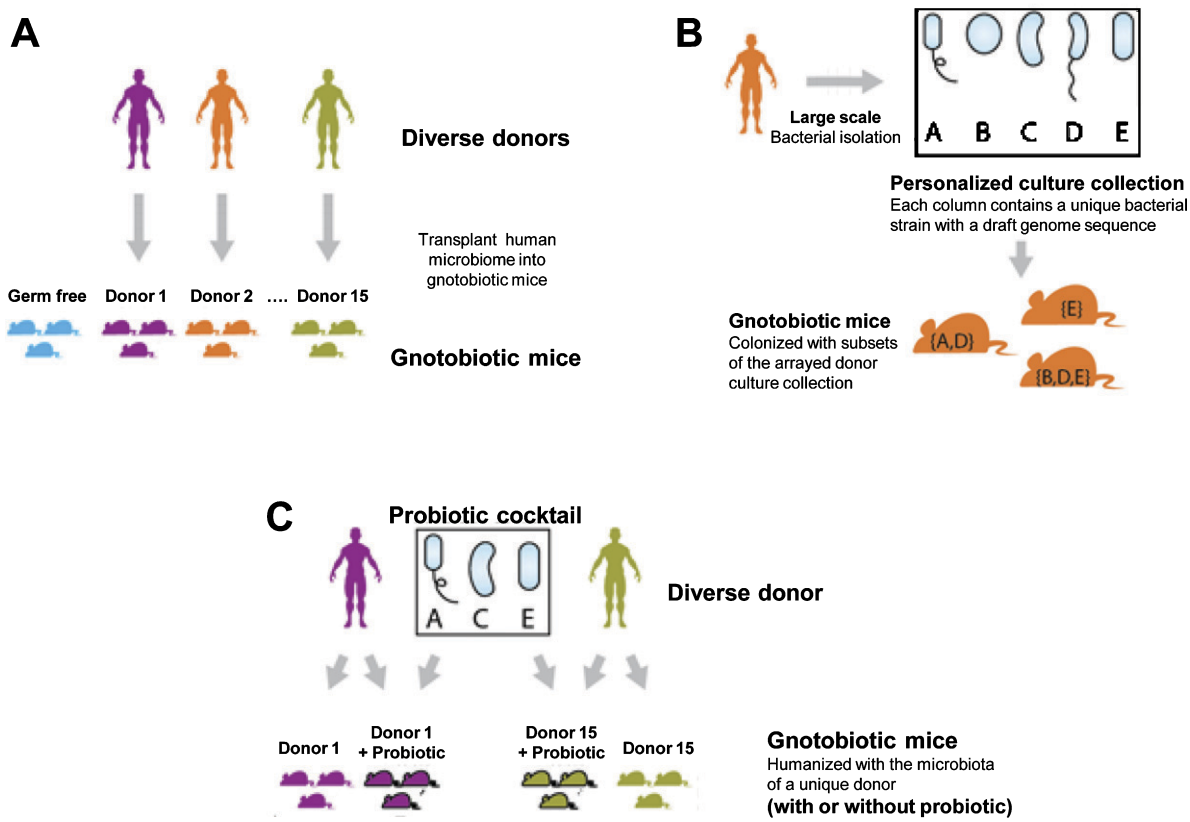


Fig. 3. The strategy used to identify interpersonal differences in the composition of the human GI microbiota. In order to understand the role individual bacteria play in the generation of polyphenol metabolites, a strategy was developed whereby (A) bacteria from a human gastrointestinal tract was transplanted to one of a gnotobiotic mouse; (B) the transplanted bacteria were analyzed through statistical methods to isolate unique strains and then transplanted into mice; (C) bacteria that were identified to produce specific metabolites were isolated, expanded, and transplanted into gnotobiotic mice to serve as a model system for next generation probiotics.

EMERGING EVIDENCE FOR THE ROLE OF THE GUT MICROBIOTA IN MOOD DISORDERS

The gut-brain axis (GBA) is a dynamic bidirectional system that is composed of the intestinal microbiota, the enteric nervous system, the peripheral and central nervous systems, humoral pathways, cytokines, neuropeptides and other signaling molecules [93]. The gut microbiota is critical not only for the production of polyphenol metabolites but also for functionality of the GBA and ultimately, neurological functions. The GI tract communicates with the brain through neuronal afferents, such as the vagus nerve, endocrine messages carried by the gut hormones and metabolic signaling, and immunological messages with cytokines [94]. With the emergence of evidence supporting a bi-directional relationship between the gut microbiota and the brain, the scientific community has begun to implicate the gut microbiota as a mediator of cognitive health [95]. The 2009 Walkerton Health Study that assessed participants with irritable bowel syndrome (IBS) over an eight-year period found that depression and anxiety were important risk factors for the persistence of IBS symptoms [96]. Studies such as the Walkerton Study, which revealed associations between gut microbes and the brain provoked an imperative to determine if psychiatric symptoms can be driven by gut dysbiosis [97–100]. One study supported this notion by showing that germ-free mice exhibited reduced anxious behavior as compared to specific pathogen free mice [101]. In spite of recent efforts and observations into a relationship between gut bacteria and mood

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disorders, explicit evidence indicating a mechanism of bidirectional communication between the gut and neuropsychiatric disorders remains elusive.

POLYPHENOLS: POTENTIAL THERAPEUTIC APPLICATIONS IN NEUROLOGICAL DISORDERS

Given the influence the gut-brain axis may have on the etiology of mood and behavioral disorders, our group set out to understand the capacity to which polyphenol metabolites produced by the gut microbiota can attenuate underlying physiological mechanisms of neurological disorders. Gut processed dietary polyphenols are plant-derived micronutrients found in high concentrations in certain diets such as the Mediterranean diet [86–88]. These compounds are associated with an array of health benefits and are a potential means to prevent a number of diseases [102–108]. Recent work from our group found that several polyphenol metabolites from grape seed extract (GSPE), concord grape juice (CGJ), and resveratrol (RSV), were able to protect against neuropathology and cognitive impairment in neurodegenerative disorders such as AD [35, 102, 103, 109–112] and related tau-mediated neurodegenerative disorders [113–116]. These studies led to the identification of 26 polyphenol metabolites from GSPE, CGJ, and RSV and each were found to accumulate in blood, and a subset in the brain, which had potential to modulate biological activities *in vivo* [30, 31, 117–120]. Moreover, we found polyphenol metabolites that were present in the brain such as 3'-O-Me-epicatechin-5-glucuronide [32] and quercetin glucuronide [31]. 3-hydroxybenzoic acid and 3-(3´-hydroxyphenyl) propionic acid were primarily responsible for inhibiting the onset and progression of AD-type pathophysiology [18]. The compounds exerted their neuroprotective effects by interfering with the generation of neurotoxic AD-type amyloid-β peptides, which can interfere with neuroplasticity and memory consolidation. In addition to interfering with AD mechanisms, studies demonstrated that multiple biologically available microbiota-derived phenolic acids including 3,4-dihydroxypenylacetic acid, 3-(3´-hydroxyphenyl) propionic acid, 3,4-dihydroxyhydrocinnamic acid and homovanillic acid from a bioactive dietary polyphenol preparation (BDPP), composed of GSPR, CGJ, and RSV, were able to modulate biological mechanisms associated with inflammation and synaptic plasticity [18, 30, 121]. These processes are known to influence psychological and cognitive resilience in, for example, animal models of social defeat and sleep deprivation. The collective evidence from our group and from other investigators [83, 122–129] implicates gut-derived biologically available phenolic metabolites as primary facilitators of the observed health benefits associated with polyphenol rich diets.

Depression and depression-like disorders are major contributors to the expanding health costs in the United States [100]. The treatment options currently available, which target neurochemical or neurobiological mechanisms identified retrospectively following discovery of the drug's initial antidepressant efficacy, produce temporary remission for approximately 50% of patients [99,100,130]. Such discouraging evidence highlights the need for novel therapeutics that can target genetic or proteomic mechanisms underlying depression. Accumulating evidence suggests that immunological abnormalities, particularly imbalances in select pro-inflammatory mediators, play important roles in the expression and continuity of depressive symptoms in vulnerable individuals [131]. These inflammatory mediators are now recognized as important biological signatures as well as key mechanistic contributory factors of depression. As such, these inflammatory mediators are also considered important novel therapeutic targets for depression [132].

Polyphenols can potentially modulate depressive disorders through their potent anti-inflammatory effects [133–139]. It has been suggested that polyphenols attenuate inflammatory conditions via its inherent anti-oxidative [134] capabilities; oxidative stress directly facilities the production of numerous proinflammatory cytokines and has been shown to contribute to the progression of cancer [140]. Furthermore, oxidative stress can either directly decrease neuronal plasticity through covalent interactions with proteins involved in axonal transport or synaptic integrity, which results in synaptic degeneration and neuronal apoptosis or indirectly through the aforementioned production of cytokines [141]. This preclinical evidence gave encouragement to our group that the administration of select bioactive polyphenol-rich preparation may intercede with the deleterious mechanisms of radical oxygen species and could attenuate depressive phenotypes in clinical conditions that often persist in spite of treatment with a serotonin selective reuptake inhibitors, such as major depressive disorder provoked anhedonia, characterized by a markedly diminished response

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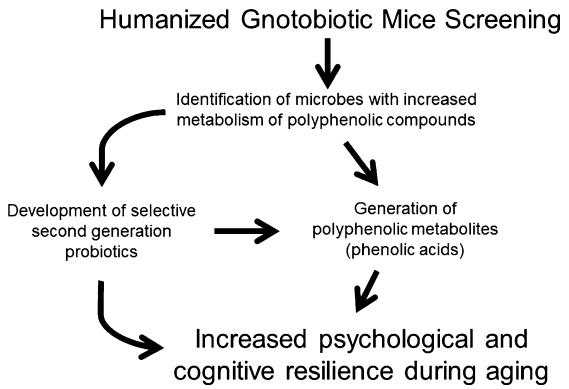


Fig. 4. Development strategy for the generation of bioactive polyphenol metabolites using probiotics. As the metabolism of polyphenols by the microbiome becomes clearer it will be possible to identify the unique bacterial populations responsible for the generation of bioactive polyphenols. Gnotobiotic mice constitute an important tool to achieve this goal; they will allow future research groups the ability to selective transplant bacterial strains into the gastrointestinal tract and to determine their unique metabolic function on polyphenols. Ultimately, the goal will be to develop therapeutic approaches to increase the bioavailability of polyphenol derivatives that have properties capable of promoting resilience against age or stress induced cognitive dysfunction, and its potential mediator inflammation.

to pleasure [131–133, 142–144] that is linked to impaired psychosocial functioning, poor treatment outcome, and suicidal behavior. Moreover, the current evidence emphasized in the review supports the potential therapeutic application of a combination of polyphenols, such as BDPP, largely reported by our group, in promoting resilience against the underlying heightened inflammatory mechanisms in stress induced depression [145]. As we expand our preclinical studies, we will look in to whether it is warranted to support further clinical development of BDPP for treating depression.

CONCLUSION

Of the numerous ways symbiotic bacteria in the GI tract influence homeostasis and affect the health of its host, a novel perspective looks at their ability to supply their host with bioactive and bioavailable compounds. As polyphenols have been shown to promote cognitive resilience against a variety of neurological disorders, work from our group, as well as others, has shown that these effects are mediated through microbiota metabolism and the subsequent generation of bioactive and bioavailable polyphenol metabolites. Further investigations should expand into understanding the specific bacteria responsible for the generation of these polyphenol metabolites. As summarized in Fig. 4, one could conjecture a strategy to develop next generation probiotic therapeutics that supplement bacteria known to promote psychological and cognitive resilience via its generation of bioactive compounds.

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Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-1151r1).

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Human Gut Microbiota in Health and Alzheimer's Disease

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Abstract. Gut microbiota plays a crucial role in human health and disease. The alterations in the composition of gut microbiota may cause the onset of certain human pathologies. One of these is Alzheimer's disease (AD). High-fat diets, administration of antibiotics, lack of probiotics and/or prebiotics in diet increase the risk of AD. On the other hand, modulation of the composition of gut microbiota may decrease the risk of AD and be able to slow down the progression of AD.

Keywords: Alzheimer's disease, dysbiosis, gut microbiota, metabolic endotoxemia, physiology

INTRODUCTION

The human gut microbiota comprises approximately 10^{14} microbes and generates a biomass of more than 1.5 kg [1]. The number of these bacterial cells is 10 times greater than the number of cells present in human body [2]. In a healthy human, these bacteria encode > 100-fold more genes than the human genome [3].

The human gut is inhabited by bacteria, archaea, unicellular eukaryotes, and viruses. The role of archaea, unicellular eukaryotes, and viruses for human health and disease has been less studied [4]. The gut microbiota is mainly composed of anaerobes.

CHARACTERISTICS OF HUMAN GUT MICROBIOTA

Composition of a healthy gut microbiota

The human gut microbiota is composed of only few phyla. The gut microbiota is dominated by only two of them: *Firmicutes* (60–80%, Gram-positive bacteria with more than 200 genera of which the most important are: *Ruminococcus, Clostridium, Eubacterium, Lactobacillus, Faecalibacterium, Roseburia,* and *Mycoplasma*) and *Bacteroidetes* (20–30%, Gram-negative bacteria with genera *Bacteroides, Prevotella*, and *Xylanibacter*). Following bacteria are present in minor proportions: *Actinobacteria* (<10%, Gram-negative bacteria with genera *Bifidobacterium*), *Proteobacteria* (<1%, Gram-negative bacteria with genus *Escherichia*, *Desulfovibrio*, and *Enterobacteriacae*), and *Verrucomicrobia* (with genus *Akkermansia*, *Fusobacteria*, and *Cyanobacteria*) [4–7]. The composition and luminal concentrations of the microbiota differ in various regions of the digestive tract (Table 1).

The three groups of bacteria are present in the healthy human gut: symbionts that have health-promoting effects; commensal bacteria that have no effect on host; and pathobionts that have negative effect on the host because they may induce pathology [8].

Role of gut microbiota in human health

Gut microbiota plays a crucial role in human health. These bacteria synthesize and release numer-

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Table 1
The composition and concentrations of microbiota in healthy human digestive tract in dependence on region. The concentration is presented as number of cells/gram of luminal contents [6, 8, 74]

Region of the digestive tract	Concentrations of bacteria	Composition of the microbiota species
Mouth	10^{12}	*Lactobacillus, Streptococcus, Helicobacter pylori, Peptostreptococcus, Veillonella, Candida*
Stomach	$0 – 10^{2}$	*Lactobacillus, Streptococcus, Helicobacter pylori, Peptostreptococcus, Veillonella, Candida*
Duodenum	$10^{2} – 10^{3}$	*Streptococcus, Lactobacillus*
Jejunum	$10^{2} – 10^{4}$	*Streptococcus, Lactobacillus*
Ileum	$10^{7} – 10^{8}$	*Streptococcus, Lactobacillus, Actinobacteria, Actinomycinaeae, Corynebacteria, Clostridium, Bacteroides*
Colon	$10^{11} – 10^{12}$	*Bacteroides, Clostridium* clusters IV and XIV, *Bifidobacterium, Enterobacteriaceae, Lachnospiraceae*

ous small molecules. Some of these molecules penetrate the circulation system and after a chemical modification may influence human health. Microflora has many metabolic functions: synthesizing vitamins (folates, K, biotin, riboflavin, cobalamin, and possibly other B vitamins) [9], and amino acids; influencing the biotransformation of bile acids [10]; and increasing the bioavailability of minerals [11].

In the cecum and colon, gut microbiota degrades non-digestible polysaccharides through fermentation resulting in short-chain fatty acids (SCFAs) production (butyrate, acetate, and propionate) [12]. The gut microbiota produces 50–100 mmol/L per day of SCFAs [13]. Up to 95% of the produced SCFAs are absorbed and they serve as an energy source for colonocytes [13]. The absorbed SCFAs stimulate the absorption of salts and water. These fatty acids also act as signaling molecules. They are ligands for two receptors of endocrine cells in gut: G-protein-coupled receptor 41 (GPR41) and G-protein-coupled receptor 43 (GPR43) [14, 15]. The binding of SCFAs to receptors stimulates the secretion of peptide YY (PYY, peptide tyrosine-tyrosine) and glucagon-like peptide 1 (GLP-1) by L-cells [15]. PYY secreted by L-cells slows intestinal transit by the inhibition of gut motility and enhancing the absorption of nutrients in the intestine [10]. GLP-1 increases energy expenditure in muscles and improves the insulin sensitivity [16]. GLP-1 also plays the role of a hunger-reducing hormone.

The increased levels of propionate of the SCFAs that induce intestinal gluconeogenesis [17] are associated with the microbiota following the gastric bypass [18], and they protect from the diet-induced obesity [19]. SCFAs, especially acetate and butyrate, act as histone deacetylase inhibitors [20, 21]. In this way, these short-chain fatty acids induce hyperacetylation of histones, which affects gene expression and results in anti-inflammatory properties, induction of growth arrest, and apoptosis [22].

Human gut microbiota also plays a role in protective function. It forms a barrier against the colonization of pathogenic bacteria and stimulates the production of substances that inhibit the adhesion of pathogens to enterocytes [11]. Gut microbiota play a key role in the development of the immune system. Microbiota releases metabolites that are signals for the development of regulatory T-cells, T-helper type 1 and type 2 cells, and T-helper 17 cells [23]. The acids, especially butyrate, have strong immunomodulatory effects by stimulating the synthesis and release of protective peptides, cytokines, chemokines, and phagocytes [24]. Secreted substances protect from commensal bacteria, as well as from the triggering of inflammatory response to pathogenic organisms and/or induction of the process of apoptosis [10]. Gut microbiota influences the building of mucus layer and the secretion of mucins in intestine [25]. Intestinal microbiota plays a protective role in the host defense against *Streptococcus pneumoniae* infection. The gut microbiota enhances alveolar macrophage function [26]. Researchers suggest that this may be a novel therapeutic strategy.

Microbiota synthesizes, from host cholesterol, primary bile acids such as cholic acid and chenodeoxycholic acid [27]. Gut microbiota metabolizes these acids and generates secondary bile acids [28, 29]. Bile acids play different roles: as naturally occurring detergents that help the absorption of dietary fats, as well as signaling molecules. They are ligands for G-protein-coupled bile acid receptor 1 (GPBAR1, also known as TGR5) and farnesoid X-activated receptor (FXR, also known as the bile acid receptor) [30]. These receptors play a role in

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the regulation of host metabolism. Gut microbiota also releases agonists that regulate TGR5 signaling [30]. Human gut microbiota also reduces the levels of tauro-β-muricholic acid, a naturally occurring antagonist, which thereby regulates FXR signaling [31]. Secondary bile acids, by binding to TGR5 and GPR receptors, stimulate the production of GLP-1 in L-cells.

Gut microbiota synthesizes and releases glycoside hydrolases that hydrolyze plant polysaccharides for releasing monosaccharides and SCFAs.

Factors influencing the composition of human gut microbiota

The composition of human gut microbiota depends on various factors: the method of delivery, feeding type of a newborn, diet, use of drugs, probiotics, prebiotics, as well as age, sex, geographical area, and microflora transplantation.

The digestive tract of a newborn is sterile. The mode of delivery influences the composition of gut microbiota in infants. Vaginal delivery causes such an environment within 20 minutes, as the newborns develop microbiota from the maternal vagina or fecal microflora (*Lactobacillus*, *Prevotella*, and *Sneathia*). For cesarean section, the composition of newborn's gut microbiota depends on the skin and hands that touch the newborn after delivery (*Staphylococcus*, *Corynebacterium*, and *Propionibacterium*) [32]. During the first days of life of infants, the composition of infant's gut microbiota is mainly formed of *Proteobacteria* and *Actinobacteria* [5, 33].

The gut microbiota remains similar to the adult profile and at approximately 2.5 years of age, the bacterial composition is similar to adult gut microbiota. In the gut microbiota composition, *Firmicutes* and *Bacteroidetes* are predominant [6], and then, from the age 7 years, these bacteria comprise of about 90% of the bacterial composition. The remaining 10% of bacteria are *Tericutes*, *Cyanobacteria*, and Proteobacteria. This composition of gut microbiota is characteristic for adults [32].

The composition of gut microbiota in breast-fed babies differs from formula-fed (bottle-fed) babies [34], as well as the introduction of solid food influences the composition of bacteria in babies [35]. For breast-fed babies, the levels of *Bifidobacterium* spp. are higher, *Bacteroides* spp. and *Lactobacillus* spp. are lower, and microbiota are less diverse [36] as compared to formula-fed babies [37].

Another factor influencing the composition of gut microbiota is the type of diet. In this instance, the effect depends on presence of fat, vegetables, fruits, saturated and unsaturated fats, etc. (Table 2). European children prefer western diet, which comprises of low dietary fiber and high animal protein and saturated fat. This diet promotes an increase in *Firmicutes* and Gram-negative bacteria [38]. The diet of African children from an African village of Burkina Faso is plant-rich. They have greater proportions of *Bacteroides* and Gram-positive bacteria [38]. In these children, the ratio of *Bacteroidetes:Firmicutes* increases, in addition to an abundance of *Prevotella*. In European children, the *Xylanibacter* is absent.

The Mediterranean diet, which is rich in fruits, vegetables, grains, and monounsaturated fats, is suggested as a gold-standard of optimum health [39]. High-fiber diet, rich in plant fibers, promotes the diversification of the microbiota [38, 40]. On the other hand, the decreased diversity in a gut microbiota has a negative influence on health [41].

Drugs are the next modifying factors of human gut microbiota. The increased introduction of antibiotics in therapy is observed. This class of drugs has an important effect on the composition of bacteria. Some taxons are not recovered even several months after the treatment of antibiotics [42, 43]. The introduction of antibiotics may cause following: the decrease of *Bifidobacteria* and *Bacteroides* (antiobesogenic bacteria), slowdown of the regrowth of *Bifidobacteria*, termination of the reestablishment of *Bacteroides* spp. [44], and reduction in the diversity of gut microbiota [45]. A 5-day antibiotic treatment changes the composition of human gut microbiota for up to 4 weeks, and after this time, microbiota reverts to the composition before the antibiotic treatment, however sometimes within 6 months [46]. There are also other negative effects of antibiotic therapy on the composition of human gut microbiota that has been described [47–49].

One of the widely used drug in patients with type 2 diabetes is metformin. It suppresses glucose output from liver. The obtained results showed that metformin changes the composition of human gut microbiota [50]. In two groups of patients treated for 2 and 4 months with metformin, the significant alterations in the relative 81 and 86 strains, respectively, were observed [51]. The significant alterations were observed especially in γ-proteobacteria (for example *E. coli*) and *Firmicutes*. In these patients, increased amounts of *Bifidobacterium* and *Escherichia coli*, and a decrease of *Intestinibacter* were observed.

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Table 2
The influence of diet type on the composition of gut microbiota [38, 40, 145–151]

Type of diet	Increased number of microbiota	Decreased number of microbiota
Western diet (High fat, low dietary fiber, high animal protein and saturated fat)	*Firmicutes, Bacteroides enterotype (Alistipes, Bilophila, Bacteroides), Lactobacillus spp., Bifidobacterium spp., Firmicutes to Bacteroidetes ratio, Enterobacteriaceae, Clostridiales, Enterobacteriales*	*Bacteroidetes,* genera within the class *Clostridia, Clostridium leptum, Enterobacter spp.*
Rich in plant-derived polysaccharides	*Actinobacteria, Bacteroidetes,* diversification of the microbiota	*Firmicutes*
Vegetarian diet	*Bacteroides thetaiotaomicron, Clostridium clostridiforme, Faecalibacterium prausnitzii*	*Bacteroides spp., Bifidobacterium spp., Escherichia coli, Enterobacteriaceae, Clostridium* cluster XIV a
Mediterranean diet (dominate of fruits, vegetables, grains, and monounsaturated fats	*Bacteroidetes, Clostridium, Prevotella*	*Bacillaceae, Proteobacteria*
High-protein diet	*Bacteroides enterotype*	*Firmicutes, Ruminococcus bromii*
Changed from a vegetarian diet to western diet	*Bacteroides*	*Prevotella*
Calorie restricted diet		*Firmicutes* to *Bacteroides* ratio
Reduced variety due to long-stay care	*Bacteroidetes*	Overall diversity

Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [52]. The key role of probiotics is the influence on the growth and/or survival of pathogenic microorganisms, which improve the mucosal barrier function or the mucosal immune system and other organs. Currently used probiotics are mostly composed of different species of lactic acid bacteria, such as *Lactobacillus salivaricus, L. paracasei, L. reuteri, L. plantarum, L. gaseri, and Bifidobacterium lactis* [11, 53]. In animal studies, the administration of *Lactobacillus* species as probiotic has an antidiabetic effect and reduces endotoxemia [54, 55]. Alzheimer's disease (AD) is treated as type 3 diabetes mellitus, of late.

Prebiotic, the next modifier of gut microbiota, is "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and the activity of one or a limited number of bacteria in the colon and thus improve host health" [56]. Prebiotics are fermented by colonic bacteria. The examples of prebiotics are: inulin, oligosaccharides, and polyphenols in red wine and coffee. The administration of prebiotics causes the following changes: increases in the levels of *Bifidobacteria* and decreases in endotoxemia; improves glucose tolerance [57] and decreases Bacteroidaceae family [58]; reduces triglyceride levels, intestinal permeability, and inflammation [56, 59]; reduces systemic and liver inflammation [60] and stimulates the secretion of gut peptides such as GLP-1 (glucagon-like peptide 1), and peptide YY (PYY) [61]. Red wine polyphenols increase the levels of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Egerthella lenta, Blautelia coccoides*, and decrease the levels of lipopolysaccharide (LPS) [62, 63].

Intestinal microflora transplantation (IMT) [11], also known as fecal microbial transplantation (FMT) [40], is a method used in therapy. In this method, fecal bacteria from a "healthy" donor are introduced into a recipient with pathological changes in the composition of gut microbiota. Using endoscopic technique, microbiota is introduced into the large intestine in the case of recurrent *Clostridium difficile* infection or into the duodenum for metabolic syndrome [11].

Composition of gut microbiota is also correlated with lifestyle. A sedentary lifestyle is associated with a high risk of developing chronic diseases such as cardiovascular disease, cancer, and diabetes. On the other hand, an active lifestyle, including physical exercise, prevents chronic disease. Results obtained from active women revealed a higher abundance of health-promoting bacteria, such as *Faecalibacterium prausnitzi, Roseburia hominis*, and *Akkermansia muciniphila*, as compared to sedentary women [64].

PATHOLOGIES

Metabolic endotoxemia

Metabolic endotoxemia is due to increased plasma levels of LPS [54, 65]. LPS is a component of the cell wall of Gram-negative bacteria [66, 67]. High-fat diet

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stimulates the overgrowth of Gram-negative bacteria. The increase of plasma LPS levels is due to fat intake and high-fat diet [54]. The relationship among fat feeding and levels of LPS has been demonstrated in human studies [66, 67].

After absorption by enterocytes, LPS binds to chylomicrons in plasma [68]. It also binds to the CD14 toll-like receptor-4 (TLR-4) in macrophages. TLRs play an important role in the innate immunity. The complex of LPS–TLR-4–CD14 triggers an inflammatory cascade [54, 69]. The complex stimulates TLR-2–mediated inflammatory response, increasing the secretion of proinflammatory cytokines by the adipose tissue [70]. In this inflammatory cascade, nuclear factor-κB (NF-κB), suppressor of cytokine signaling-3 (SOCS-3), interleukin 6, and tumor necrosis factor-α (TNF-α) are also involved. As a result, diseases such as type 2 diabetes mellitus and insulin resistance occurs [71].

Dysbiosis

The change of the gut microbiota composition is termed "dysbiosis". Several factors alter the balance of gut microbiota creating dysbiosis. The factors of dysbiosis are as follows: diet, antibiotics, proton pump inhibitors, nonsteroidal anti-inflammatory drugs, iron, methotrexate, metformin, chronic stress, and gastrointestinal tract infection [11]. In diseases due to dysbiosis, the shift from predominant symbionts to potential pathobionts is observed [16, 72–75]. For example, in patients with type 2 diabetes a reduced proportion of *Firmicutes* and *Clostridia* was observed [76]. In overweight people, the ratio of *Bacteroidetes:Firmicutes* is disturbed [77]. Dysbiosis causes diseases such as autoimmune and metabolic diseases, bacterial infections, cancers, etc. Dysbiosis also causes psychiatric diseases/disorders such as autism spectrum disorder (ASD) [78–80], schizophrenia [81, 82], major depressive disorder (MDD) [83, 84], and other neuropsychiatric disorders [85]. There are also diseases of central nervous system (CNS) due to dysbiosis, such as multiple sclerosis [86], AD [87], and Parkinson's disease [88, 89]. ASD and MDD depend on the gut–brain–axis [9]. Gut–brain–axis is also involved in stress, behavior, and stroke [4, 90].

GUT–BRAIN–AXIS

The gut–brain–axis consists of the bidirectional communication between the gastrointestinal system and CNS. It plays a role in the homeostasis of neural, hormonal, and immunological signaling [91]. As mentioned earlier, gut microbiota causes the release of bioactive signaling molecules: SCFAs, catecholamines, neurotransmitters, and neuropeptides such as serotonin and gamma-aminobutyric acid (GABA) [92, 93]. For example, GABA is produced by *Lactobacillus* sp. and *Bifidobacterium* sp.; acetylcholine is produced by *Lactobacillus* sp.; serotonin is synthesized by *Escherichia*, *Streptococcus*, *Enterococcus*, and *Candida*; dopamine – by *Escherichia, Bacillus* sp., and *Serratia*; norepinephrine is released by Bacillus sp. Escherichia, and *Saccharomyces* [94, 95]. Microbial enzymes may also produce neurotoxins. D-lactate is a product of gut bacterial fermentation of carbohydrate. In patients with chronic fatigue syndrome and neurodegenerative dysfunction, increased levels of D-lactate producing bacteria were found. The next neurotoxin produced by bacterial enzymes is ammonia. This neurotoxin may cause hepatic encephalopathy. SCFAs contribute to ASD [96]. For details, see [39, 97–100]. The release of metabolites and neurometabolites by intestinal microbiota causes the human intestine to act as an endocrine organ.

In the microbiota–gut–brain axis, the vagus nerve is also involved. It plays an important role in the bidirectional signaling between gastrointestinal (gut microbes) and nervous systems [91]. In this instance, as a neural mechanism of this communication it causes the induction of c-Fos in vagal sensory neurons [97].

The activation of immune system by gut microbiota also plays a crucial role in the network between gut and brain. It is due, for example, by synthesis and release of proinflammatory cytokines, interleukin 6 (IL-6), and chemokine ligand 2 (CCL2) [97].

These substances, SCFAs, neurotransmitters, neurotoxins, etc., penetrate the gut mucosa are transported by blood and can cross the blood–brain–barrier (BBB) and affect the CNS. On the other hand, CNS sends signals to gut which regulate gastrointestinal functions like motility, secretion, and mucin production. Neural response influences the secretion of gastrointestinal hormones that regulate the functions of gastrointestinal tract.

The interactions between the gut microbiota and CNS need further search. They are not fully elicited. This is due to different neural, endocrine, and immune, processes, which may be affected by gut microbiota and metabolites produced by the microbiota [95].

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Clearly, functions of brain, its "health", or diseases/disorders, depends on diet, drugs used, probiotics, prebiotics, age, bacterial infections, etc. These modulators influence the composition of human gut microbiota [101, 102]. Human gut microbiota also influences the hypothalamic–pituitary–adrenal axis [103].

GUT MICROBIOTA AND ALZHEIMER'S DISEASE

AD is the most common neurodegenerative disorder. It is the cause of dementia one of its most common symptoms in older people. The estimated prevalence of AD in 2015 was 44 million people [104]. A progressive loss of cholinergic function is observed in AD patients. Neuronal cell death in the hippocampus and cerebral cortex causes this pathology.

There are two forms of AD. Over of 95% of patients with AD have sporadic or late-onset AD. Environmental factors and genetic predisposition contributes to the pathology of this form. The second form of AD is familial, called early-onset AD, in less than 5% of AD patients, due to mutations in any of the following three genes: the amyloid precursor protein (APP), presenilin 1 (PSEN-1), and presenilin 2 (PSEN-2) [105–107]. Recently, an accepted proposition is that the neuropathological features of both forms of AD are due to: abnormal extracellular accumulation of amyloid-β peptide (Aβ), senile plaques, and intracellular neurofibrillary tangles [108].

Peptide Aβ derives from a membrane-spanning, ~770 amino acid amyloid-β protein precursor (AβPP), distributed in the neuron membrane. It is a cleavage by β- and γ-secretase [109] (amyloidogenic pathway). The monomers of the peptide Aβ are soluble, highly flexible and have an aggregation propensity. $A\beta_{40}$ peptides associate with microvessel endothelial cells that line the cerebral vasculature. $A\beta_{42}$ peptides form the central core of the senile plaque. They are insoluble, proinflammatory parenchymal lesions that are progressively deposited during AD [110, 111]. In healthy subjects, α-secretase produces nontoxic αAβPP fragments (nonamyloidogenic pathway). Aβ deposition and clearance are regulated by the ubiquitin-proteasome system and autophagy. In AD neurons, the proteolysis causes impairment. Therefore, the oligomeric structures of Aβ are accumulated and change the functions of proteasome and autophagy.

Animal studies with germ-free "gnotobiotic" mice showed that commensal gut microbiota is critically essential for signaling between gut microbiome and autonomic nervous system, enteric nervous system, CNS, and neuro-immuno-neuroendocrine system [112, 113].

Gram-positive bacteria *Lactobacillus* brevis and *Bifidobacterium dentium* produce γ-aminobutyric acid. GABA is the major inhibitory neurotransmitter in human CNS [92]. The concentrations of GABA in CNS are correlated with its concentrations in the gastrointestinal tract. Decreased amounts of enteric *Lactobacillus* and *Bifidobacterium* decrease the levels of GABA in the gut, and then decrease the levels of GABA in the CNS. The dysfunctions in CNS due to GABA-mediated neuromodulation and neurotransmission are linked to the development, for example, AD. Postmortem study of patients with AD showed that the GABA levels were decreased in frontal, temporal, and parietal cortex of AD patients as compared to patients without AD [114, 115].

Serotonin (5-hydroxytryptamine, 5-HT) plays a crucial role in the regulation of cognitive function. Gut microbiota plays an important role in the synthesis of serotonin. Germ-free (GF) animal studies have shown that in the blood of a GF mice the content of 5-HT was about 60% lower as compared to a specific–pathogen–free mice with a normal gut microbiota. After the reconstruction of gut microbiota in GF mice, the concentration of 5-HT was increased [116]. The selective serotonin reuptake inhibitors reduce the Aβ protein synthesis in the brain. Therefore, increased extracellular 5-HT levels can reduce the formation of Aβ plaque, and in this manner, reduce the risk of AD [117]. These results were obtained in animal and human studies. The changes in the biosynthesis of 5-HT due to disturbances in gut microbiota influence the development of AD.

Glutamate, the excitatory neurotransmitter in the human CNS, and N-methyl-D-aspartate (NMDA) glutamate receptor play a role in the physiology of human brain. NMDA glutamate receptor plays an important role in learning and memory [118]. The relationship between the expression of NMDA glutamate receptor and the gut microbiota has been demonstrated. In GF mice and after the administration of antibiotics, the expression of NMDA mRNA in hippocampus and the level of NMDA receptor are significantly reduced [119, 120].

In the brain of AD patients, an elevated level of neurotoxic β-N-methylamino-L-alanine (BMAA)

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amino acid was found. This neurotoxin, influenced by NMDA, is generated by *Cyanobacteria* in the human intestine. BMAA may contribute to the neurologic dysfunction [118], such as inflammatory neurodegeneration observed in AD [121]. BMAA normally never incorporates into the brain proteins. But it can be incorrectly incorporated into the polypeptide chains of brain, which may lead to protein misfolding. This Aβ plaque pathology is characteristic of AD [121, 122]. Chronic exposure to BMAA triggers intracellular neurofibrillary tangles and Aβ deposits increase the risk of AD [123].

Cyanobacteria also produce other neurotoxins such as saxitoxin and anatoxin-α. These toxins may further contribute to neurological diseases, over the course of aging [124]. Therefore, the risk of AD may depend on the number of *Cyanobacteria* in human intestine [125].

A protein called brain–derived neurotrophic factor (BDNF) is distributed in the CNS. This protein is involved in processes such as neuronal survival, differentiation, prevention of neuronal damage and death, etc. In GF mice, the expression of BDNF protein is decreased [126], as well as the expression of mRNA BDNF in hippocampus of these mice is downregulated [127, 128]. In AD patients, the levels of BDNF are decreased in the brain and serum [129].

Bacteria and fungi, as a component of human gut microbiota, secrete amyloid protein in CNS, resulting in Aβ accumulation and increased risk of AD [130].

The infection of pathogens also increases the risk of AD. *Chlamydiophila pneumoniae*, the Gram-negative bacteria, may cause pneumonia. Postmortem investigations of brain of AD patients showed the presence of *C. pneumoniae* in astrocytes, microglia, neurons, and in infected cells close to senile plaques and intracellular neurofibrillary tangles [131]. *Helicobacter pylori*, the Gram-negative bacteria live in gastric epithelial cells. These bacteria cause chronic gastritis, gastric ulcer, and gastric cancer. The correlation between *H. pylori* infection and AD has been demonstrated. The pathophysiology of AD for *H. pylori* may be due to the release of proinflammatory cytokines and induction of oxidative stress [132, 133]. *Toxoplasma gondii*, a protozoan intracellular parasite, can cause encephalitis and nervous system dysfunction. This parasite stimulates chronic inflammation in the brain and CNS. In AD patients, the levels of serum anti-*T. gondii* antibodies are increased. This observation suggests a link between AD and *Toxoplasma gondii* infection [134].

Viruses are also present in gut microbiota. There are described coordination between viruses and AD. For example, HSV-1, a neurotropic virus, can enter the CNS and cause herpes simplex encephalitis; and brain pathologies due to HSV-1 are like pathologies in the brain of AD patients [135]. Very important observation is the presence of HSV-1 DNA in 90% of the amyloid plaques of AD patients [136]. Pathologies observed in brain due to HIV are similar to pathologies observed in the brain of AD patients [137]. The infection by human cytomegalo-virus and hepatitis C virus also increase the risk of AD [138, 139]. For details, see [125].

At the end of this paper, it is necessary to include the sentence written by Bonfili et al. [140] "... modulation of the microbiota induces positive effects on neuronal pathways that are able to slow down the progression of Alzheimer's disease".

FUTURE DIRECTIONS

The correlation between the composition of gut microbiota and AD is not fully understood. There are several unanswered questions. It was found that modulation of gut commensal microflora may be a promising therapy for AD [140]; however, it is not clear the nature of the pathophysiological mechanism of AD due to composition of gut microflora [141]. What is a way in which intestinal microbiota affect the pathogenesis of AD? Is modulation of the gut microbiota due to probiotics or prebiotics, a potential therapy in AD? What are the associations between theory on role of gut microbiota in pathogenesis of AD and other theories, such as inflammation, energy metabolism, and so on, as the cause of AD? There are also other, important questions [142–144].

CONCLUSIONS

Gut microbiota influences the human health and diseases. Intestinal bacteria can modulate gut–brain interactions. It is also important to understand the role of dysbiosis in brain physiology and pathology. Investigations of different factors—such as diet, drugs, probiotics, prebiotics, etc.—on the composition of human gut microbiota are also very important. Human lifestyle may have an influence on the development of AD.

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DISCLOSURE STATEMENT

The author's disclosure is available online (https://www.j-alz.com/manuscript-disclosures/17-0908r2).

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Association between Alzheimer's Disease and Oral and Gut Microbiota: Are Pore Forming Proteins the Missing Link?

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Abstract. Alzheimer's disease (AD) is a neurodegenerative condition affecting millions of people worldwide. It is associated with cerebral amyloid-β (Aβ) plaque deposition in the brain, synaptic disconnection, and subsequent progressive neuronal death. Although considerable progress has been made to elucidate the pathogenesis of AD, the specific causes of the disease remain highly unknown. Recent research has suggested a potential association between certain infectious diseases and dementia, either directly due to bacterial brain invasion and toxin production, or indirectly by modulating the immune response. Therefore, in the present review we focus on the emerging issues of bacterial infection and AD, including the existence of antimicrobial peptides having pore-forming properties that act in a similar way to pores formed by Aβ in a variety of cell membranes. Special focus is placed on oral bacteria and biofilms, and on the potential mechanisms associating bacterial infection and toxin production in AD. The role of bacterial outer membrane vesicles on the transport and delivery of toxins as well as porins to the brain is also discussed. Aβ has shown to possess antimicrobial activity against several bacteria, and therefore could be upregulated as a response to bacteria and bacterial toxins in the brain. Although further research is needed, we believe that the control of biofilm-mediated diseases could be an important potential prevention mechanism for AD development.

Keywords: Alzheimer's disease, biofilms, infection, periodontal diseases, porins

INTRODUCTION

Alzheimer's disease

In recent years, considerable progress has been made regarding the understanding of the pathogenesis, diagnosis, and treatment of Alzheimer's disease (AD). However, it still remains a fatal condition with significant negative impacts on economic and

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social issues. Until now, there are no effective therapeutic approaches to prevent, delay, or even reverse the condition, which starts with an initial mild loss of cognitive function and progresses toward language problems, memory loss, motor difficulties, and ultimately death [1]. The shift in demographics toward a highly prevalent elderly population is further increasing the prevalence of AD worldwide and has increased the financial burden of the disease (e.g., medications, nursing care). Currently, it is estimated that in the US alone, more than 5 million people aged 65 or older suffer from AD, and the costs for treating the disease are estimated at over US$180 billion per year [2, 3].

AD is a neurodegenerative disease, which is associated with neuronal loss in several areas of the brain, such as frontal cortex and hippocampus, and can be classified as either "early-onset" (also known as "familiar") or "late-onset" [4]. The late-onset form of the disease is the most prevalent and is considered to be the main worldwide cause of dementia in the elderly population [5]. Although it is believed that late-onset AD is mostly determined by environmental factors, the presence of the apolipoprotein E (APoE) $\varepsilon 4$ allele has been demonstrated to increase the risk of developing AD [6].

In the present review, we focus on the emerging issues of brain bacterial infection and AD, and on the existence of antimicrobial peptides having pore-forming properties that function in a similar way to the pore formed by amyloid-β (Aβ) in a variety of cell membranes. Thus, it is possible that brain microbial infection might accelerate/precipitate the onset of AD and that Aβ might act as an antimicrobial or as an enhancer of the neurodegenerative process.

The amyloid cascade as a cause of Alzheimer's disease

There is abundant evidence, based on genetic and animal data, suggesting that Aβ peptide accumulation first initiates and then might be acting as a causative agent of the disease. For instance, the existence of mutations in genes for the amyloid precursor protein (*APP*) (chromosome 21) and presenilins 1 (chromosome 14) and 2 (chromosome 1) [7] has been reported in a small number of AD patients. Furthermore, as mentioned above the ApoE E4 allele has been associated to sporadic AD [8]. On the other hand, environmental factors and lifestyle habits such as diet, physical exercise, and education are also believed to affect the onset of AD [9]. One of the main histological markers of AD are senile plaques that are made up mainly of Aβ [10]. Although its function is still unknown [11], Aβ is a 39–42 amino acid peptide generated by the enzymatic processing of amyloid-β protein precursor (AβPP), which has sites for α-β- and γ-secretases, of which β− and γ− secretases produce Aβ [12]. Aβ is mainly found in the extracellular matrix in the brain and cerebrospinal fluid at nanomolar concentrations and is widely accepted as the main neurotoxic agent in the disease [13]. Early manifestations of AD are associated with the synaptotoxic effects produced by soluble oligomeric forms of Aβ [14].

The central dogma is that monomers of Aβ aggregate to form small molecular weight species, also referred to as oligomers [14], which are believed to be highly neurotoxic [15]. Although there is no final conclusion, diverse forms from dimers to 56 kDa oligomers appear to be the most important causal agents in the disease [16, 17]. It is believed that before affecting neuronal function, the peptides have to associate with the cell membrane. It was recently shown that Aβ association to the membrane resulted in the leakage of important cellular components, as well as entry of calcium into the cell [15]. The unregulated entry of calcium can by itself cause alterations in synaptic transmission, cell toxicity, and death [18]. This experimental evidence points to the idea that Aβ toxic events are multiple and that one/several of them might serve as a therapeutic target. Nowadays, there is a reasonable consensus that Aβ will provide the first, early cellular toxic stress and that tau hyperphosphorylation will subsequently orchestrate the final deadly blow to the neuron [10].

Pathogenesis of AD

In terms of pathogenesis, AD shows two distinct structures in affected neurons known as neurofibrillary tangles (NFT) and the previously described senile plaques [19, 20]. NFT are a characteristic structure found in the brain of AD patients and correspond to intracellular accumulations of hyperphosphorylated and misfolded tau protein [21, 22]. Tau protein is normally found in the cytoplasm of neuronal cells and along their axons within the CNS [23] and when phosphorylated has the tendency to form paired helical filaments that accumulate as NFT [24]. There are six isoforms of the tau protein, and all six are hyperphosphorylated in AD (reviewed in [25]). Therefore, potential therapies

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against tau pathogenesis are mostly directed against hyperphosphorylation, although they also include approaches to inhibit aggregation, as well as tau-directed immunotherapies [26]. Despite the above knowledge, the consequence of NFT accumulation in the central nervous system (CNS) is not yet fully understood.

On the other hand, and as previously mentioned, senile plaques are constituted by Aβ deposits generated by the cleavage of the AβPP. Reasons for the presence of the precursor protein in the CNS and why it is cleaved to Aβ are not well understood. However, it is known that Aβ is able to rupture the neuronal plasma membrane by the formation of pores leading to cytoplasmic leakage and cell death [27, 28], by either direct lipid disruption or by its interaction with ion channels in the membrane [15, 29]. Some studies have shown that reduced cerebrospinal fluid levels of $A\beta_{42}$ in patients showing mild cognitive impairment is strongly indicative of disease progression [30–32].

Interestingly, it has been recently shown that Aβ functions as an antimicrobial peptide, which has the capacity to kill various strains of bacteria such as *Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Streptococcus pneumoniae (S. pneumoniae), Enterococcus faecalis (E. faecalis),* and the fungus *Candida albicans (C. albicans)* [33]. The mechanism of action of Aβ as an antimicrobial peptide is believed to stem from its ability to entrap microbial cells and avoid their adhesion to host cells [34]. There has also been evidence that Aβ has a protective activity against certain viruses and may be overexpressed in patients with viral infections [35, 36].

Similarities between AD and infectious dementia

Some neurodegenerative diseases present strong infectious components in their pathogenesis, and therefore are commonly referred to as infectious dementia. One of these pathologies is Creutzfeldt-Jakob disease, which is a fatal brain disorder characterized by behavioral changes, memory loss and progressive dementia, among other symptoms [37, 38]. All these symptoms are very similar to the ones observed in AD patients. One of the causes of Creutzfeldt-Jakob disease is believed to be prion accumulation which can be acquired by exposure to brain tissue from an infected person [39]. The variant form of Creutzfeldt-Jakob disease (i.e., bovine spongiform encephalopathy) is a similar disease, but in this case the transmission is from cows to humans [40]. Other forms of infectious dementia include toxoplasmosis, syphilis, and human immunodeficiency virus (HIV) [41]. Interestingly, the symptomatology and pathology of general paresis in advanced stages of neurosyphilis is remarkably similar to AD [42]. Although AD is not traditionally considered as infectious, there has been increasing evidence that microorganisms may play a central role in the disease [43]. There is also recent evidence that Parkinson's disease may have an infectious influence, as it has been shown to be associated to influenza virus [44].

THE ROLE OF BIOFILMS IN SYSTEMIC HEALTH AND DISEASE

Currently, it is known that biofilms are associated to a wide range of chronic diseases in the human body [45]. A biofilm refers to a community of different microorganisms (i.e., bacteria, virus, fungi) embedded in an extracellular matrix that confers resistance to antibiotic attack and facilitates nutrient exchange and adhesion to substrates [46, 47]. Biofilms are initiated by the irreversible adhesion of microorganisms onto a surface, and factors promoting this attachment include surface roughness, hydrophobicity and presence of bacterial appendages (i.e., fimbriae) [48–50]. These biofilms are not only present on biological tissues, but also on surfaces of biomedical and prosthetic devices and are involved in the development of diseases such as tooth cavities, periodontal disease, urinary tract infections, and bacterial vaginosis [51–54]. However, they are not only responsible for site-specific infection because single cells can detach from the biofilm mass and migrate to other sites and spread infection (reviewed in [55]).

In humans, biofilms can be found in different regions of the body, with the most important areas being the skin, mouth, gut, and urogenital tract [56]. Typically, the group of microorganisms that inhabit one of these regions is referred to as microbiota (e.g., gut microbiota). Recently, it has been demonstrated that the microbiota is an important factor in maintaining homeostasis in healthy patients, and that an imbalance in microbiota composition (i.e., increased concentrations of pathogenic bacteria) can lead to disease [57]. For the purpose of this review, focus will be placed on discussing the oral and gut microbiota, as they are currently believed to be the most implicated in the pathogenesis of AD.

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Oral biofilms: An important reservoir of infections

The oral cavity is believed to contain between 500–700 bacterial species, although less than half of these species can be cultivated in the laboratory [58–60]. In recent years, there has been increasing evidence that oral bacteria are associated to the onset and maintenance of a wide range of systemic diseases, including cardiovascular disease, pneumonia, atherosclerosis, and infective endocarditis [61]. For example, oral biofilm on the surface of infected removable dentures can generate a local inflammatory disease known as denture-related stomatitis [62, 63]; however, it may also act as a reservoir for pathogens involved in respiratory diseases. Recently, O'Donnell et al. isolated six respiratory pathogens from denture surfaces, including *S. pneumoniae*, *Pseudomonas aeruginosa (P. aeruginosa)*, and *Haemophilus influenzae B* (*H. influenzae* B) [64]. Furthermore, a study by Iinuma et al. demonstrated that in elderly patients, increased microbial load and inflammation is associated to higher risks of pneumonia [65]. Also, oral cariogenic bacteria such as *Streptococcus mutans (S. mutans)* have been isolated from infected heart valves [66, 67], and a study by Nakano et al. demonstrated the presence of *S. mutans* in both heart halves and atheroma plaques [68]. Therefore, bacteria are able to propagate from the oral cavity to other tissues and disseminate infection. How does this process occur? It is believed that bacteria from the oral cavity can enter into the bloodstream during procedures that generate transitory bacteremia such as dental treatment, tooth extraction, and tooth brushing [69–71]. The most relevant oral biofilms involved in dissemination are therefore the ones closest to the vascularized tissues surrounding the tooth, such as the periodontal biofilm.

Periodontal disease is a pathology that causes the inflammation and destruction of the tissues surrounding teeth, such as the periodontal ligament, alveolar bone, gingiva, and tooth cementum [72]. Periodontal disease is initiated and maintained by a specific biofilm on the surface of the tooth, which are mostly comprised of Gram-negative anaerobic strains such as *Prevotella intermedia (P. intermedia)*, *Porphyromonas gingivalis (P. gingivalis)*, *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)*, *Fusobacterium nucleatum (F. nucleatum)*, and *Treponema denticola (T. denticola)*. If left uncontrolled it can lead to tooth loss and potentially progress toward total edentulism [73].

Although the association between periodontal disease bacteria and systemic diseases is well known, the mechanisms underlying this process are still not fully understood. Overall, it is now believed that periodontal disease induces an inflammatory response that is not confined to the local site of infection. Instead, microorganisms and pro-inflammatory mediators are able to enter the bloodstream and affect other tissues and organs [74, 75] (Fig. 1). This process is facilitated by the direct contact between the periodontal ligament and vascular components, as well as the large area of inflamed gingival tissue in periodontal disease patients (believed to be between 8–20 cm^2) [76]. Increased levels of systemic C-reactive protein, IL-6, IFN-γ, TNF-α, and IL-1β have been found in patients suffering from periodontal disease [77–79], which can lead to endothelial dysfunction and altered immune responses [80]. Ultimately, this combination of bacterial infection and immune modulation originating from periodontal tissues is able to create a systemic response that may lead to chronic inflammatory diseases such as rheumatoid arthritis [81] (Fig. 2). Therefore, the control of oral and dental infections is currently an important strategy against the prevention of a broad range of systemic conditions [82–84].

Gut microbiota and chronic diseases

Trillions of microorganisms reside in the human gut where their composition varies due to nutritional factors, as well as chronic diseases such as inflammatory bowel syndrome and ulcerative colitis [85–88]. They are of extreme importance for the host as they participate in nutrient digestion, influence gene expression, and regulate the mucosal immune system [89]. During early lifehood, the gut microbiota is determined by factors such as delivery method (vaginal delivery or caesarean) [90], cessation of breastfeeding [91], and introduction of complementary feeding [92]. Therefore, it is no surprise that alterations in the gut microbiota composition have been associated to several chronic diseases in humans, such as obesity, diabetes, and cancer (reviewed by [93]). Ley et al. observed that the ratios between *Bacteroidetes* and *Firmicutes*, two important taxa of bacteria, are significantly different between obese and lean humans and mice. The authors found that the relative proportions of *Bacteroidetes* are decreased in obese subjects when compared to lean controls [94, 95]. Furthermore, Ajslev et al. observed that early exposure to antibiotics during childhood (before 6 months of age) was associated with future

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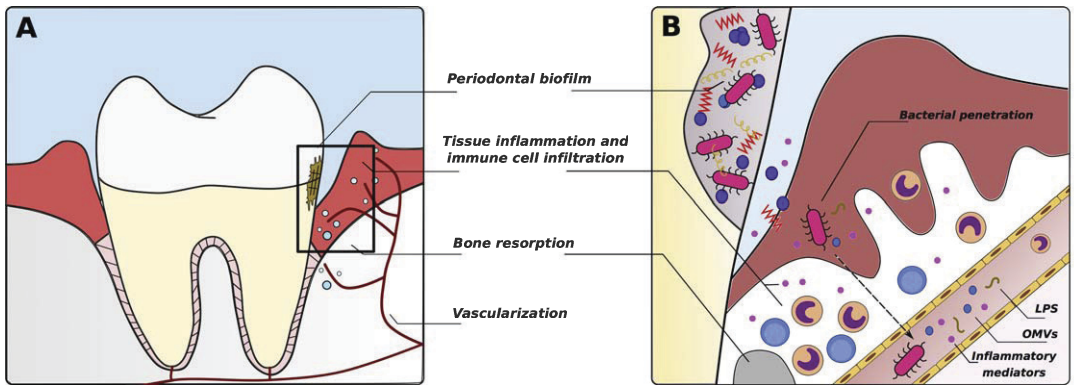


Fig. 1. Periodontal disease as a source of systemic infection and inflammation. A) Periodontal disease is a chronic, infectious disease caused by the accumulation of a specific biofilm on the surface of the tooth that causes inflammation of the soft tissues surrounding the tooth and bone resorption. B) Biofilm accumulation generates a chronic immune response in the periodontal tissues releasing inflammatory mediators such as IL-6, IFN-γ, TNF-α, and IL-1. Other mediators such as RANKL are involved in osteoclastic activation and bone resorption. Bacteria from the periodontal biofilm, as well as lipopolysaccharide (LPS) and outer membrane vesicles (OMVs), are able to migrate into the bloodstream and affect distant tissues and organs.

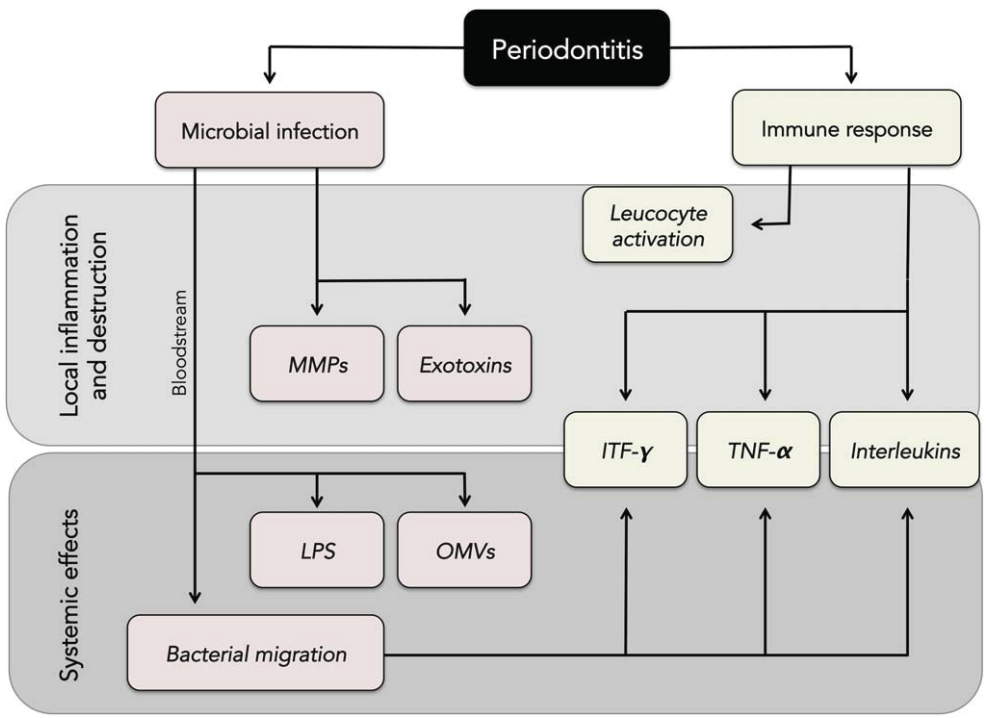


Fig. 2. Overview of the local and systemic inflammatory effects of periodontal disease. MMPs, metalloproteinases; LPS, lipopolysaccharide; OMVs, outer membrane vesicles; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha.

obesity [96], which could be explained by the effect of antibiotic treatment on the establishment of gut microbiota at early stages of development. Also, the species *Bilophila wadsworthia (B. wadsworthia)* has been associated with bowel inflammation in rat models, which is probably a consequence of H_2S production by this particular strain of bacteria [97]. Another important gut pathogen is *Helicobacter pylori (H. pylori)*, which is a causative agent of stomach ulcer and gastric cancer. This is due to its capacity to locally harm mucosal epithelium via proteases and VacA cytotoxins [98], and also affect the immune response in the gastric tissue by production of TNF-α and IL-1β [99–102].

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EVIDENCE OF MICROBIAL INFECTION AS A FACTOR FOR AD DEVELOPMENT

Despite the abovementioned studies suggesting a link between microorganisms and a number of chronic diseases in humans, there is still no consensus regarding the potential effect that microbial infections may have on the initiation and progression of AD. Some early studies showed viral and bacterial strains, as well as parasites, in brains of AD patients; more recent experimental approaches, such as next generation sequencing of 16S ribosomal RNA, have demonstrated the presence of increased levels of bacteria in affected brain tissue [103]. Another study has also suggested the presence of poly-microbial infections in AD brain tissue [104]. Overall, these findings have strengthened the hypothesis that pathogens play an important role in the development of AD. This hypothesis is supported by research linking the presence of microorganisms with increased Aβ deposition, tau hyperphosphorylation, and glial cell inflammation and activation [105–107]. Although the list of potential pathogens is growing, this review will focus on the strongest data regarding spirochetes, and oral and gut biofilms.

Spirochetes

One of the most relevant pathogens associated with the presence of AD are spirochetes, which are helical-shaped, highly motile Gram-negative bacteria involved in the pathogenesis of a wide range of human diseases [108, 109]. In a recent meta-analysis, Maheshwari and Eslick found a ten-fold increase of AD occurrence when spirochete infection was found in either the cerebral cortex or serum of patients [110]. Among these, species such as *Treponema pallidum (T. pallidum)*, *Borrelia burgdorferi (B. burgdorferi)* and a number of dental spirochetes, which have shown the strongest associations to AD [43, 111, 112].

B. burgdorferi, the causative agent of Lyme disease, has been isolated from AD brains in several occasions [113, 114]. It is believed that *B. burgdorferi* may persist in tissues after chronic Lyme neuroborreliosis, and be associated with the onset of AD in a number of patients [115]. *In vitro*, Miklossy et al. found that upon exposure to *B. burgdorferi*, primary rat neurons developed morphological changes similar to the ones seen by Aβ deposition in AD, as well as increased levels of AβPP and hyperphosphorylated tau protein [116]. However, other groups have not been able to find evidence of this particular pathogen in brain tissue of AD patients, although these authors considered only *B. burgdorferi* in their research and ignored the predominant oral spirochetes [117, 118]. In an earlier study, Miklossy also found evidence of spirochetes in the brains of AD patients, which were not present in a group of paired healthy control samples [119]. Furthermore, Riviere et al. found strong evidence of six oral *Treponema* species (*T. amylovorum, T. denticola, T. maltophilum, T. medium, T. pectinovorum*, and *T. socranskii)* in brains of AD patients, supporting the idea that more than one species of spirochete may be involved in brain invasion. Analysis of the frontal cortex demonstrated oral *Treponema* invasion in 14 out of 16 AD brains, but only in 4 out of 18 non-AD patients [120]. Another study also showed that senile plaques from AD patients contained spirochete-specific DNA and that pure *B. burgdorferi* biofilms were able to produce both AβPP and Aβ, which suggests that senile plaques contain a combination of both host and bacterial produced Aβ [121].

T. pallidum, the spirochete responsible for syphilis and neurosyphilis, also has a well-known ability to colonize the brain and generate a disease known as general paresis, which is similar to AD in terms of symptoms and pathogenesis [122]. The similarities between AD and neurosyphilis have led to the assumption that both diseases may share a common bacterial background (reviewed in [42]). Although *T. pallidum* is sexually transmitted, it has been found in the brain of patients even at early stages of infection [123], and its accumulation over time leads to the development of general paresis, which shares many hallmarks of AD such as Aβ plaque deposition, NFT, progressive dementia and cortical atrophy [42, 124].

Oral bacteria and AD

There is a growing range of evidence strengthening the link between periodontal bacteria and AD. One of the most relevant species involved in periodontal disease is *P. gingivalis* [125]. Kamer et al. showed that AD patients present increased levels of TNF-α and antibodies against periodontal pathogens, including *P. gingivalis*, compared to normal controls [126]. In another recent study, Poole et al. demonstrated the presence of *P. gingivalis* derived lipopolysaccharide, an important bacterial endotoxin, in brain samples from AD patients [127]. This data is in line with previous work linking lipopolysaccharides with increased Aβ deposition [128, 129]. By comparing

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gene expression data of *P. gingivalis* to microarray datasets of the hippocampus of AD patients, Carter et al. demonstrated that upregulated genes in *P. gingivalis*-infected macrophages matched the ones found in AD brains [130]. They also suggested that *P. gingivalis*, as well as periodontitis, can influence AD either directly by inflammation, or indirectly by promoting other AD-related risk factors such as diabetes, atherosclerosis and hypertension [130]. Furthermore, Noble et al. utilized a logistic regression model to suggest that levels of *P. gingivalis* IgG are associated to cognitive decay in patients over 60 years of age [131]. This idea was explored further in an animal model demonstrating that ApoE–/– mice infected for 24 weeks with the oral pathogen *P. gingivalis* showed actual infections in the brain, and particularly in hippocampal neurons that are critical for memory formation [132]. This direct bacterial colonization in the brain is interesting since it suggests a direct effect of bacteria in the brain parenchyma, as opposed to a long-distance effect via inflammatory mediators. Alternatively, inflammatory factors such as LPS might also be involved by increasing the permeability of the blood-brain barrier (BBB) and thus facilitating bacterial penetration into the brain [133]. Under this setting, it is possible to consider that bacteria might induce local inflammation and release peptides that can act as porins.

In a subsequent study, it was reported that high serum IgG levels for *Actinomyces naeslundii (A. naeslundii)*, another periodontal pathogen, was associated to increased risks for AD onset in patients over 65 years old [134]. Increased antibody levels for other important periodontal pathogens such as *P. intermedia* and *F. nucleatum* have also been found in patients with AD [135]. Interestingly, serum antibody levels for these bacteria were already elevated at baseline in the group that developed AD, even before cognitive impairment had occurred. This data suggests that periodontal disease and the presence of high serum antibody concentrations might occur prior to AD and may play a role as a causative factor. Another pathogen that has been associated to AD is *E. faecalis*, which is an oral pathogen implied in root canal infection and chronic periodontal disease [136], which has been shown to migrate into the brain and form abscesses [137]. Underly et al. found that *E. faecalis* infection increased expression of tau changes in primary rat cortical neurons, suggesting a potential link to AD pathogenesis [138]. The fact that AD seems to be associated to the presence of several periodontal bacteria instead of a unique species suggests that the bacterial factor may be non-specific, strengthening the idea that overall inflammation and infection play a part in the pathogenesis of AD.

Finally, it is important to consider the associations found between tooth loss and the development of AD. In a recent study, Batty et al. found that tooth loss was associated with increased risk of dementia in a group of 55–88 year old adults [139]. Similarly, in a 13-year long longitudinal study, Li et al. observed that tooth loss was associated to cognitive decline in elderly Chinese patients [140]. It is important to note that in both studies the number of teeth was self-reported by the patients and not confirmed by clinical examination. However, a study by Steward found no association between tooth loss and development of dementia, although they did find an association between dementia and gum inflammation [141]. It is necessary to emphasize that there are many potential causes for missing teeth (i.e., trauma, dental cavities) and therefore, the parameter of tooth loss may not be the optimal indicator for periodontal disease or the levels of periodontal bacteria in the oral cavity [142].

Gut microbiota

Among gut microorganisms, *H. pylori* appears to be one of the most relevant pathogens associated with AD. This is particularly worrying as it is estimated that 4.4 billion people are infected with *H. pylori* worldwide [143]. In a clinical study, Kountouras found that *H. pylori* was found in 88% of AD patients versus 47% of age-matched controls at baseline, and eradication of *H. pylori* improved cognitive parameters in the AD group [144]. In a recent publication, Felice demonstrated that an important *H. pylori* peptide modulates a large number of genes, some of which are directly related to AD [145]. The peptide, Hp(2-20), was found to alter the expression of 77 AD genes, 30 of which modulate inflammatory pathways. The specific mechanisms behind *H. pylori* pathogenesis in AD remains unknown, although it may be related to increased production of pro-inflammatory mediators such as TNF-α, INF-γ, and several interleukins [146], all of which have been shown to be important in AD pathogenesis [147].

Despite the apparent importance of *H. pylori*, there are other potential gut microbes believed to also play a role in AD. In a recent paper by Minter et al. mice that underwent long-term antibiotic treatment showed significant reduction in Aβ plaque deposition, which was correlated to a shift in the composition of the overall gut microbiota. This work is interesting as it

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demonstrates that gut microbiota can be altered to prevent the progression of AD pathogenesis [148]. Also, Liang et al. showed that treatment with probiotic *Lactobacillus helveticus* NS8 improved cognitive functions in rats subjected to stress [149]. Furthermore, Wang et al. demonstrated that treatment of rats with ampicillin for a month disrupted their gut microbiota and led to symptoms such as increased anxiety and spatial memory impairment, but further treatment with *Lactobacillus fermentum* NS9 reverted these effects [150]. Many other animal studies have also demonstrated an association between the administration of probiotic bacteria and improved cognitive function [151–153]. A recent clinical study showed that *Bifidobacterium longum 1714* improved visuospatial memory performance and reduced stress in 22 healthy patients [154], suggesting that specific strains of microorganisms have the ability of directly modulating brain function in humans. Furthermore, recent data has shown that strains such as *E. coli* and *Salmonella enterica* have the ability to secrete bacterial Aβ, which is used to promote biofilm stability as it is resistant to denaturing and degrading conditions (reviewed in [155–158]); however, the effect of this bacterial-derived Aβ on the host is not yet understood.

Another topic of interest is the way in which gut microbiota may influence the brain by means of the gut-brain microbiota axis [159]. It has been shown that alterations of this microbiota can lead to changes in behavior, and are implied in the regulation of anxiety, pain, obesity, and autism [160, 161]. It is believed that the gut-brain microbiota axis is not influenced by direct bacterial infection of the brain, but via the homeostatic control of innate immunity [162]. Furthermore, disturbances in the gut epithelium barrier, or in other factors such as mucin production, may increase intestinal permeability and allow the passage of antigens and pathogens into the host (reviewed in [163]). This increased permeability has been denominated 'leaky gut', and it has been associated to multiple autoimmune diseases such as celiac disease, lupus erythematosus and diabetes type I [164, 165]. The residing microbiota is important in regulating the function of the epithelium layer and plays a key part in the maturation and maintenance of the intestinal barrier [166–168]. Interestingly, a recent study by Braniste et al. demonstrated that the gut microbiota also has a direct impact on the permeability of the BBB because germ-free mice developed increased BBB permeability that was reduced once the gut microbiota was restored [169]. Increased permeability in both the intestinal barrier and BBB would provide easy passage of microorganisms and bacterial toxins not only into the bloodstream, but also into the brain and contribute to the pathogenesis of AD.

Other relevant microorganisms

Besides the abovementioned bacteria, there is also evidence suggesting that other microorganisms may be implied in the pathogenesis of AD. Recent work by Pisa et al. demonstrated the presence of several strains of fungi in brain tissue of 10 AD patients, whereas no fungal cells were found in the control group [170]. Also, Alonso et al. found evidence of increased serum levels of fungal polysaccharides and disseminated fungal infection in AD patients [171]. Interestingly, cases of infection by the fungus *Cryptococcus neoformans* have been clinically mistaken for AD, and in these cases dementia was reversed after proper antimycotic treatment [172, 173]. Furthermore, the fact that Aβ has shown to be antimicrobial against *C. albicans* supports the association between fungal infection and AD pathogenesis [33]. Also, it is important to mention that infection with HIV can lead to HIV-associated dementia, characterized by the infection of macrophages, microglia and astrocytes in the brain [174]. These infected cells shed viral proteins such as Tat and gp120, which in turn promote neuroinflammation by the production of inflammatory cytokines, as well as glutamate accumulation due to the down-regulation of the EAAT2 glutamate transporter in astrocytes [175–177].

POTENTIAL LINK BETWEEN MICROBES AND Aβ: PORE FORMATION IN CELL MEMBRANES

In the microbe world, ion channels are critical for controlling cellular homeostasis and defense. Porins, for example, are outer membrane proteins found in many Gram-negative bacteria that allow the passage of small solutes important for various metabolic processes (<650 Da) [178]. Interestingly, like Aβ, porins present a secondary structure having predominantly β sheets that can form complex association with at least trimeric conformation [179–182]. The oral Gram-negative anaerobe *F. nucleatum* is of particular interest since it not only causes periodontal disease but also can produce clinical infections in other body sites [183]. A recent study showed that people that developed cognitive impairment presented higher

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levels of serum antibodies for *F. nucleatum* [135]. However, it is unknown if the impairment was related to the presence of the bacteria in the brain. This oral bacterium is also interesting because it expresses a protein in the outer membrane, known as FomA, that functions as a non-specific porin in lipid bilayer membranes [184]. Furthermore, Zilm et al. described an upregulation of SSP 3802 (belonging to the Omp IP family of porins) in *F. nucleatum* as a response to a pH increase from 7.2 to 7.8, which is believed to be the pH of diseased periodontal sulcus [181]. *Neisseria meningitidis* expresses two major outer membrane porins, PorA and PorB, while *Neisseria gonorrhoeae* only expresses PorB, which is found to play multiple roles during infection [186, 187]. Novak and Cohen found that *P. gingivalis* are able to induce membrane depolarization of polymorphonuclear leukocytes following bacteria-cell contact, which is consistent with pore formation or activation [188]. Some studies have demonstrated that porins can translocate from bacteria to foreign membranes [189, 190], which suggests a potential mechanism for bacterial-induced cell death. Thus, it is possible that bacteria in the brain cause neuronal membrane leakage producing Aβ movement from/to the neuron, initiating a process of increased damage now by the toxic Aβ peptide.

Ion channel formation in microorganisms also serves a defensive role when released to the extracellular medium. Gramicidin, a classical ion channel forming antibiotic is secreted by *Bacillus brevis* and it corresponds to a hydrophobic pentadecapeptide, consisting of an alternating sequence of D- and L-amino acids forming a β-bonded helix in a hydrophobic environment (reviewed in [191]). This property leads to both ion channel formation and anti-microbial activity against other strains of bacteria [192, 193]. Similarly, nystatin derived from *Streptomyces noursei* is a polyene antifungal agent which in presence of ergosterol forms channels in the cell membrane disrupting the normal cellular environment and ultimately leading to cell death [194].

Furthermore, porin-like membrane proteins have been described in relevant strains of Treponema, as well as certain important periodontal pathogens. *T. pallidum* expresses TprC/D and TprI as outer membrane proteins, both of which have been identified to display porin-like activity [195]. Furthermore, *T. denticola* has been found to possess a 53 kDa surface antigen (Msp) on its outer sheath that demonstrated dual adhesin and porin activity [196, 197]. Outer membrane vesicles of *Eikenella corrodens*, another periodontal pathogen, displayed porin-like properties and induced cytotoxicity against mouse macrophages [198]. It is possible that the above-mentioned ion channel-forming peptides and the complex molecules normally found in the microbial environment are important in the development of AD. Similar to the Aβ peptides, these microbial-derived peptides might be causing membrane damage and ion dyshomeostasis leading to neuronal death. As discussed throughout this review, porin producing bacteria such as *T. pallidum*, *T. denticola*, and *F. nucleatum* are frequently observed in the brains of AD patients. Also, Aβ was reported to have antimicrobial activity against several bacteria [33], and therefore could be upregulated as a response to bacteria and bacterial toxins in the brain.

HOW COULD BACTERIAL OUTER MEMBRANE VESICLES CONTRIBUTE TO PORIN FORMATION?

Almost every cell (mammalian, bacterial, plant) has the ability to secrete extracellular vesicles, and until recently, it has been considered to be mainly a cellular waste system. However, it was found that extracellular vesicles can carry a variety of cargos such as DNA, RNA, proteins and lipids [199], and play key roles in cellular communication, even between kingdoms [200]. Bacteria shed periplasmic fluid containing extracellular vesicles or outer membrane vesicles (OMVs), which are 20 nm to 250 nm in size, in an active process during cell growth [201].

Over the last years, OMVs have become increasingly interesting in the study of both pathology and regeneration. Since OMVs are derived from the bacterial membrane, they also carry specific active membrane proteins. Several studies have shown the involvement of β-lactamase carrying OMVs in antibiotic resistance [202, 203] and also delivery of bacterial virulence factors by OMVs in bacteria such as *E. coli* [204], *P. aeruginosa* [205], and *P. gingivalis* [206]. Also, OMVs—especially ones derived from *P. gingivalis*—have been shown to modulate immune responses. One mechanism involves gingipain, an endopeptidase involved in nutrient collection. In the initial phase of *P. gingivalis* infection, gingipain activates the C1 complex, which creates a local inflammatory response improving nutrient supply and colonization. In later phases, with increasing presence in and around the biofilm, gingipain has the ability to inactivate complement factors (C3, C4, C5) leading to resistance to the complement system [207].

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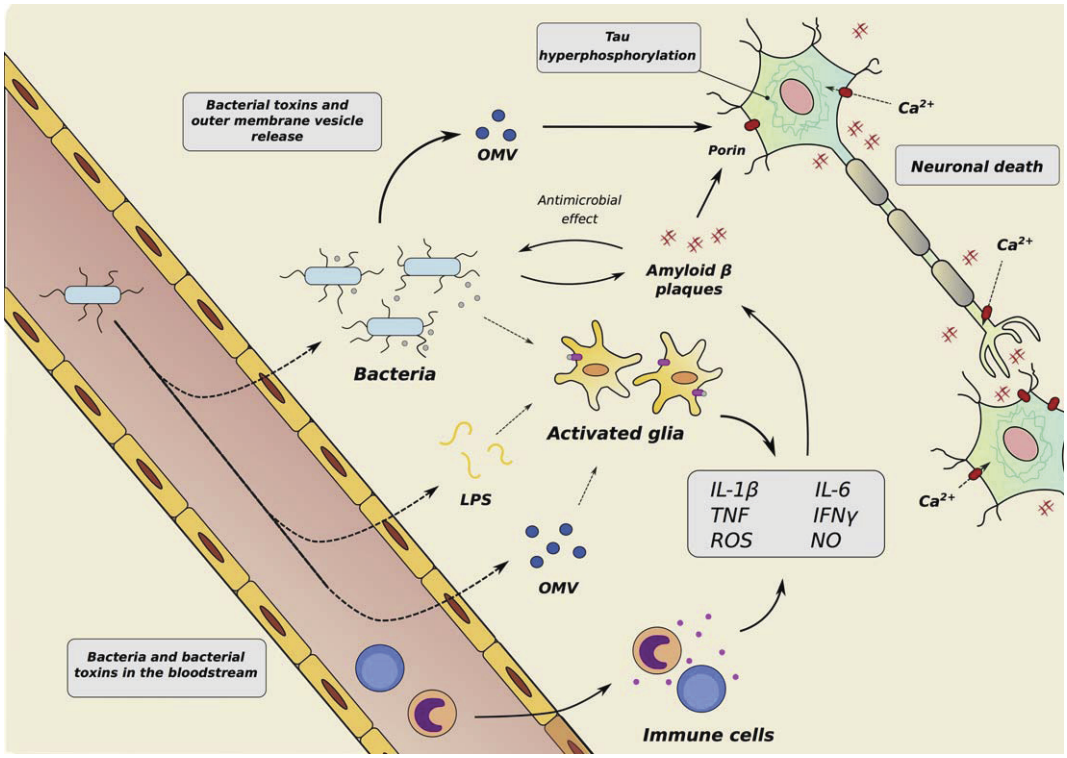


Fig. 3. Proposed link between bacterial infection and Aβ in AD pathogenesis. Bacteria and released components (such as LPS and OMVs) can enter brain tissue and generate a local immune response that initiates plaque deposition and tau hyperphosphorylation, as well as upregulate inflammatory mediators such as TNF-α and IL-1β that potentiate neuroinflammation. Furthermore, bacteria could directly damage neurons by the release and incorporation of porin-like proteins with the potential of causing unregulated calcium entry, synaptic toxicity and neuronal death. The pore-induced membrane damage could act synergistically with the Aβ induced pore formation to enhance neurotoxicity. Alternatively, Aβ could be released by neurons and act as an antibacterial agent because of its pore forming action.

Furthermore, *P. gingivalis* [208], *H. pylori* [209], and *P. aeruginosa* [210] share an interesting trait: they are not only porin-forming bacteria, but they have been shown to produce outer membrane vesicles that enter mammalian cells via lipid rafts [205, 211, 212]. This is interesting since Malchiodi-Albeli et al. demonstrated that disruption of lipid rafts protects neurons from amyloid oligomer-induced toxicity [213], suggesting a similar entry route of both amyloid and porin-containing OMVs. Therefore, it is possible that bacterial OMVs could be a key factor regarding neuronal death, although further research is necessary in order to understand this potential association.

CONCLUSIONS

As discussed in this review, there is a broad amount of research demonstrating an association between AD and infectious processes such as periodontal disease [214]. This association is not limited to a specific type of microorganism, but rather to a wide range of bacteria, fungi, and viruses. This suggests that instead of being associated to a particular individual pathogen, the mechanisms behind the development of AD are more general and may be mostly derived from generalized microbial injury and its consequent chronic inflammatory response within the brain tissue. This microbial effect is probably a combination of both direct damage caused by the migration of microorganisms into the CNS due to increased BBB permeability in older patients [215], and an indirect effect mediated by the systemic release of bacterial toxins, OMVs and pro-inflammatory molecules into the bloodstream. For example, *Treponema* species can potentially migrate to the brain from the oral cavity (where around 60 species of *Treponema* have been identified, many of them invasive [216]), and cause local damage due to a combination of porin production and generalized neuroinflammation. In either case, the presence of microbial components in the brain may potentiate Aβ plaque deposition and generate neuronal dysfunction by the action of porin-like

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molecules that increase membrane permeability and calcium leakage.

From the above, it is necessary to begin redefining the current understanding of AD to include a microbial component (Fig. 3). This does not mean that the genetic background or other potential environmental factors should not be considered. However, several studies have provided evidence supporting the idea that infection might be related to AD pathogenesis, and further efforts should be placed on exploring this possibility [214]. Until recently, diseases such as gastric cancer were believed to be non-infectious, but nowadays they are known to be associated to pathogens. Thanks to this discovery, in the last decades antibacterial treatment against *H. pylori* has reduced the incidence of gastric cancer in the population [217]. In the same way, it is possible that novel therapeutic approaches to prevent and treat AD could include antibacterial management in the future. In any case, the importance of maintaining control of biofilm-mediated diseases must be strongly highlighted. In summary, the reviewed data suggests that the link between bacteria and AD resides in the capacity of the bacteria to act directly and indirectly on the brain, and future research should be encouraged in order to further explore the specific mechanisms behind the infectious component of AD.

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Gut Microbiota Alteration and Its Time Course in a Tauopathy Mouse Model

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Abstract. Emerging evidence suggests that gut microbiota dysbiosis plays a role in neurodegenerative disorders. However, whether the composition and diversity of the gut microbiota are altered in tauopathies remains largely unknown. This study was aimed to examine the diversity and composition of the gut microbiota in tauopathies, as well as the correlation with pathological changes in the brain. We collected fecal samples from 32 P301L tau transgenic mice and 32 age- and gender-matched littermate mice at different ages. The 16S ribosomal RNA sequencing technique was used to analyze the microbiota composition in feces. Brain tau pathology levels were measured by immunohistochemistry. The diversity and composition of the gut microbiota significantly changed with aging. At the phylum level, the relative abundance of *Bacteroidetes* was increased, while *Firmicutes* were decreased in P301L mice compared with that in Wt mice after 3 months of age. In addition, *Actinobacteria* was decreased in P301L mice at 3 and 6 months of age, meanwhile *Tenericutes* was decreased in P301L mice at 10 months of age. Moreover, several specific macrobiota were highly associated with the levels of AT8-tau or pT231-tau protein in the brain. Our findings suggest that gut microbiota changed with aging, as well as in the tauopathy mice model. Modulation of the gut microbiota may be a potential strategy for treatment of tauopathy.

Keywords: 16S ribosomal RNA sequencing, gut microbiota, tau, tauopathy

INTRODUCTION

Tauopathies are progressive neurodegenerative disorders associated with Alzheimer's disease (AD) [1], frontotemporal lobar degeneration (FTLD) [2], Pick's disease [3], corticobasal degeneration [4], progressive supranuclear palsy (PSP) [5], and chronic traumatic encephalopathy [6]. The accumulation of pathological tau is the hallmark of these diseases and is closely correlated with cognitive decline. Despite substantial progress in recent years, the comprehensive pathogenesis of tauopathies remains unclear. Moreover, thus far, no therapeutic interventions are currently available.

Bidirectional communication between the brain and gut has long been recognized. Recent studies have revealed the vital role of the gut microbiota in the function of the central nervous system [7, 8].

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Alterations in gut microbiota composition can be related to the development of several neurological disorders, including AD [9], Parkinson's disease [10], and amyotrophic lateral sclerosis [8]. The dyshomeostasis in the gut microbiota is proposed to be a crucial cause of the onset of AD [11]. In contrast, metabolites derived from gut microbes protect neurons from amyloid-β (Aβ)-induced neurotoxicity and paralysis in an AD invertebrate model [12]. These results indicate the complex interaction between the gut microbiota and brain dysfunction. As a complex and multisystem disease, a series of mechanisms have been identified in tauopathies. However, the alteration in gut microbial composition and diversity has not yet been clearly confirmed. This study was aimed to examine the diversity and composition of the gut microbiota in tauopathies, as well as the correlation with pathological changes in the brain. Our findings suggest that gut microbiota diversity and compositions changed with aging, as well as in the tauopathy mice model. Moreover, several specific macrobiota are highly associated with pathological changes in the brain of P301L mice, indicating that modulation of the gut microbiota may be a potential strategy for tauopathy treatment.

MATERIALS AND METHODS

Animals and sample collection

P301L tau transgenic mice, expressing the longest human tau isoform (2N4R) with the FTDP-17 mutation P301L in neurons, were provided by Professor Juergen Gotz from Queensland Brain Institute, Queensland University (Australia) and raised in Daping Hospital Animal Laboratory Center. Mice food, provided by Xietong Organism (Jiangsu, China), was sterilized with irradiation (60Coγ). The sterilized water was produced by pure water system (LT-DW1500/D, Nico, China). All mice were raised under specific pathogen-free conditions with the sterilized food and water provided. A 12-h light-dark cycle was maintained. Young mice were weaned from their mothers after 1 month and raised under the same conditions. Age- and gender-matched wild-type (Wt) littermate mice were chosen as the controls. Fresh fecal samples were collected from mice of both genders at 1, 3, 6, and 10 months of age ($n = 8$, per group). A total of 64 fecal samples were collected in the morning to avoid interference from any possible circadian rhythm effects and stored at -80°C until subsequent DNA extraction. This study was approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Science of Daping Hospital.

DNA extraction and sequencing

Microbial DNA was extracted from 64 fecal samples using the standard E.Z.N.A. ® Stool DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to standard protocols. The concentration and purification of extracted DNA were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The V3-V4 region of the bacterial 16S ribosomal RNA gene was amplified to evaluate bacterial diversity by a thermocycler PCR system (GeneAmp 9700, ABI, USA). DNA was amplified by using the 338F and 806R primer set (338F: 5'-ACTCCTACGGGAGGCAGCAG-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3'). PCRs were conducted according to previous protocols [13], and the resulting products were further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) to remove failed and low quality products. Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2×300 bp) on an Illumina MiSeq platform (Illumina, San Diego, USA) by Majorbio Bio-PharmTechnology Co. Ltd. (Shanghai, China) according to the manufacturer's instructions.

Processing of sequencing data

Raw fastq files were demultiplexed and quality filtered by Trimmomatic and then merged by FLASH. The criteria were followed as previously described [14]. The obtained sequences were clustered into operational taxonomic units (OTUs) at the 97% sequence similarity level using UPARSE (version 7.1). Then, UCHIME (version 4.2.40) was used to identify and remove chimeric sequences. The taxonomy of each 16S rRNA gene sequence was analyzed by the Ribosomal Database Project Database Classifier algorithm (http://rdp.cme.msu.edu/) using a confidence threshold of 70%. All of the sequencing data in the present study can be accessed from NCBI with the accession number SRP188916.

Bioinformatic analysis

Alpha diversity analysis, including the Shannon index, Simpson, Chao1, ACE index and Sobs index, was carried out in QIIME on rarefied OTU

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tables. Beta diversity between samples was generated on the basis of bray_curtis or weighted_unifrac algorithms and reported according to principal coordinate analysis (PCoA) using the R package (Version 2.15.3). LEfSe (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload) was used to identify the taxa that were significantly different between groups. The area under the receiver operating characteristic curve (ROC) of the random forest (RF), namely AUC-RF, was carried out to identify and validate key predictive biomarkers using the R package. Briefly, AUC-RF used the ROC curve and the AUC as the predictive accuracy of RF and then selected the set of variables with the highest AUC value. The process of AUC-RF for key predictive biomarkers selection consists of the following steps: step 1) iterative elimination process; step 2) visual representation of the elimination process; step 3) selection of the optimal set of predictor variables; and step 4) predictive accuracy and probability of selection.

Immunohistochemistry

Free-floating immunohistochemistry methods were used for phosphorylated tau immunohistochemical staining. Phospho-tau at Thr231 (pT231) antibody was used to stain phosphorylated tau in the pre-neurofibrillary tangle (NFT) stage, while AT8 (phospho-tau at Ser202 and Thr205) antibody was used to stain hyperphosphorylated-tau deposited in intra-NFTs and extra-NFTs at the later stage of tangles [15]. Brain tissue sections were treated with 0.5% Triton X-100 (Sangon Biotech, Shanghai, China) mixed with 0.3% H_2O_2 in PBS for 30 min and then blocked with 3% BSA (Solarbio, Beijing, China) for 30 min at room temperature. The primary antibody was diluted with 3% BSA in a proper proportion (pT231 1:200, Signalway Antibody, USA; AT8 1:100, Invitrogen, USA.) and incubated overnight at 4°C. Secondary antibodies conjugated with HRP were diluted 1:200 with PBST to incubate the sections for 1 h at 37°C the next day, followed by visualization with DAB solution. Three brain slices (bregma –2.1 mm) per mouse were selected for pT231-tau staining, and another three brain slices (bregma –2.1 mm) per mouse were selected for AT8-tau staining in different groups of P301L mice. A 40-fold objective was used to capture images for pT231-tau staining from hippocampal CA1 and AT8-tau staining from amygdala. ImageJ software (National Institutes of Health, Bethesda, USA) was used to quantify the positive staining fraction of each image by another experimenter manually. Tau levels in the brain were quantified by the average positive staining fraction of the three brain slices and used for subsequent correlation analysis.

Statistical analysis

All data are represented as the mean ± SD. One-way ANOVA and Tukey's test were used to compare observed species among different groups. Two-tailed Student's *t* test or a Mann-Whitney *U* test was used to compare the differences in bacterial communities between P301L and Wt as applicable. Correlations between brain pathology and the abundance of microbiota were calculated using Spearman's rank correlation. To correct for multiple testing, *p* values were adjusted using Benjamini-Hochberg false discovery rate, and significant association was considered below a threshold of 0.05. All analyses were conducted with GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Characteristics of high-throughput sequencing data

A total of 2,995,005 qualified reads were filtered for downstream analysis. An average of 46797 reads per sample was obtained for this study (the minimum of one sample was 30920, and the maximum was 72203). The gradually flattening rarefaction curves and coverage index (99.78%) indicated an adequate depth of sequencing (Supplementary Figure 1a).

Changes in bacterial communities in mice with aging

The Chao1 index showed higher diversity and richness in 3-month-old and 6-month-old Wt mice than in 1-month-old Wt mice, and no significant difference was observed in P301L between different ages (Fig. 1a). The relationships between the community structures were examined by PCoA and bray_curtis analysis. The community structures of the gut microbiota differed as a function of age in both Wt (Fig. 1b, ANOSIM $r = 0.4568$, $p < 0.001$) and P301L mice (Fig. 1c, ANOSIM $r = 0.2281$, $p < 0.001$).

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The difference in bacterial communities between P301L and Wt mice during the course of tauopathy development

Alpha diversity, including the Shannon index, Simpson index, ACE index, and Sobs index, showed no significant difference between P301L and Wt mice at any age (Supplementary Figure 2). Based on the distribution of the sample points, weighted_unifrac analysis showed no significant difference in PCoA between P301L and Wt mice at 1 month (ANOSIM $r = -0.0184$, $p = 0.516$, Supplementary Figure 1b). By contrast, the P301L and Wt groups were obviously distinct after 3 months (Fig. 1d, ANOSIM $r = 0.6356$, $p < 0.001$, Supplementary Figure 1c and d), and the total explanation rate of PC1 and PC2 reached 56.61% at 3 months.

At the phylum level, no significant difference was observed in the gut microbiota at 1 month between P301L and Wt mice (Fig. 2a). After 3 months of age, the relative abundance of *Bacteroidetes* was increased, while *Firmicutes* was decreased in P301L mice compared with that in Wt mice (Fig. 2b-d). In addition, *Actinobacteria* was decreased in P301L mice at 3 and 6 months of age (Fig. 2b, c); meanwhile *Tenericutes* was decreased in P301L mice at 10 months of age (Fig. 2d). From the phylum to genus level, the statistically significant differences in microbiota between P301L and Wt mice at 3, 6, and 10 months of age are presented in Figures 3, 4, and 5, respectively. At 3 months of age, the relative abundance of *norank_f_Bacteroidales_24_7_group, Phascolarctobacterium, Parabacteroides*, and *unclassified_o_Bacteroidales* were increased in P301L mice, while a total of 22 bacteria, including *Lactobacillus, Enterorhabdus*, and *Staphylococcus were* significantly decreased in P301L mice compared with those in matched Wt littermates at the genus level. At 6 months of age, the relative abundance of *Ruminococcaceae_NK4A214_group, Bacteroides, Anaeroplasma*, and *norank_f_Clostridiales_vadinBB60_group* were increased in P301L mice, while *Staphylococcus, Bifidobacterium*, and *Gemella* were significantly decreased in P301L mice at the genus level. At 10 months of age, the relative abundance of *norank_f_Bacteroidales_24_7_group Anaerovorax, Peptococcus, unclassified_o_Bacteroidales, Caproiciproducens, Ruminiclostridium, norank_f_Ruminococcaceae, norank_f_Clostridiales_vadinBB60_group*, and *Oscillibacter* were increased in P301L mice, while *Roseburia, norank_f_Mycoplasmataceae, Lachnoclostridium, Klebsiella, Streptococcus*, and *unclassified_f_Coriobacteriaceae* were significantly decreased in P301L mice at the genus level.

Association between the gut microbiota and tau pathologies in the brain

To investigate whether gut microbiota are associated with tau pathologies in the brain of P301L mice, we used pT231 and AT8 to investigate the levels of different types of phosphorylated tau proteins. According to immunohistochemical assays, pT231-positive staining was observed as early as 1 month in p301L mice, while AT8-positive staining was observed until the animal reached 3 months (Supplementary Figure 3). The correlations among the abundance of microbiota and tau pathologies in the brain of P301L mice at different months of age were presented in Table 1. At the genus level, no statistically significant correlation was observed between gut microbiota and tau pathologies (pT231-tau) in mice at 1 month of age, while statistically significant correlations were observed between specific gut microbiota and tau pathology in mice at 3, 6, and 10 months of age. *Marvinbryantia* was negatively correlated with the abundance of pT231-tau in P301L mice at 3 months of age. For P301L mice at 6 months of age, *Lactobacillus* and *Desulfovibrio* were negatively correlated with the abundance of pT231-tau, meanwhile *Streptococcus*, *Anaerotruncus*, *Lactococcus*, *unclassified_f_Erysipelotrichaceae*, and *Eubacterium_brachy_group* were negatively correlated with the abundance of AT8-tau. For P301L mice at 10 months of age, *Candidatus_Saccharimonas*, Alistipes, *Rikenella*, *Odoribacter*, *Blautia*, *Ruminococcaceae_UCG_014*, *Eubacterium_xylanophilum_group*, *Paraprevotella*, *Butyricicoccus*, *Ruminiclostridium_6*, and *Parvibacter* were negatively correlated with the abundance of pT231-tau, meanwhile *Bacteroides*, *Parabacteroides*, *Escherichia-Shigella*, and *Clostridium_innocuum_group* were positively correlated with the abundance of pT231-tau. These results suggest that changes in bacterial communities may be associated with tau pathologies in the brain of P301L mice.

Predictive value of microbial markers for tauopathies

Based on the AUC-RF algorithm [16], the optimal model with the maximum area under the

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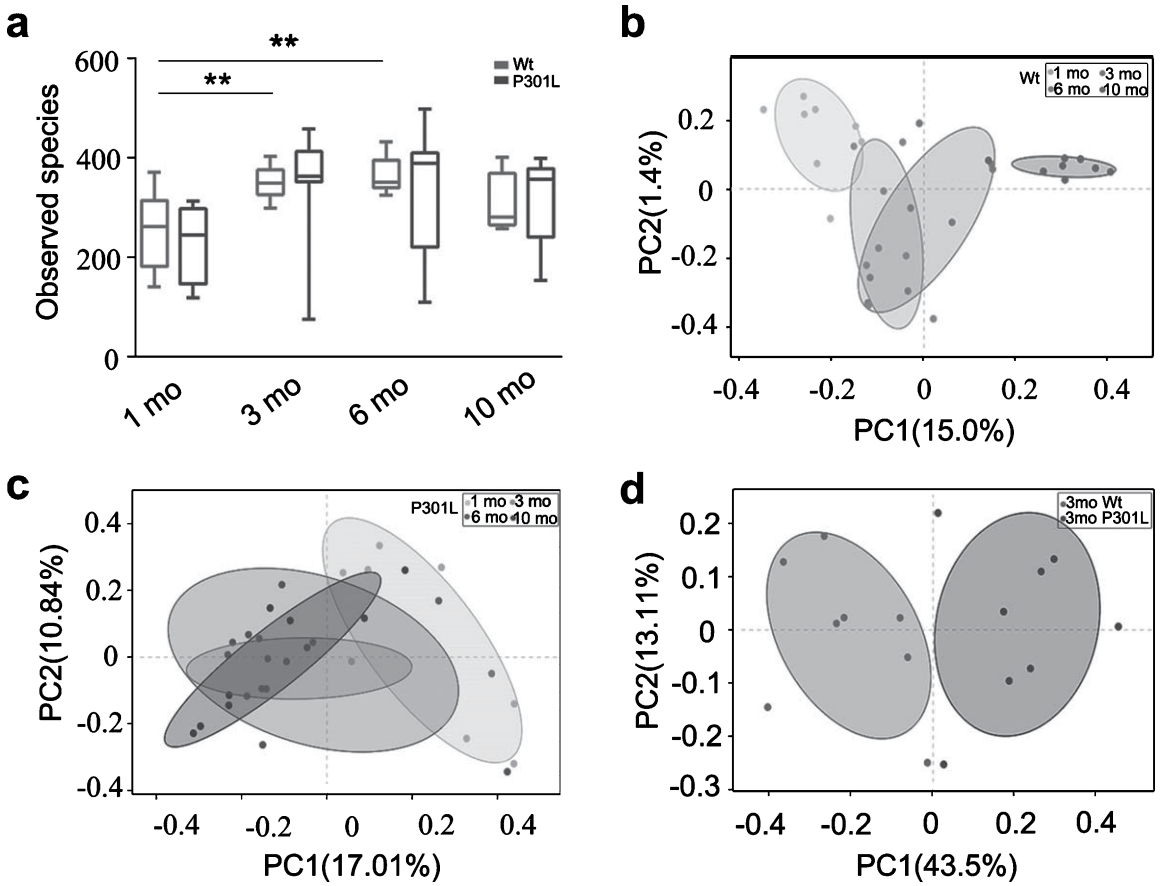


Fig. 1. Diversity of the gut microbiota between P301L and Wt mice. a) The Chao1 index displays the microbial diversity of each group. Box represent 5-95 percentile. Principal coordinate analysis (PCoA) for the gut microbiota composition in the Wt (b) and P301L groups (c) at 1, 3, 6, and 10 months of age. d) PCoA for the gut microbiota composition between P301L and Wt mice at 3 months of age. The first two principal components are plotted ($n = 8$ per group). One-way ANOVA and Turkey's test, $^{**}p < 0.01$.

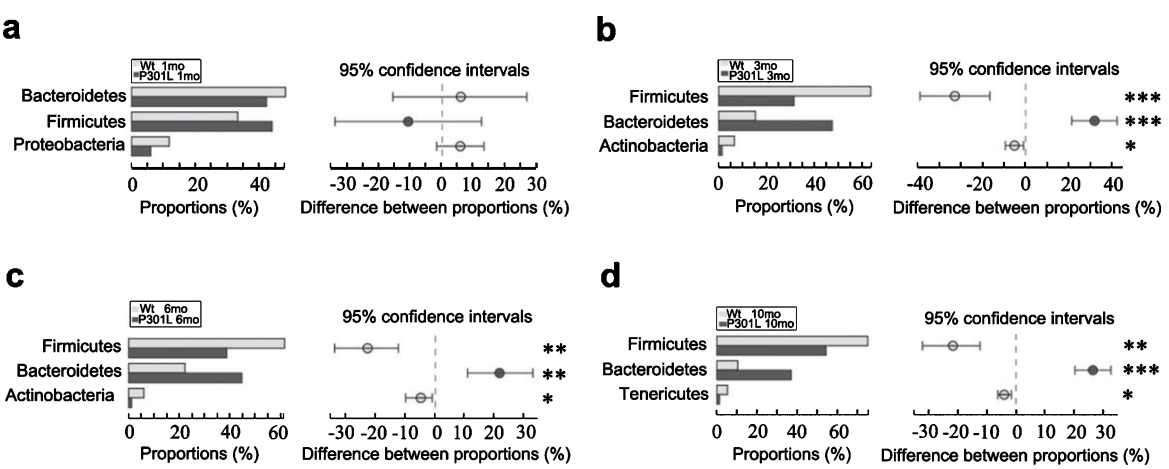


Fig. 2. Different bacterial communities between P301L and Wt mice at different months of age. a) Different bacterial communities on the phylum level at 1 month of age, (b) 3 months of age, (c) 6 months of age, and (d) 10 months of age. ($n = 8$ per group). Mann-Whitney U test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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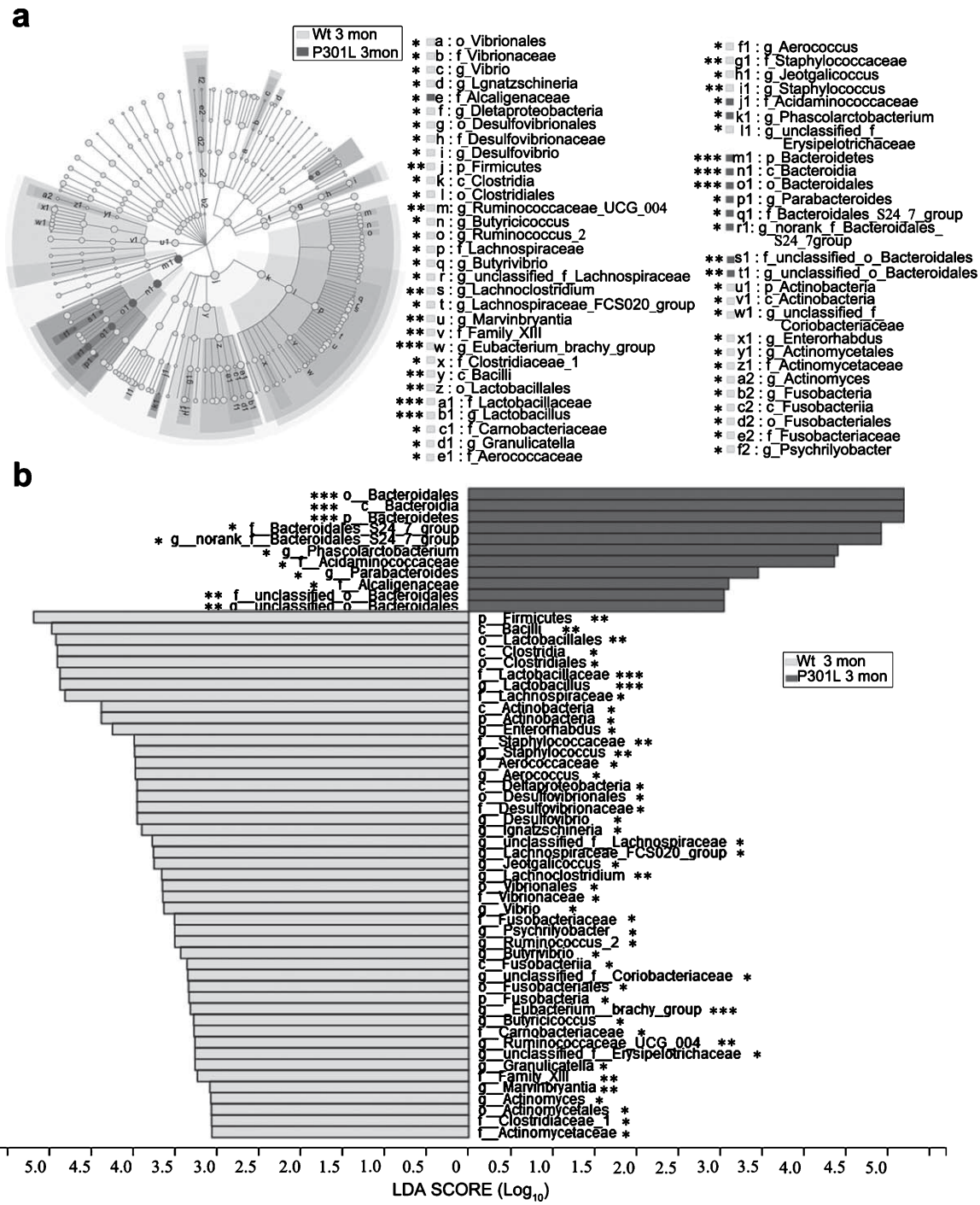


Fig. 3. Taxonomic differences of microbial communities between P301L and Wt mice at 3 months of age. a) LEfSe identified the most differentially abundant taxons between P301L and Wt mice from the phylum to genus level. (Blue) taxa enriched in P301L mice; (Red) taxa enriched in Wt mice; (Yellow) no significant difference in taxa between P301L and Wt mice. The size of each dot is proportional to its effect size. ($n = 8$ per group). b) P301L mouse-enriched taxa are indicated with a positive LDA score (blue), and taxa enriched in Wt mice are indicated with a negative score (red). The LDA score threshold is ≥ 2. ($n = 8$ per group), non-parametric factorial Kruskal-Wallis sum-rank test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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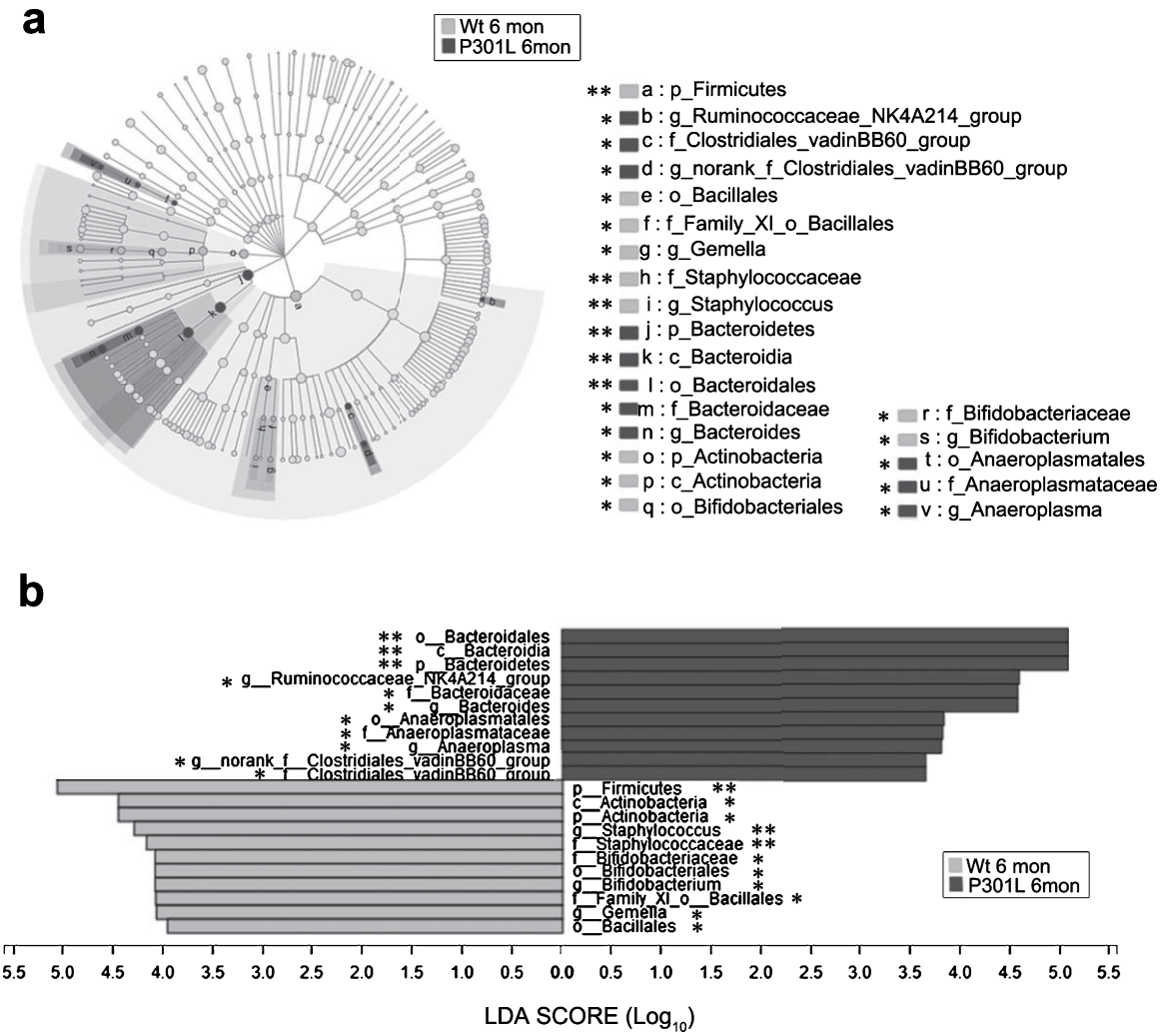


Fig. 4. Taxonomic differences of microbial communities between P301L and Wt mice at 6 months of age. a) LEfSe identified the most differentially abundant taxons between P301L and Wt mice from the phylum to genus level. (Blue) taxa enriched in P301L mice; (Red) taxa enriched in Wt mice; (Yellow) no significant difference in taxa between P301L and Wt mice. The size of each dot is proportional to its effect size. ($n = 8$ per group). b) P301L mouse-enriched taxa are indicated with a positive LDA score (blue), and taxa enriched in Wt mice are indicated with a negative score (red). The LDA score threshold is ≥ 2. ($n = 8$ per group), non-parametric factorial Kruskal-Wallis sum-rank test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ROC curve utilized 3 crucial bacterial populations (*Eubacterium_brachy_group, Staphylococcus*, *and Prevotellaceae_UCG_001*). For the whole cohort, the AUC for a single bacterium ranged from 0.641 to 0.781 (Supplementary Figure 4a), while for multi-biomarker analysis, the ROC curve for the model had an AUC of 0.835 (Supplementary Figure 4b). For the young cohort (1 month), the maximum AUC was 0.703 (Supplementary Figure 4c), while for the adult cohort (3–10 months), the AUC reached 0.872 (Supplementary Figure 4d).

DISCUSSION

In the present study, gut microbiota composition and diversity changed with aging. Moreover, significant differences were observed in gut microbiota composition between P301L tau transgenic mice and Wt littermates from 3 months of age onward. The relative abundance of *Bacteroidetes* was increased, while *Firmicutes* were decreased in P301L mice compared with that in Wt ones at 3, 6, and 10 months of age. In addition, *Actinobacteria* was decreased

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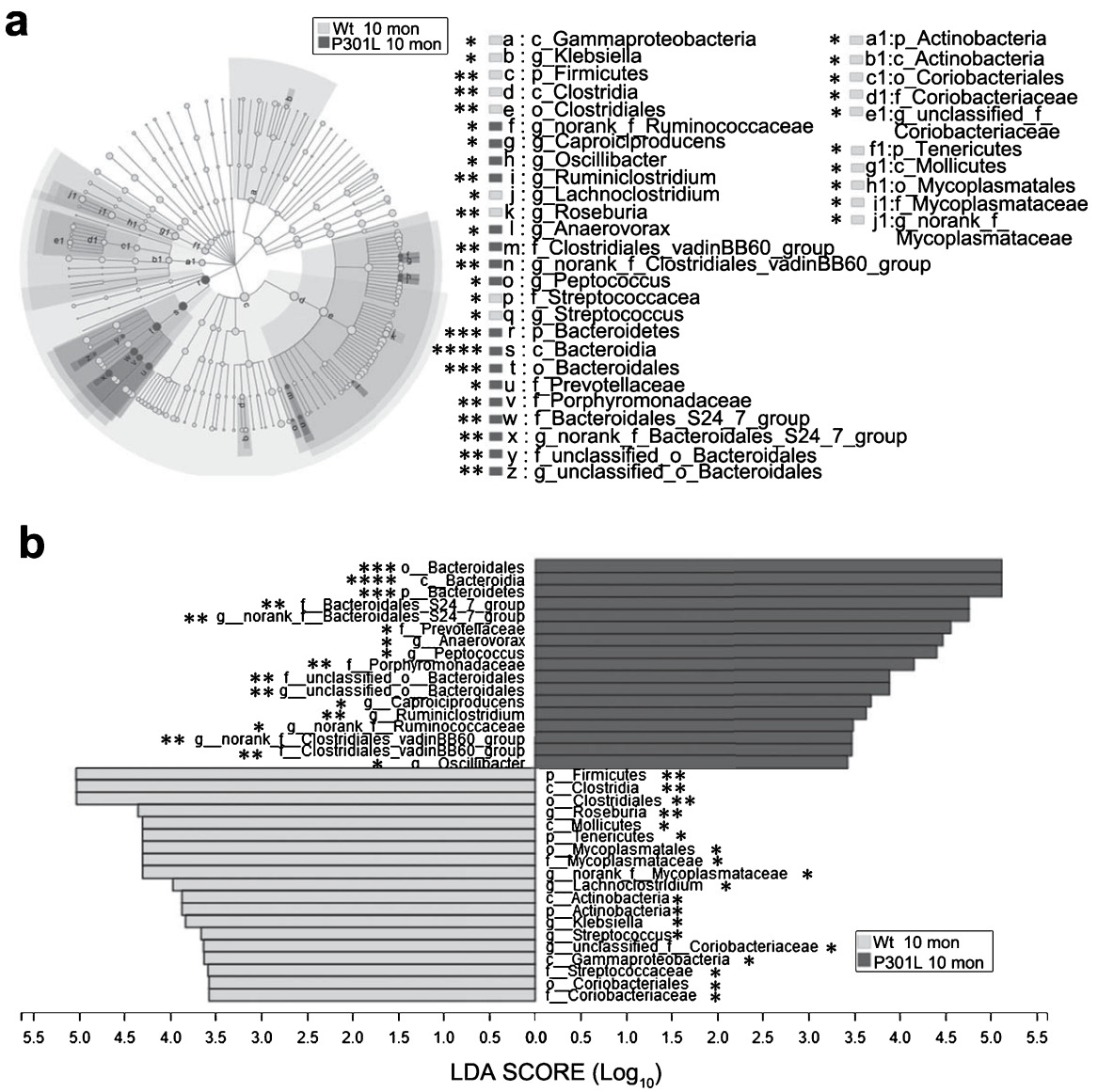


Fig. 5. Taxonomic differences of microbial communities between P301L and Wt mice at 10 months of age. a) LEfSe identified the most differentially abundant taxons between P301L and Wt mice from the phylum to genus level. (Blue) taxa enriched in P301L mice; (Red) taxa enriched in Wt mice; (Yellow) no significant difference in taxa between P301L and Wt mice. The size of each dot is proportional to its effect size. ($n = 8$ per group). b) P301L mouse-enriched taxa are indicated with a positive LDA score (blue), and taxa enriched in Wt mice are indicated with a negative score (red). The LDA score threshold is ≥ 2. ($n = 8$ per group), non-parametric factorial Kruskal-Wallis sum-rank test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

in P301L mice at 3 and 6 months of age, meanwhile *Tenericutes* was decreased in P301L mice at 10 months of age. Notably, several specific bacterial genera were highly correlated with the tau pathology in the brain of P301L mice at 3, 6, and 10 months of age.

Emerging evidence indicates a link between the gut microbiome and aging and age-related diseases, although correlations between specific patterns of the gut microbiota and specific disease entities are undisputed [9, 17]. Recent investigations indicate that neurodegeneration and cognitive disorders might directly interfere with the neuronal network of the gut-enteric nervous system [18, 19] or may even arise from the enteric nervous system via the microbiota-gut-brain axis [20], indicating the complexity of the bidirectional interaction network between the central nervous system and gut microbiota. Based on this

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Table 1
Correlations between the abundance of gut microbiota and tau pathologies in the brain of P301L mice at different months of age*

Age (months)	Microbiota	pT231		AT8	
		r	*q* value#	*r*	*q* value
3	Allprevotella	–0.714	0.103	–0.381	0.430
	norank_f_Mycoplasmataceae	–0.286	0.493	–0.738	0.102
	Enterorhabdus	–0.357	0.424	–0.833	0.110
	Blautia	–0.738	0.102	–0.643	0.135
	Mucispirillum	–0.381	0.430	–0.810	0.083
	Staphylococcus	–0.546	0.255	–0.764	0.099
	unclassified_f_Erysipelotrichaceae	–0.452	0.358	–0.738	0.081
	Marvinbryantia	–0.916	0.011	–0.506	0.276
	Caproiciproducens	–0.784	0.116	–0.316	0.491
	Eubacterium_nodatum_group	–0.786	0.077	–0.214	0.610
	Bilophila	–0.646	0.152	–0.729	0.073
6	Lactobacillus	–0.905	0.036	–0.143	0.736
	Helicobacter	–0.238	0.789	–0.714	0.071
	Desulfovibrio	–0.881	0.036	–0.405	0.384
	Enterorhabdus	–0.595	0.360	–0.786	0.054
	Odoribacter	–0.571	0.357	–0.762	0.056
	Streptococcus	–0.253	0.892	–0.831	0.040
	Anaerotruncus	–0.238	0.733	–0.905	0.036
	Mucispirillum	–0.810	0.068	–0.333	0.473
	Lactococcus	0.144	0.826	–0.838	0.041
	Ruminiclostridium_5	–0.833	0.060	–0.214	0.646
	Quinella	–0.252	0.822	–0.755	0.056
	unclassified_f_Erysipelotrichaceae	–0.024	0.955	–0.910	0.018
	Butyricicoccus	0.263	0.950	–0.719	0.074
	norank_f_Ruminococcaceae	–0.238	0.684	–0.714	0.065
	Eubacterium_brachy_group	–0.300	0.940	–0.901	0.012
	Ruminiclostridium_6	–0.342	0.918	–0.781	0.050
	Ruminococcaceae_UCG_004	0.096	0.869	–0.790	0.060
	Caproiciproducens	–0.778	0.083	–0.419	0.387
10	Bacteroides	0.786	0.027	0.405	0.363
	Candidatus_Saccharimonas	–0.970	0.017	–0.731	0.136
	Alistipes	–0.810	0.026	–0.571	0.263
	Parabacteroides	0.810	0.023	0.571	0.236
	Rikenella	–0.898	0.007	–0.347	0.400
	Odoribacter	–0.826	0.021	–0.515	0.272
	Blautia	–0.905	0.006	–0.595	0.255
	Ruminococcaceae_UCG_014	–0.738	0.042	–0.452	0.316
	Escherichia–Shigella	0.933	0.009	0.376	0.381
	Lactococcus	–0.415	0.307	–0.732	0.166
	Eubacterium_xylanophilum_group	–0.946	0.006	–0.683	0.176
	Paraprevotella	–0.898	0.005	–0.683	0.151
	Butyricicoccus	–0.790	0.028	–0.826	0.094
	Ruminiclostridium_6	–0.903	0.004	–0.854	0.119
	Clostridium_innocuum_group	0.764	0.033	0.483	0.294
	Parvibacter	–0.951	0.004	–0.551	0.243
	Ruminococcaceae_UCG_005	–0.452	0.277	–0.764	0.153

*r values : negative value, microbiota was negatively correlated with tau pathology; positive value, microbiota was positively correlated with tau pathology. #q value, adjusted by Benjamini – Hochberg false discovery rate.

study, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are the dominant components of the gut microbiota in mice, consistent with previous reports [17, 18]. Clear differences were observed in gut microbiota composition between mice of different ages, suggesting some parallel shifts during aging. Indeed, the gut bacterial composition and ecological network change with aging and is proposed to play a crucial role in basic neurogenerative processes and maintain normal functions at different life stages [21]. For term infants, most of the early gut microbiota colonizers are derived from the mother; the early gut microbiota is mainly affected by the mode of delivery and feeding patterns [22, 23] and then gradually matures into an adult-like structure until the age of 1 to 3 years old [23, 24]. Likewise, gut microbiota compositions

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in an AD mouse model were also proved similar to Wt mice at the young age (3 months old) and started to diverge with aging [25]. Similar to these previous studies, no significant difference was observed in the gut microbiota between the matched littermate mice at 1 month in this study, which might be due to the same delivery mode and feeding patterns.

Microtubule associated tau protein is abnormally phosphorylated in AD and aggregates as paired helical filament (PHF)-tau in NFTs. In mice expressing normal human tau isoforms (Htau), young Htau mice with early stages of tau pathology showed no cognitive deficits, while in old Htau mice, with tau accumulation progresses and the presence of NFTs, cognitive function also declines [26], suggesting highly association between PHF-tau and cognitive impairment. In AD patients, the burden of PHF-tau measured by [F-18]-T807-PET significantly elevated and correlated with the severity stages defined by Braak staging. Interestingly, in a small normal cohort, aged subjects also presented a slightly higher PHF-tau burden than that in younger subjects, suggesting that PHF-tau may be associated with aging and cognitive damage [27]. In this study, pT231-tau protein was observed at 1 month in P301L mice, which is earlier than that of the alteration in the gut microbiota. However, AT8-tau protein was observed at 3 months of age, which was consistent with a previous report [28].

Homeostasis between *Bacteroidetes* and *Firmicutes* reportedly changes with aging [29]. According to a previous report, the relative abundance of *Bacteroidetes* decreased in patients with AD [30]. In an AD mouse model (APP/PS1), the ratio of *Firmicutes* to *Bacteroidetes* dynamically changing with aging, although no significant difference was observed when compared with their Wt littermates [17]. However, in another AD mouse model (5xFAD), the amount of *Firmicutes* was increased while *Bacteroidetes* was decreased in comparison with the Wt littermates at the age of nine weeks [18]. Undoubtedly, these composition changes are due to various factors. In our study, *Bacteroidetes* were increased while *Firmicutes* were decreased in P301L mice compared with those in their matched littermates at 3, 6, and 10 months of age. This difference may be partly due to the tau transgenic background. In mThy1.2 promoter-driven P301L mice, NFTs mainly accumulate in the amygdala, which is involved in mediating the effects of emotion and stress on learning and memory [31], as well as increased exploratory behavior [32]. In our previous study, significantly increased total distance and average speed were confirmed in P301L mice [2]. In fact, exercise has a complex impact on gut microbiota diversity and abundance. In mice, exercise could significantly increase the percentage of *Bacteroidetes* and decrease *Firmicutes* through several metabolic pathways [33]. In this sense, these species-disparate findings may be partly due to the behavioral dysfunction in P301L mice caused by tau pathology in the brain, indicating that tau pathology in the brain may affect the homeostasis of the gut microbiota. One possible mechanism may be the intestinal dysfunction caused by the presence of tau in the gut, resulting in gut microbiota disorder ultimately. As we know, enteric nervous system (ENS) is modulated by central nervous system. In APP transgenic AD mice, ENS alterations in molecule and function have been proved. The ENS undergoes significant changes during the early onset of APP expression. Notably, the high amount of Aβ within the ENS leads to neuronal tissue losses and significant disturbances in the gut wall [34]. It is reported that certain tau species are present in peripheral tissues, including skin, liver, and sigmoid colon, but the significance of its presence is still unclear [35, 36]. Thus, pathological changes in the brain may affect gut microbiota by regulating ENS. In addition, amygdala appears to play a prominent role in the development of stress-related gastrointestinal disorders [37]. In this study, accumulation of the PHF-tau in the area of amygdala may cause intestinal dysfunction and eventually lead to alteration of gut microbiota. Another possible mechanism may be infections associated with tauopathies. In AD patients, tissue from the brain was proved to exist fungal infection [38]. The dysbiosis of fungal was also proved to be correlated with intestinal dysfunction [39], suggesting that the existence of brain infection may be an ignored mechanism underlying the dyshomeostasis of the gut microbiota.

Notably, increasing evidence proposed that gut microbiota may be involved in the pathogenesis of brain diseases, especially in neurodegenerative diseases and aging [40–42]. Germ-free AD mice showed a drastic reduction of Aβ pathology in the brain when compared to those with gut microbiota, and implantation of microbiota from conventionally-raised AD mice increased brain Aβ levels [43].The results suggested that gut microbiota may contribute to the development of AD. Moreover, the germ-free condition may be another influencing factor for the reduction of Aβ pathology in the brain. In our study, all mice were raised under specific pathogen-free conditions to minimize the influences of other factors

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on the results as much as possible. This may lead to a decrease of tau pathological level in the brain, and the influence of the cleanliness of the living environment to tauopathies should be further explored.

Recently, increasing interest has been focused on the phyla *Actinobacteria* because of its proven involvement in the modulation of gut homeostasis, inflammatory immune changes, autoimmune responses, metabolism, and the gut-brain axis [44]. Potent metabolic activity against pathogens, especially its role in systemic diseases as well as its possible therapeutic applications, has also been proposed. In this study, *Actinobacteria* was decreased in P301L mice at 3 and 6 months of age. In an AD mouse model, the relative abundance of *Actinobacteria* was also proved to be decreased with age [17]. *Bifidobacterium* is a crucial class of this phylum that can produce γ-aminobutyric acid (GABA) in the human intestine [45]. As mentioned earlier, GABA, the major inhibitory neurotransmitter in the human CNS, has been implicated in a number of tauopathies, including AD [46], FTLD [47], and PSP [48]. The levels of GABA were decreased in the brains of patients with AD, FTLD, and PSP compared to those in normal controls [48–51]. This dysfunction of the brain GABAergic system has also been associated with cognitive impairment [52]. In fact, GABA concentrations in the CNS and gastrointestinal tract are linked. Decreased amounts of enteric *Bifidobacterium* can decrease the levels of GABA in the gut, ultimately leading to decreased GABA in the CNS [46]. However, the number of *Bifidobacteria* substantially decreases after weaning and continues to decrease with age [53, 54]. Similar changes were observed in *Lactobacilli*, a pivotal component of the *Firmicutes* phylum; together with *Bifidobacteria*, these changes represent the probiotic therapeutic approach. In middle-aged rats, *Lactobacillus* and *Bifidobacterium* dietary supplementation could enhances robust improvements in long-term object recognition memory by altering brain metabolites [55]. In this study, all mice were raised under specific pathogen-free conditions with free feeding and drinking water. The food and water provided were sterilized. No significant differences were observed in body weight, food intake, and water intake between the two groups of mice, indicating that the differences in gut microbiota may not be caused by abnormal feeding behavior or body weight.

In the current study, *Lactobacillus* and *Bifidobacterium* were significantly decreased in P301L mice at 3 and 6 months of age, respectively. Moreover, *Lactobacillus* was negatively correlated with the pathological changes in the brain of P301L mice at 6 months of age. These reduced *Bifidobacterium* and *Lactobacilli* in the gut might contribute to tau pathogenesis and cognitive impairment, suggesting that dyshomeostasis in the gut microbiota may contribute to the progression of tauopathy. The neuronal degeneration caused by gene mutation may be a crucial reason for the reduction of *Bifidobacterium* and *Lactobacilli* mediated by ENS as discussed above. The dysfunction of amygdala caused by the abnormal aggregation of tau protein may be another ignored mechanism. In addition, tau pathology caused by gene mutation may also cause dietary dysfunction and lead to the reduction of *Bifidobacterium* and *Lactobacilli* ultimately.

Staphylococcus, a genus of the *Firmicutes* phylum, has traditionally been considered harmful to the health of the host. Interestingly, *Staphylococcus* is an early and enriched gut colonizer of infants, especially in those residing in high hygienic areas recently. Additionally, this high colonization of *Staphylococcus* was proposed to have a potential role in the development of the microbiota and immune system [56]. In our study, *Staphylococcus* was decreased in P301L mice at 3 and 6 months of age when compared with that in Wt mice. Thus, it is becoming apparent that the abundance and physiological function of *Staphylococcus* in the gut microbiota is probably more complex and diverse than previously thought.

In this study, most of the bacteria taxa, such as *Marvinbryantia*, *Lactobacillus, Streptococcus, unclassified_f_Erysipelotrichaceae*, and *Eubacterium_brachy_group* were negatively correlated with tau pathology in the brain, suggesting the decrease of these microbiota may be closely related to the severity of tauopathies. However, the mechanism needs to be further studied.

Tauopathies encompass different disorders that may manifest with various clinical syndromes. Differential diagnosis with other proteinopathies remains challenging. In this study, a significant difference was observed in the gut microbiota between P301L and Wt mice, suggesting that the alteration in the gut microbiota may have potential value in the differentiation and early prediction of tauopathies. When used as a single microbial marker to predict tauopathies, *Eubacterium_brachy_group* showed higher AUC in comparison with *Staphylococcus* and *Prevotellaceae_UCG_001*. However, combination of *Eubacterium_brachy_group*, *Staphylococcus*, and *Prevotellaceae_UCG_001* substantially represent

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the highest AUC for prediction of tauopathies among multiple markers. Early diagnosis and differentiation are of great clinical value for the prevention and treatment of tauopathies. For the young cohort at the early stage of tauopathies, combinations of *Eubacterium_brachy_groupaphylococcus* with *Prevotellaceae_UCG_001* represent the highest AUC for prediction of tauopathies. Although more human-based studies should be explored, the above results suggested that the alteration in the gut microbiota may have potential value in the differentiation and early prediction of tauopathies.

This study was based on a mouse model; further investigations based on human tauopathy should be conducted to confirm our findings. Nevertheless, by recruiting well-matching subjects and controlling the conditions, our study identified alterations in the gut microbiota composition in a tauopathy model that were highly associated with tau pathology in the brain, although the causality remains unclear. As genome-wide association studies have revealed that genetic locus variation and epigenetics are highly associated with both familial and sporadic tauopathy [57], our study still has important value for human sporadic tauopathy research. Additionally, modulation of the gut microbiota may be a potential strategy for tauopathy treatment.

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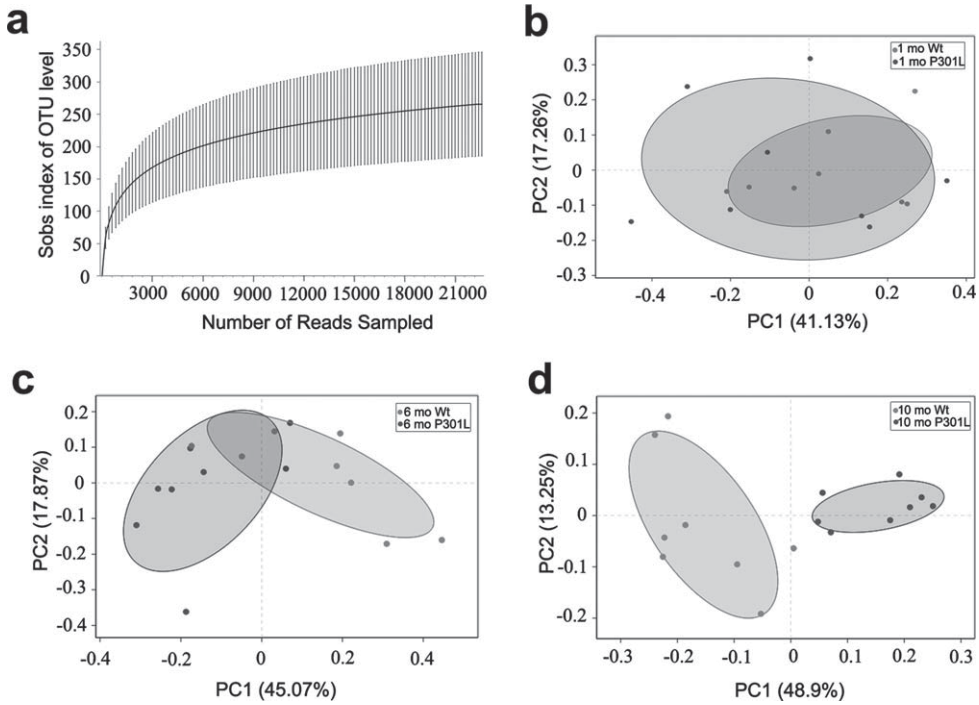
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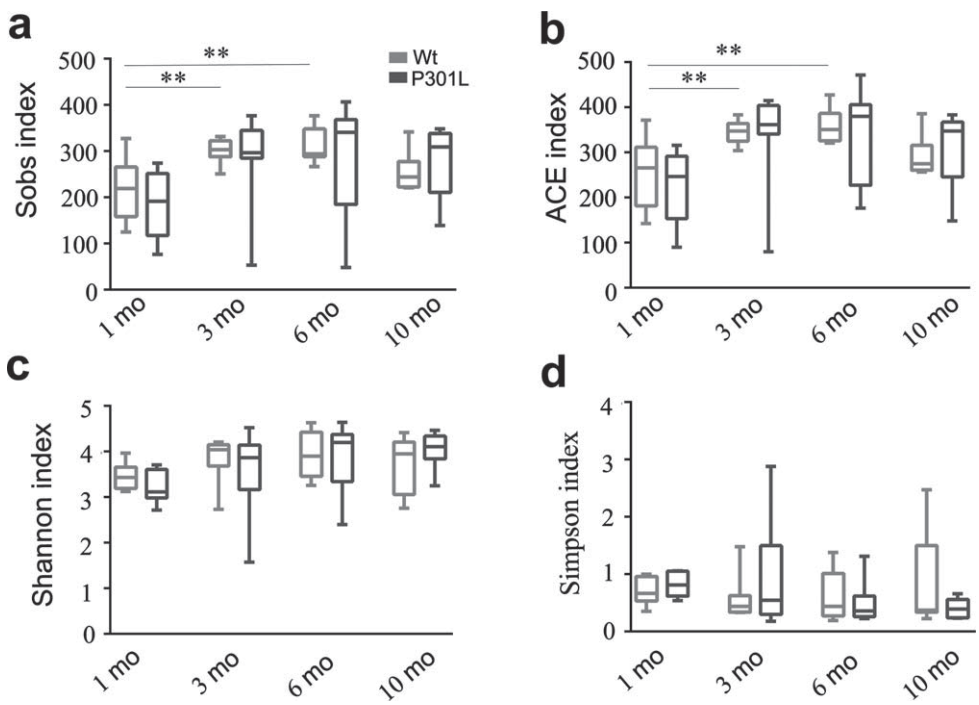
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SUPPLEMENTARY MATERIAL

Gut Microbiota Alteration and Its Time Course in a Tauopathy Mouse Model

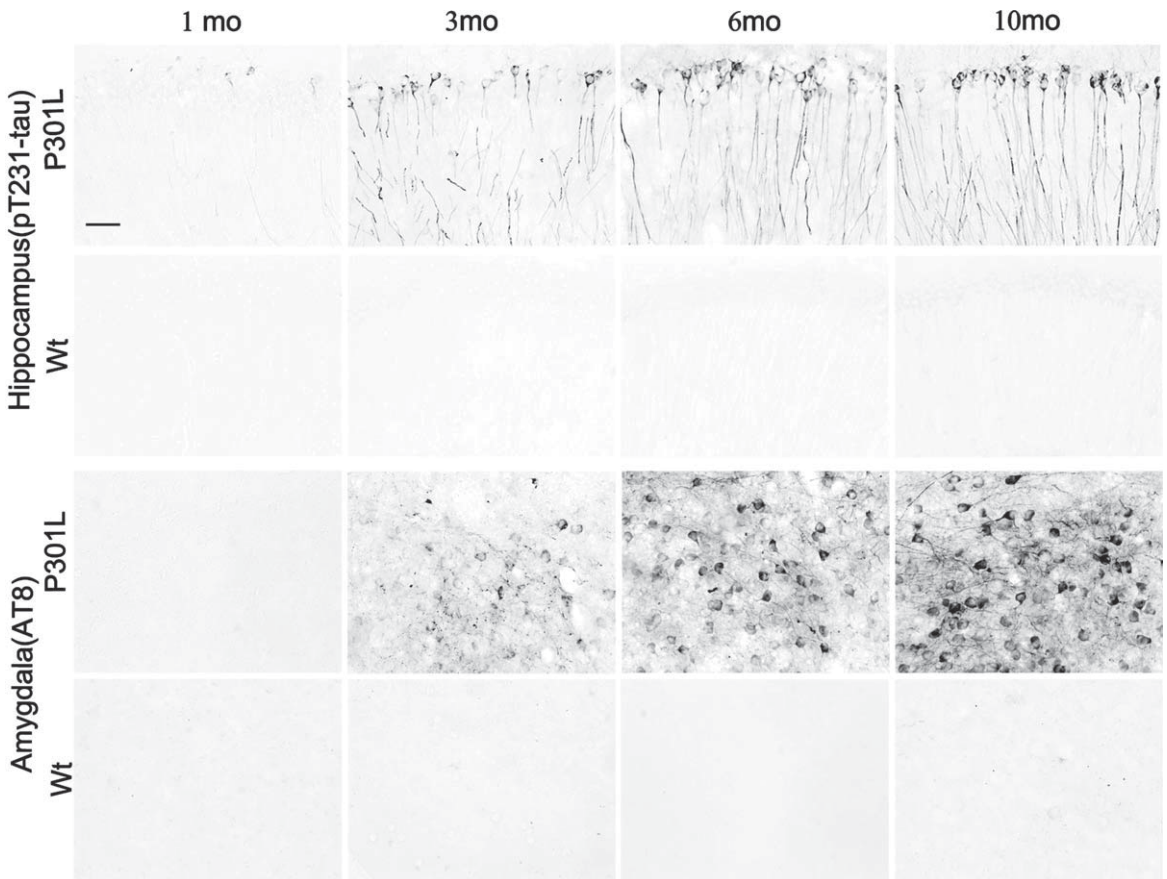


Supplementary Figure 1. Diversity and richness of the gut microbiota in mice. a) The Sobs index curve (smooth tendency) indicates a near-complete sampling of the community. b-d) PCoA for the gut microbiota composition between P301 L and Wt mice at 1, 6, and 10 months of age, respectively. The first two principal components are plotted.



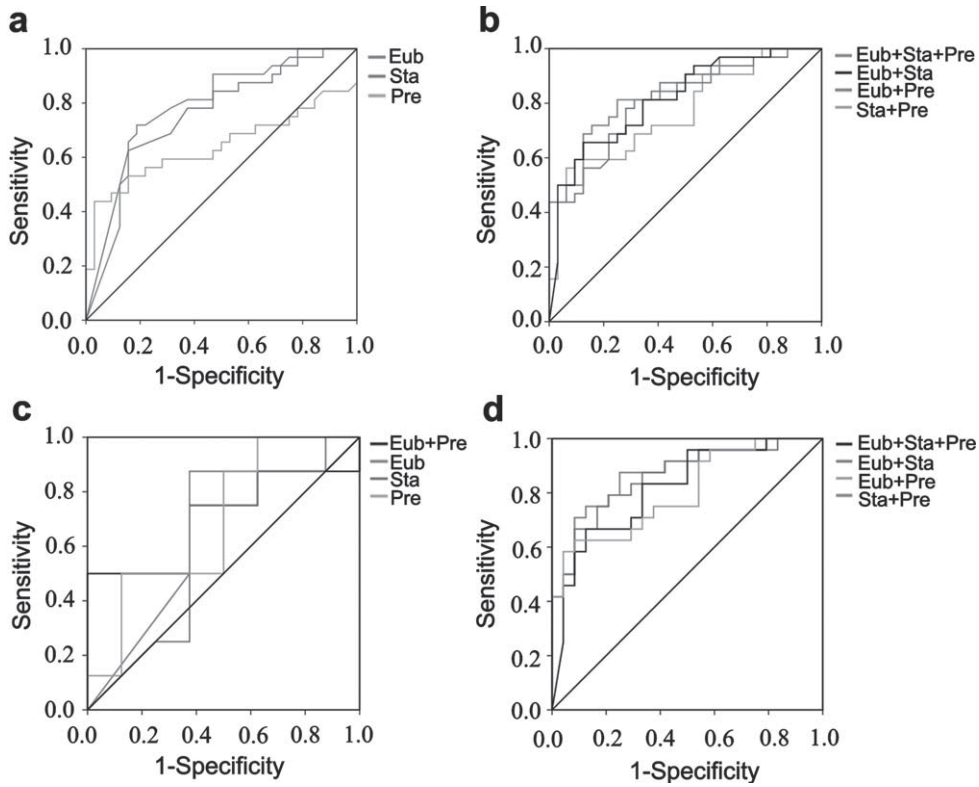
Supplementary Figure 2. Alpha diversity between P301L and Wt mice at different ages. a-d) Sobs index, ACE index, Shannon index and Simpson index between P301L and Wt mice at 1, 3, 6, and 10 months of age, respectively. $n = 8$ per group, $^{**}p < 0.01$.

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Supplementary Figure 3. Immunohistochemical staining of tau pathologies in the brain. Representative images of pT231 and AT8 immunohistochemical staining in mice at different months of age. Scale bars 50 μm.

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Supplementary Figure 4. The predictive value of microbial markers for tauopathies. Receiver operating characteristic curve (ROC) for predictive of tauopathies with a specific bacterial population utilized on the AUC-RF algorithm. (a) and (b) show the ROC for a single bacterium and multi-biomarkers in the whole cohort, respectively. (c) and (d) show the ROC in the young cohort (1 month) and adult cohort (3-10 months), respectively. Eub, Eubacterium_brachy_group; Sta, Staphylococcus; Pre, Prevotellaceae_UCG_001.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Bioactive Food Abates Metabolic and Synaptic Alterations by Modulation of Gut Microbiota in a Mouse Model of Alzheimer's Disease

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Abstract. Recent investigations have demonstrated an important role of gut microbiota (GM) in the pathogenesis of Alzheimer's disease (AD). GM modulates a host's health and disease by production of several substances, including lipopolysaccharides (LPS) and short-chain fatty acids (SCFAs), among others. Diet can modify the composition and diversity of GM, and ingestion of a healthy diet has been suggested to lower the risk to develop AD. We have previously shown that bioactive food (BF) ingestion can abate neuroinflammation and oxidative stress and improve cognition in obese rats, effects associated with GM composition. Therefore, BF can impact the gut-brain axis and improved behavior. In this study, we aim to explore if inclusion of BF in the diet may impact central pathological markers of AD by modulation of the GM. Triple transgenic 3xTg-AD (TG) female mice were fed a combination of dried nopal, soy, chia oil, and turmeric for 7 months. We found that BF ingestion improved cognition and reduced Aβ aggregates and tau hyperphosphorylation. In addition, BF decreased MDA levels, astrocyte and microglial activation, PSD-95, synaptophysin, GluR1 and ARC protein levels in TG mice. Furthermore, TG mice fed BF showed increased levels of pGSK-3β. GM analysis revealed that pro-inflammatory bacteria were more abundant in TG mice compared to wild-type, while BF ingestion was able to restore the GM's composition, LPS, and propionate levels to control values. Therefore, the neuroprotective effects of BF may be mediated, in part, by modulation of GM and the release of neurotoxic substances that alter brain function.

Keywords: Lipopolysaccharide, microbiota, neuroinflammation, pro-inflammatory bacteria, propionate, sirt1

INTRODUCTION

Alzheimer's disease (AD) is a degenerative brain disease and the most common cause of dementia. In AD, aggregation of amyloid-β (Aβ) protein and hyperphosphorylation of tau represents the major

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pathological hallmarks [1, 2]. On the one hand, misfolded and aggregated proteins are recognized by astroglia and microglia cells and trigger an innate immune response, characterized by the release of inflammatory cytokines and neuroinflammation, which contribute to disease progression and severity [3, 4]. On the other hand, it has been postulated that soluble Aβ may cause neuronal membrane damage, producing reactive oxygen and nitrogen species [5]. This oxidative damage would alter synaptic membrane structure, causing alterations in dendritic spines with a subsequent cognitive decline [6]. In addition, data obtained from clinical and preclinical AD studies have observed the presence of non-convulsive epileptic patterns, as well as cerebral hyperactivity long before amyloid plaques are detected [7–10]. Thus, AD pathogenesis is a multifactorial disease that conveys different mechanistic pathways. This may explain the fact that currently, there are no effective pharmacological therapies to cure or stop the progression of this devastating disease.

Gut microbiota (GM) consists of all the microorganisms living in our intestine and its composition is associated with host-health and disease [11, 12]. GM dysbiosis is functionally linked to host immune system. Lipopolysaccharides (LPS), components of the outer membrane of gram-negative bacteria [13], are increased in dysbiosis [14, 15] along with systemic [16] and central [15] inflammation. LPS administration to mice results in memory impairment, Aβ aggregation, and astrocyte activation [17]. Notably, LPS can be detected in parenchyma and blood vessels of non-demented aged and AD brain samples, but LPS levels were greater in diseased subjects [18–20]. Moreover, recent reports indicate that AD patients [21, 22] and transgenic (TG) mice [23–25] present different GM composition compared to controls.

The composition of GM depends on various factors, diet being one of its main modulators [26]. It is particularly interesting to note that some risk factors linked to the development of AD are ingestion of high-fat diets [27–29], metabolic disorders [30], and systemic infections [31–34], all related to alterations of GM composition. On the contrary, ingestion of a healthy diet can decrease the risk of developing dementia and brain atrophy [35–39]. Moreover, direct administration of probiotics in TG mice decreases amyloid aggregation and neuroinflammation [23], while improving cognitive functions in AD patients [40].

Recent studies illustrated the role of GM in amyloid aggregation, as GM depletion by a germ-free condition [41], or by antibiotic treatment [42, 43] reduced the amyloid burden and microglia activation in TG mice. However, it is unclear which factors may be linked to the gut-brain axis in AD. Short-chain fatty acids (SCFAs) are produced by the GM after the degradation of non-digestible polysaccharides, with butyrate, acetate, and propionate the most abundant fermentation products [44]. TG mice present different concentrations of SCFAs in feces [23, 25], and in brain tissue [25] compared to control mice, while probiotics administration to TG mice improves cognitive performance, restores SCFAs concentration in feces, and reduced pro-inflammatory cytokines in plasma [23]. We have previously demonstrated that the ingestion of bioactive food (BF), food that besides its nutritional value offers additional benefits against a disease condition [45], was able to modify GM, restoring glucose levels, oxidative stress in liver and brain, reducing LPS plasma levels, neuroinflammation, and causing improved cognitive abilities in obese rats [14, 15]. In this study, we aimed to elucidated whether diet-induced GM modifications in TG mice can impact not only the amyloid aggregation, but also synaptic and metabolic alterations, both events observed in early stages of AD. We found two bacterial associated-products that can be related to AD pathogenesis in 9-month-old female TG mice, and we describe the positive effect of BF ingestion on several pathological markers of AD.

MATERIALS AND METHODS

Experimental design

Female 3 × Tg-AD transgenic mouse (TG) (RRID: MMRRC_034830-JAX) harboring the APPSWE and TauP301L transgenes on a PS1M146V knock-in background (homozygous mutant APPSWE, PS1M146V, and TauP301L), and female wild-type (WT) B6129SF1/J (RRID:IMSR JAX:101043) from same genetic background as PS1M146V knock-in mice, but harboring the endogenous wild-type mouse PS1) (both Jackson Laboratory, Bar Harbor, ME, USA) were used for the study. All mice were housed with access to food (Purina RodentChow5001) and water *ad libitum*, and under optimal vivarium conditions (12 h/12 h light–dark cycle, 20°C, and 40–50% relative humidity). Animal management was supervised by a licensed veterinarian in accordance with the principles set forth in the NIH guide for the care

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and use of laboratory animals, and was approved by the Bioethics Committee of the Instituto de Neurobiología, UNAM. This work was carried out in accordance with the EU Directive 2010/63/EU for animal experiments and the ICMJE Uniform Requirements for manuscripts submitted to biomedical journals. Once animals reached two months of age, they were housed individually and feed with two types of diet: 1) **Control diet** (TG-AIN, $n = 7$; WT-AIN, $n = 9$), prepared according to the American Institute of Nutrition recommendations [46]; 2) **Bioactive Food diet** (TG-BF, $n = 10$), with similar composition as AIN-93 diet, except that cellulose was exchanged by dried nopal (5%), casein by soya protein (19.4%), soy oil by chia seed oil (9%), adding 0.1% turmeric (Table 1). Both diets offer equal nutritional requirements and same calories/g (Table 1). Diets were administered in a dry form (5 g/day, per animal) during 7 months. On average, animals ate both diets without any preference, with an average of 2.2 g/day. Body weight and food intake was monitored over the course of the protocol.

Cognitive assessment

Three days before finalizing dietary intervention, behavioral testing was performed. Spatial and short-term working memory was evaluated by the T-maze [47]. It is an ideal test to examine differences in the cognitive outcome of behavioral tasks without stress components (i.e., water maze) even in early stages of AD pathology [48]. This is an appropriate test to evaluate hippocampal and prefrontal cortex (PFC) functions [49], both regions heavily affected in AD. In order to habituate the animals for the behavioral test, animals were placed in the behavioral room during 1 h, one day before testing. When animals are faced with T-maze apparatus, they display a tendency to alternate into the maze arm that was previously ignored. Briefly, a T-shaped maze Plexiglas apparatus with starting arm ($8.5 \times 10.5 \times 33.0$ cm), and the two choice arms ($8.5 \times 10.5 \times 30.0$ cm) was used as previously described [47]. T-maze consisted of two phases: 1) Sample phase: animals were placed at the start arm, and allowed to choose between one of the two choice arms. A central divider was inserted into the start arm to create a start box. Once animals have chosen an arm, they were confined there for 30 s, thereafter the door was reopened and allow mice to return to the start arm. Animals were finally removed and returned to their cage. 2) Choice phase: Two minutes later, animals were placed again in the start arm, but without the central divider, and allowed them to choose an arm. If the animal chooses two different arms in both phases, this is counted as spontaneous alteration. Two hours later the procedure was repeated. Three trials were given on each day, during two days, with a total of 6 trials per animal. Percentages of spontaneous alternations were calculated from total of correct arm entries. Experiments were recorded with a Sony DCR TRV280 camera, which was connected to a computer equipped with in-house develop software to track animal's trajectory.

Tissue preparation

One day after behavioral testing mice were sacrificed by cervical decapitation, plasma and brain were immediately collected for further analysis. Brain was divided into two halves. Right hemisphere was dissected out and PFC and cortex (CX) were separated. Tissues were snap frozen in liquid nitrogen and stored at –70°C until processing for western blot and SCFAs analysis. Left hemisphere was post fixed in 4% paraformaldehyde for 48 h at 4°C for immunohistochemistry and immunofluorescence analysis. For this, brain tissue was cryoprotected by immersion in 30% sucrose/PBS for 4 days. Coronal brain slices (40 μm thickness, from Bregma –2.18 mm to Bregma –2.54 mm) were obtained with a sliding microtome (Leica Jung histoslide 2000 R). All sections were immersed in cryoprotectant solutions for light microscopy and immunofluorescence, as described previously [50], and stored at –20°C until use.

Immunohistochemistry

Detection of hyperphosphorylated tau was achieved by use of AT100 antibody (1:250), and Iba-1 antibody (1:100) for microglia detection. Briefly, sections were thoroughly washed with PBS, and permeabilized with 0.2% Triton in PBS (0.2% PBS-triton) for 20 min. Endogenous peroxidases were inhibited by incubation in 0.3% H_2O_2 in PBS for 10 min. Tissue sections were washed three times for 10 min each, in 0.2% PBS-triton. Non-specific binding was blocked by incubation in 5% bovine serum albumin (BSA, Sigma) in PBS for 5 min. Subsequently, sections were incubated with primary antibodies, all diluted in 0.2% PBS-triton, washed and incubated with respectively secondary peroxidase-conjugated antibodies (Table 2) in 0.2% PBS-triton for 2 h at room temperature. Hydrogen peroxide (0.01%) and 3,30-diaminobenzidine (DAB)

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Table 1
Composition of experimental diets (g/kg diet)

CONTROL (AIN-93)		BIOACTIVE FOOD (BF)	
Ingredients	g/kg	Ingredients	g/kg
Cornstarch	397.486	Cornstarch	373.30
Casein	200	Soy protein	194
Maltodextrin	132	Maltodextrin	132
Sucrose	100	Sucrose	100
Soy oil	70	Chia seed oil	90
Cellulose	50	Nopal	50
Mineral mix	35	Mineral mix	35
Vitamin mix	10	Vitamin mix	10
L-Cysteine	3	L-Cysteine	3
Choline	2.5	Choline	2.5
TBHQ	0.014	TBHQ	0.014
		Turmeric	1
Energy (Kcal/g)	3.9		3.9
Anti-oxidant activity (Equivalent Trolox: μm/g of sample)	166.4 ± 34.61		$1540 \pm 110.2^{***}$

The data for anti-oxidant activity was analyzed by an unpaired *t*-test. Data represents mean ± S.EM. $^{***}p < 0.001$.

(0.06%) in 0.2% PBS-triton was used to develop the horseradish peroxidase enzymatic reaction. The enzymatic reaction was stopped with 0.2% PBS-triton and then sections were mounted on glass slides and left to dry overnight. Dry sections were cover slipped with mounting medium Entellan (Merck).

To evaluate the presence of amyloid aggregates, we used BAM-10 antibody that recognized $A\beta_{1-40}$ peptide. Briefly, coronal sections were washed three times in PBS during 5 min, then incubated in formic acid (89.8%) for 10 min, followed by three times washing in PBS for 5 min each. Sections were incubated in solution of H_2O_2 (30%)+Methanol (10%) for 30 min followed by washing in PBS and then incubated in 0.1% PBS-tween for 15 min, followed by incubation in 5% goat serum (Vector) in PBS-triton for 30 min. Thereafter, sections were incubated with BAM-10 (1:1500) in 5% goat serum in PBS-triton for 18 h at 4°C. After washing in PBS, sections were incubated with secondary antibody (Table 2) in 0.2% PBS-triton for 2 h at room temperature. Subsequently, sections were washed and incubated with the avidin-biotin complex (ABC Kit; Vector Laboratories) in 0.2% PBS-triton for 2 h according to the manufacturer's instructions. Finally, antibody binding was visualized with the chromogen DAB (DAB Peroxidase Substrate Kit; Vector Laboratories) 0.025%, with 0.01% H_2O_2 as a catalytic agent. Sections were washed with PBS, dehydrated by serial dilution in ethanol, and mounted with Eukit (Fluka). Control sections for all antibodies were processed without the primary antibody.

Immunofluorescence

For immunofluorescence staining, sections were washed in PBS and pre-treated with citrate buffer 20X (Sigma), at 94°C for 10 min, and then permeabilized with 0.2% PBS-triton for 20 min. Thereafter, sections were treated with 5% BSA for 5 min, and incubated with primary antibodies (Table 2): anti-Glial Fibrillary Acidic Protein (GFAP, 1:500), anti-Sirtun-1 (SIRT1, 1:500) (Table 2), all diluted in 5% horse serum (Vector Laboratories, S-2000) during 48 h at 4°C. Then, tissue was washed with 0.2% PBS-triton, and incubated with respective secondary antibodies (Table 2) in 0.2% PBS-triton. All sections were co-incubated with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen) in 0.2% PBS-triton for 30 min, washed and mounted on glass slides. They were cover slipped with mounting medium Vecta shield (Vector Laboratories). Control sections were processed without the primary antibody.

Image acquisition and analysis

Leica DMI6000 inverse microscope was used to acquire bright-field images under 40xobjective for BAM-10, AT100, and iba-1. For fluorescent labeling, a laser scanning microscope (Leica TCS-SP8) with

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Table 2
List of Primary antibodies and secondary antibodies used in the current study

Antibody	Host/Isotype	Brand	Catalog #	RRID
Primary antibodies				
Anti p-AMPK	Rabbit/IgG	Santacruz biotechology	sc-33524	AB_2169714
AMPK	Rabbit/ IgG	Santacruz biotechology	sc-74461	AB_1118940
ARC	Mouse/IgG	Santacruz biotechology	sc-17839	AB_626696
AT100	Mouse/IgG	Jackson ImmunoRes	MN1060	AB_223652
BAM-10	Mouse/IgG	Sigma	A3981	AB_1078153
FFAR 3	Rabbit/ IgG	My biosource	MBS7003084	AB_2732033
GFAP	Mouse/IgG	Cell Signaling	3670	AB_561049
GLUR1	Rabbit/ IgG	GeneTex	GTX 61101	AB_10619776
p-Gsk3-β	Rabbit/IgG	Cell signalling	9331	AB_329830
Gsk3-β	Rabbit/IgG	Santacruz biotechology	sc-9166	AB_647604
Human Tau	Mouse/IgG	Thermo scientific	MN1000	AB_2314654
Anti-Iba-	Rabbit/	Wako Chemicals	019–19741	AB_839504
1MDA	IgGRabbit/IgG	Abcam	AB6463	AB_305484
PSD 95	Mouse/IgG	Thermo scientific	MA1-046	AB_2092361
SIRT1	Rabbit/IgG	Santacruz	sc -15404	AB_2188346
Synaptophysin	Rabbit/IgG	Abcam	ab32127	AB_2286949
Tau 231/AT180	Mouse/IgG	Thermo scientific	MN1040	AB_223649
Secondary antibodies				
Peroxidase-conjugated anti-mouse	Goat/IgG	Jackson ImmunoRes	115–035–146	AB_2307392
Peroxidase-conjugated anti-rabbit	Goat/IgG	Jackson ImmunoRes	111–035–144	AB_2307391
Biotinylated anti-rabbit	Horse/IgG	Vector Laboratories	BA-1100	AB_2336201
Cy3-conjugated anti-rabbit	Donkey/ IgG	Jackson ImmunoRes	711–165–152	AB_2307443
Cy5-conjugated anti-mouse	Goat/ IgG	Jackson ImmunoRes	115–175–166	AB_2338714
ALEXA488-conjugated anti-mouse	Goat/IgG	Jackson ImmunoRes	115–545–166	AB_2338852
ALEXA488-conjugated anti-mouse	Donkey/IgG	Jackson ImmunoRes	715–545–150	AB_2340846
ALEXA647-conjugated anti-goat	Donkey/IgG	Jackson ImmunoRes	705–605–147	AB_2340437

argon (488 nm), and helium/neon (543 nm) lasers and with optimized pinhole diameter was used. For GFAP and SIRT1, we used a 20X objective (SIRT zoom 4), whereas for double labeling of SIRT1 and GFAP, a 40X objective was used. Images were obtained by using system optimized z-stacks parameter (10 stacks, each stack 1.385 μm) in Z axis, projected and analyzed in a two-dimensional plane. Stacks were superimposed as a single image by using the Leica LAS AF 2.6.0 build 7268 software.

Iba-1 + cells located in the *striatum (st.) radiatum* from CA1 hippocampal region were quantified based on their morphological characteristics, according to previous reports [50–52]. Microglia cells were classified as: inactive (displaying a slight ramified morphology and small rounded soma), or active (hypertrophic soma and with extensively thick and branched processes). Number of astrocytes labeled with GFAP were quantified in *st. radiatum, st. oriens* of CA1 hippocampal region, and layers I-V from entorhinal cortex (Ent. cx). We have differentiated in sub-areas of CA1 region because previous reports indicate *stratum*-specific alterations in hippocampus of TG mice [53].

Brightfield images were acquired at the same exposure light, and tissue background staining was subtracted from optical density values from fiber tracts within the same section, e.g., corpus callosum [53]. Threshold was determined by identifying best signal-to-noise ratio from at least 4–5 images, and averaging threshold values from those images. All images were analyzed with that averaged threshold [54]. Immunoreactive signal was evaluated by using the plugin "*measure*" in Image J 1.49v (NIH, open access) as previously reported [50].

Immunoreactivity against BAM-10 showed scarce amyloid aggregates (i.e., plaques) in subiculum (Fig. 2). BAM-10 staining was mainly as intracellular aggregates (Fig. 2). Thus, images obtained from subiculum were used to quantify number of plaques, while *st. pyramidale* of CA1 region of the hippocampus, and Ent. cx (layers III–V) were used for quantification of intracellular aggregates.

For BAM-10 and AT100, percentage of area stained was calculated in a determined area (BAM-10:0.00614357142 mm^2 in CA1, and 0.0933945 mm^2 in cortex; AT100:0.001314 mm^2). The percentage of area stained was calculated as the sum of areas with aggregates divided by the total area analyzed, and multiplied by 100.

For quantification of microglia cells and astrocytes, the total number of iba1 + and GFAP + cells

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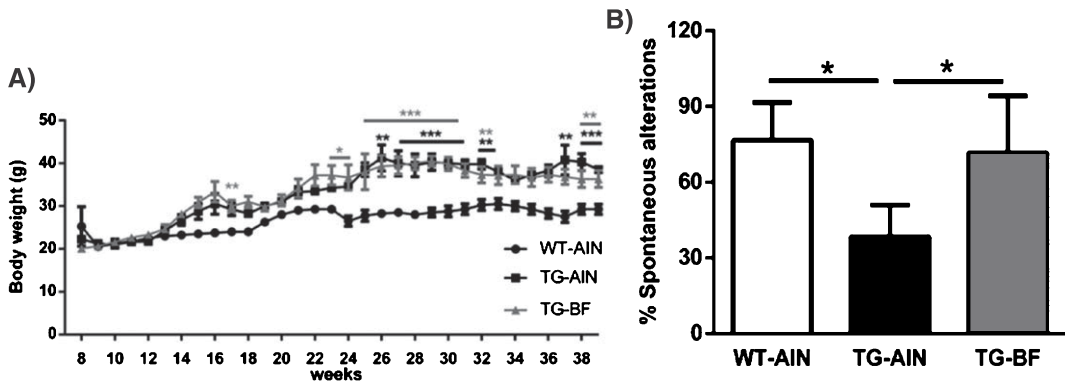


Fig. 1. Effect of bioactive food on body weight and spatial memory. A) Body weight (g) in TG-AIN, TG-AIN, and TG-BF mice. TG mice gained more body weight compared to WT-AIN mice starting from the 17th experimental week (black asterisk: WT-AIN versus TG-AIN; grey asterisk: WT-AIN versus TG-BF). At the end of experiment, WT-AIN mice gained less body weight (9.66 ± 0.66 g), compared to TG-AIN (16.25 ± 1.03 g) ($p < 0.001$) and TG-BF mice (16.16 ± 2.71 g) ($p < 0.01$), but there were no differences in final weight gain among TG mice. B) Working memory and spatial learning. Spontaneous alterations were recorded during 2 days-test on the T-maze. TG-AIN mice alternated less than WT-AIN mice ($p > 0.05$), while TG-BF showed same values as control mice ($p < 0.05$ versus TG-AIN). Data in A are expressed as the mean ± S.E.M., in B as mean ± S.D. WT-AIN, $n = 9$; TG-AIN, $n = 7$; TG-BF, $n = 10$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

were measured as number of cells / number of images × single image area (Iba1:0.089355 mm^2; GFAP: 0.0338578125 mm^2).

SIRT1 immunofluorescence was evaluated in hippocampal region. We used the plug-in "*Analyse particles*" of Image J 1.49v (NIH, open access) adjusting size and circularity of particles immunereactive to SIRT1. We summed the total number of particles quantified per area (*st. radiatum*: SIRT; 0.00185165 mm^2; *st. oriens*: SIRT:0.0015mm^2) in at least 3 images per animal. For detection of SIRT1 in GFAP+ cells, we first located SIRT1 immunoreactivity in the nucleus of GFAP + cells labeled with DAPI in *st. radiatum* of CA1 hippocampal region. Then, SIRT1 signal intensity was analyzed by Image J plugin "*measure*" only in the nucleus, as described above. In all staining, one image (with 20x objective) or two images (with 40x objective) per slice, 3 slices per animal, and at least 5 animals per group were analyzed, yielding 5 values for statistical analysis.

Western blot

Cortex was homogenized in RIPA buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0) (10% weight/volume). Homogenates were centrifuged at 13000 rpm for 15 min at 4°C. The purified total protein was quantified by the Lowry assay (Bio-Rad DC Protein Assay Kit) method. Samples (40 μg proteins) were separated on SDS-PAGE (10–12%) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% blotting grade nonfat dry milk (Bio-Rad, Hercules CA, USA) or with 1% casein (C3400, Sigma) for p-AMP, p-GSK 3β, p-Tau231, for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies as described in Table 2: ARC (1:250), GLUR1 (1:500), PSD 95 (1:1000), Synaptophysin (1:1000), AMPK (1:1000), p-AMPK (1:1000), Gsk3-β (1:250), p-Gsk3-β (1:500), FFAR 3 (1:250), Human Tau (1:250), Tau 231/AT180 (1:250), Malondialdehyde (MDA) (1:1000). Membranes were washed with TBS-T (TBS+0.05% Tween20), and then incubated with a horseradish peroxidase linked to secondary antibody (Table 2). As loading control, we used α-Actin (1:1000). Immunoreactive bands were visualized by enhanced chemiluminescence ECL for routine immunoblotting [55] using Chemidoc (Bio rad XRS+SYSTEM). The western blots were performed at least 3 times using independent blots. Densitometry analysis was performed using NIH image J software. Values were normalized with actin and expressed as fold increase.

Fecal microbiota analysis

DNA isolation and sequencing

Fecal samples were collected for each animal during the habituation day (three days before sacrifice). Animals were placed during 20 min in a Plexiglas arena (50 cm × 50 cm) previously cleaned and disinfected. At least 100 gm of feces pellets

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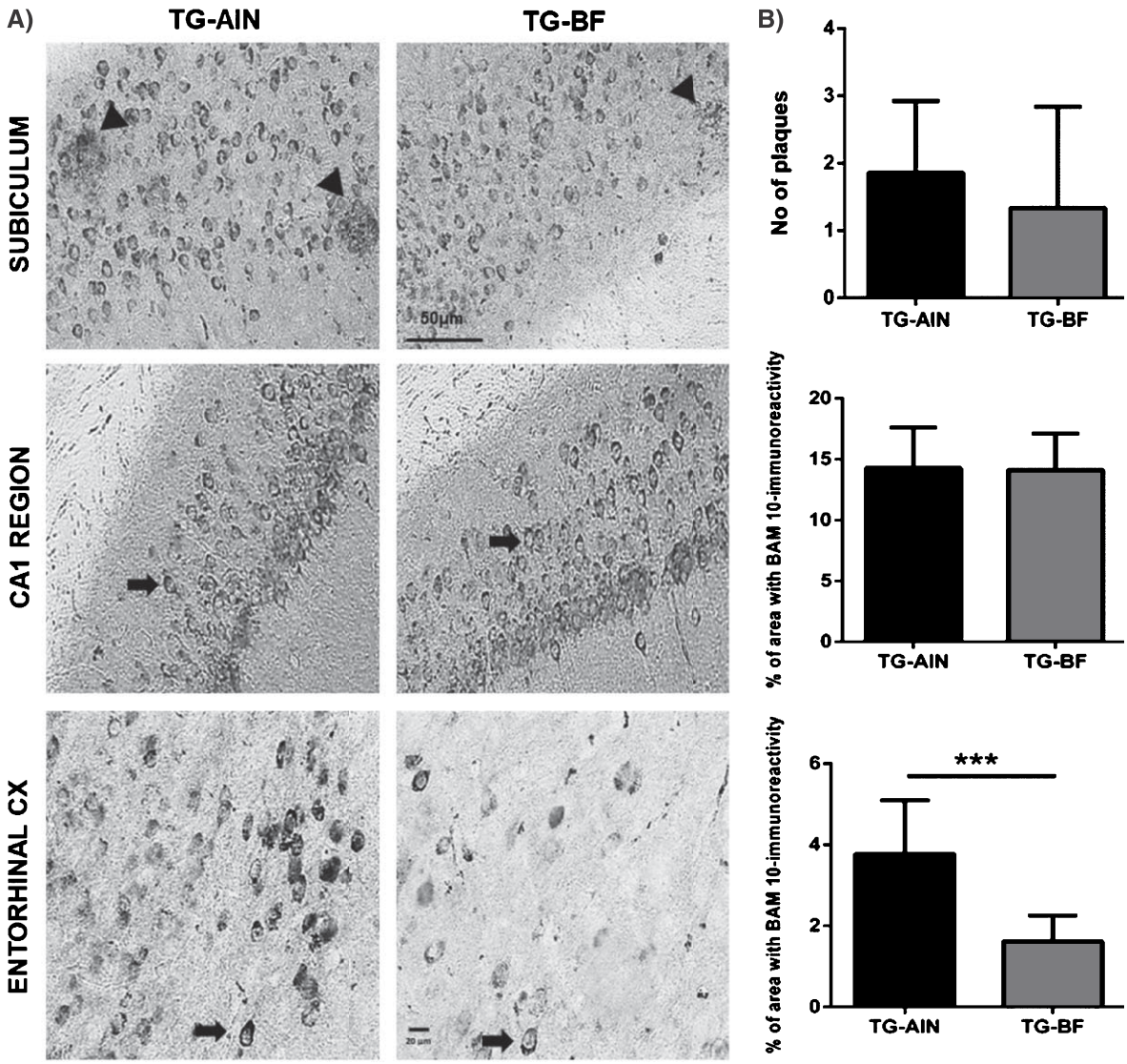


Fig. 2. Evaluation of amyloid pathology. A) Representative images of BAM-10 staining in hippocampus and Ent. cx. Scale bar 50 µm (hippocampus) and 20 µm (cortex). B) Quantification of BAM-10 staining: Upper panel, in subiculum there were few large aggregates with no differences between groups. Middle panel, in CA1 region there were no differences between groups. Lower panel, in Ent. cx. TG mice treated with BF showed a decreased BAM-10 staining compared to TG-AIN mice ($p < 0.001$). $N = 5$ for each group. Data are expressed as the mean ± SD. ***$p < 0.001$.

were collected per animal, immediately frozen and stored at –70°C until use. Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. MiSeq platform was used for the sequencing of the samples and then genomic libraries of the regions V3 and V4 of the 16 S gene were generated, using primers for those regions that also contained an overhang adapter specified by Illumina (F: 5′-TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGCCTACGGGNGGCWGCAG-3′ and R: 5′-GTCTCGTGGGCTCGGAGA TGTGTATAA-GAGACAGGACTACH VGGGTATCTAATCC-3′). The amplicons of the V3 and V4 regions were generated by PCR reactions containing genomic DNA (5 ng/µL in 10 mM Tris pH 8.5), high Fidelity DNA polymerase 2 × KAPA HiFi HotStart ReadyMix and primers (1 µM). This mixture was placed into the thermal cycler and run through the following program: 3 min at 95°C, followed by 25 amplification cycles consisting of denaturation (30 s at 95°C), alignment (30 s at 55°C) and elongation (30 s at

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72°C). The final elongation consisted of 5 min at 72°C. The amplicons were purified using AMPure XP beads and their size was verified on capillary electrophoresis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA), with an approximate size of 550 bp. Once passed the quality control, the samples were indexed using the Illumina Nextera XT Index Kit (v.2, Set A). For this process 5 μL of the first PCR product, High Fidelity DNA polymerase 2 × KAPA HiFi HotStart ReadyMix and primers (Index) were mixed and returned to the thermocycler, using the following program: 3 min at 95°C, followed by 8 amplification cycles which consisted of denaturation (30 s at 95°C), alignment (30 s at 55°C) and extension (30 s at 72°C). The final extension consisted of 5 min at 72°C. This product was purified and the integrity was analyzed. The amplicons had an approximate size of 610 bp. The concentration of double-stranded DNA was determined by fluorometry (Qubit fluorometer 3.0, high sensitive kit). The final library was mixed equimolarly and sequenced on the Illumina MiSeq platform (MiSeq Reagent Kit V.3, 600 cycles) following the supplier's instructions. Sequence Analysis: For taxonomic composition analysis, Custom C# and python scripts, as well as python scripts in the Quantitative Insights into Microbial Ecology (QIIME) software pipeline 1.9 were used to process the sequencing files. The sequence outputs were filtered for low-quality sequences (defined as any sequences that are 620 bp, sequences with any nucleotide mismatches to either the barcode or the primer, sequences with an average quality score of 0). Sequences were chimera checked with Gold.fa, and chimeric sequences were filtered out. Analysis started by clustering sequences within a percent sequence similarity into Operational taxonomic units (OTUs). 91% of the sequences passes filtering, resulting in 83,906 sequences/sample with a 97% similarity threshold. Operational taxonomic units (OTUs) picking was performed using tool set Qiime-tools, using Usearch method. OTUs were picked against the Green Genes 13.9. 97% OTUs reference database. After the resulting OTU results files were merged into one overall table, taxonomy was assigned based upon the gg v13.9 reference taxonomy. Thus, 99.76%, 99.69%, 99.64%, 86.8%, 50.48%, and 10.86% of the reads were assigned to the phylum, class, order, family, genus, and specie level, respectively. Species richness (Observed, Chao1) and alpha diversity measurements (Shannon) were calculated, and we estimated the within-sample diversity at a rarefaction depth of >17351 reads per sample. Weighted and unweighted UniFrac distances were used to perform the principal coordinate analysis (PCoA). Microbial sequence data were pooled for OTUs comparison and taxonomic abundance analysis but separated by batch in PCoA to have clear PCoA figures. For even sampling, a depth of 17,351 sequences/sample was used. PCoAs were produced using Emperor. Community diversity was determined by the number of OTUs and beta diversity, measured by UniFrac unweighted and weighted distance matrices in QIIME. ANOSIM, a permutational multivariate analysis of variance was used to determine statistically significant clustering of groups based upon microbiota structure distances.

Lipopolysaccharide analysis

LPS content in plasma was determined by ELISA kit (CEB526Ge, Cloud-Clone Corp) according to manufacture instructions.

Short chain fatty acids

Short-chain-fatty acids (SCFA) content in feces and brain samples were analyzed by gas chromatography (GC). We collected fecal samples from WT-AIN, TG-AIN one day before finalizing the experiment, and from WT and TG mice of 3-, 6-, and 11-months of age (WT, $n = 3$ each age; TG, $n = 4$ each age) for the ontogeny study. Feces samples were immediately frozen and stored at –70°C until analysis. Fifty mg of feces was suspended in 5 mL of water and homogenized to get fecal suspension. The pH of the suspension was adjusted to 2–3 by adding 5M HCl, and then kept at room temperature for 10 min with occasional shaking. The suspension was centrifuged for 20 min at 5000 rpm and the supernatant was collected. The internal standard, 2-Ethylbutyric acid solution (1 mM in formic acid), was spiked into the supernatant. Supernatant was injected in GC (Agilent technologies-6850 series 11, Agilent, Santa Clara, CA, USA) with flame ionization detection (Agilent), by using Agilent J&W DB-225 ms column as previously described [56].

To evaluate the levels of propionic acid in brain we used GC-Mass Spectrophotometry (MS) using an Agilent Intuvo 9000 gas chromatography system coupled to an Agilent 5977B mass spectrometric detector (MSD, Agilent Technologies, Santa Clara, CA). No sample preparation was required as Thermal separation Probe accessory (5990-8715EN Thermo Sep Probe.indd - Agilent) was used for introduction of the sample into GC-MS. Briefly, samples were weighed

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and loaded into the vial for propionic acid extraction at the inlet, followed by the standard GC analysis. Propionic acid was separated using an Agilent Column (part number 122-7033UI-INT: Serial number US16460202), phase DB-WAX_UI (Dimensions 30 m × 250 μm × 0.5 μm). 0.4 μl of derivatives was injected in split mode with a ratio of 20:1, and the solvent delay time was set to 6 min. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The initial oven temperature was held at 60°C for 0.05 min, ramped to 250°C at a rate of 900°C/min, and finally held at this temperature for 3 min. Data analysis was performed using MassHunter Quantitative software. (Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis

Data are expressed as the mean ± S.E.M. for body weight and microbiota analysis at species and genus level, otherwise we expressed data as mean ± S.D. For analyzing BAM-10, AT100 TAU231, and propionate concentration in feces we used an un-paired *t*-test. To assess body weight changes we used a two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test. One-way ANOVA followed by Holm-Sidak *post-hoc* test was used for T-maze data, and Tukey's test for the rest of the analysis (GFAP, iba1, SIRT1, SIRT1 in GFAP, GluR-1, PSD-95, Arc, synaptophysin, pGSK-3β/GSK-3β, pAMPK/AMPK, microbiota, propionate level and FFAR3 in brain and LPS levels) as recommended by the Software (GraphPad v.6). All results were considered statistically significant at $p < 0.05$.

RESULTS

Body weight and behavior

We observed that TG groups had significantly higher body weight compared to WT mice (Fig. 1A). However, BF ingestion by TG mice did not modify food intake, nor weight gain with respect to the control group. Body weight changes in TG mice have been associated with glucose intolerance [57, 58].

Spatial and working memory were assessed using a T-maze test (Fig. 1B). Percentage of spontaneous alterations decreased in TG-AIN compared to WT-AIN mice, but BF ingestion for 7 months significantly improved cognition in TG-BF mice (Fig. 1B).

Amyloid-β

We analyzed Aβ load using BAM-10 antibody. At 9 months of age, only a few amyloid plaques were observed in the subiculum of 3xTg-AD mice. We did not detect a significant effect of BF on the number of plaques in TG mice (Fig. 2A, B). However, intracellular Aβ was readily identified in neurons from the hippocampal region and Ent.cx. No significant differences in the degree of intracellular Aβ aggregation were detected in the CA1 subfield of the hippocampus as a function of diet. In Ent. cx, however, TG-BF mice had fewer aggregates than the TG-AIN group ($p < 0.001$) (Fig. 2A, B).

Tau phosphorylation

Tau pathology was analyzed by immunohistochemistry (AT100) and immunoblot (TAUp231). AT100 staining in the pyramidal layer of the CA1 region decreased in TG-BF compared to TG-AIN mice (Fig. 3A, B). Moreover, protein levels of p-TAU231/TAU in the cortex correlated to those changes as p-TAU231 decreased significantly in TG-BF compared to TG-AIN mice, resulting in a lower p-TAU231/TAU ratio in TG-BF mice (Fig. 3C).

Inflammation

Glial cells were labeled with iba1 and GFAP antibodies. We classified the microglia phenotype as inactive or active, according to previous reports [50, 51]. Representative images of the microglia are shown in Fig. 4A. The quantification of microglia in *st. radiatum* showed more activated cells in TG-AIN compared to WT-AIN mice, while the inclusion of BF abated those increases. The number of inactive microglia did not differ between any of the three groups (Fig. 4B, left panel). Astrocytes labeled with GFAP were quantified in hippocampus (*st. radiatum* and *st. oriens*) and in Ent.cx; representative images are shown in Fig. 4A. We observed that TG-AIN mice had more GFAP + astrocytes in both *strata* and Ent. cx compared to WT-AIN mice. Contrastingly, the ingestion of BF resulted in a lower number of astrocytes in both *strata* from hippocampus, but there was not a significant recovery in Ent. cx in TG-BF mice (Fig. 4B, right panel).

Metabolic and synaptic proteins

To evaluate if the anti-inflammatory effect of BF could be associated with changes in cellular metabolism, we analyzed SIRT1, GSK-3β, and

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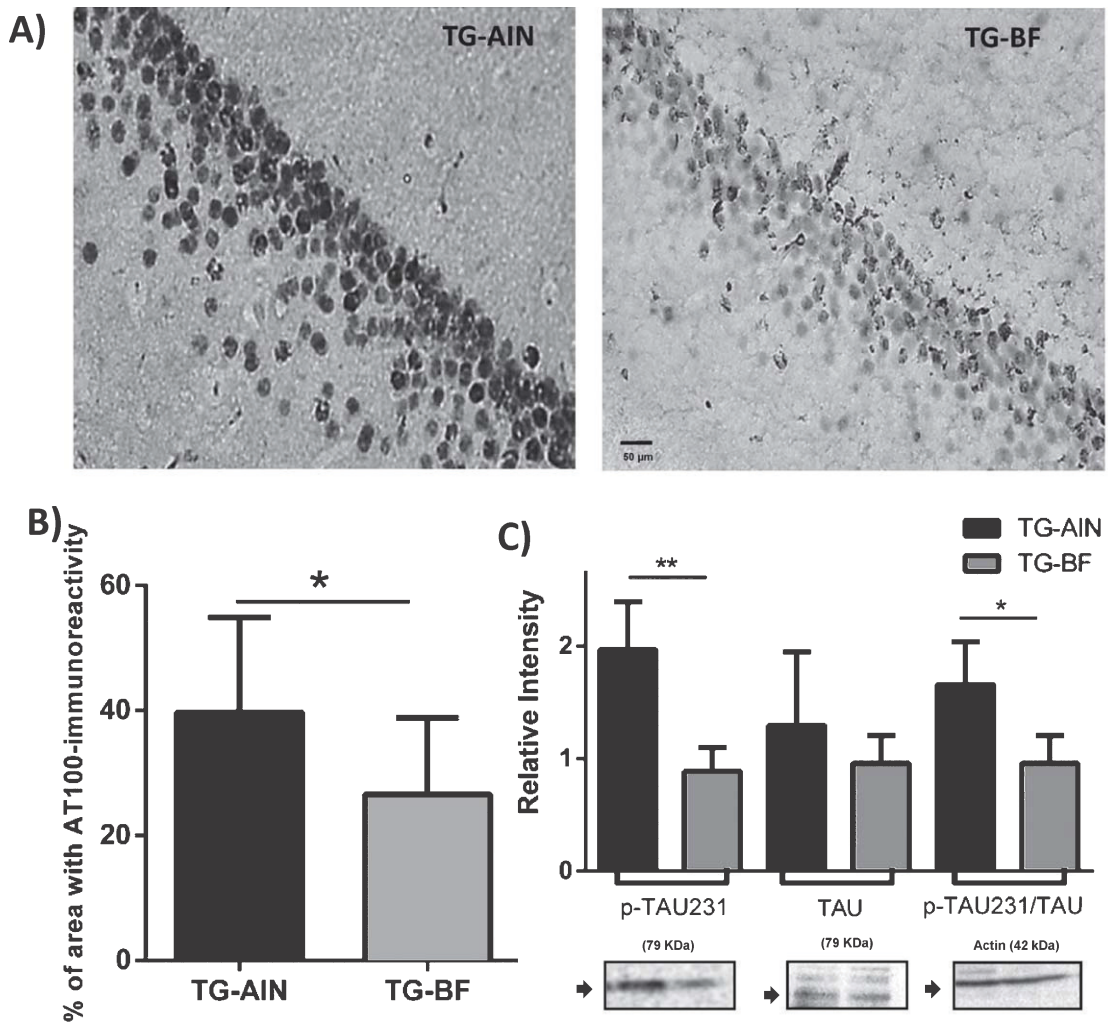


Fig. 3. Tau hyperphosphorylation in TG mice. A) Representative photomicrographs of AT100 staining in CA1 hippocampal region. B) Quantification of AT100 indicates a reduction in the percentage of area occupied by tau aggregates in TG-BF mice compared to TG-AIN ($p < 0.05$). C) Western blot analysis of brain cortex corroborates that tau hyperphosphorylation (p-TAU231) decreased in TG-BF mice compared to TG-AIN ($p < 0.01$). Blot were normalized with α-actin. Images below graphs are representative blots for each protein done by quadruplicate in pool samples. Scale bar 50 µm. N = 5 for each group. Data represents mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.01$.

AMPK proteins in brain tissue. We evaluated the immunoreactivity against SIRT1 in *st. radiatum* and *st. oriens* (Fig. 5). In *st. radiatum*, a layer with an increased number astrocytes and microglia cells, SIRT1 was higher in TG-AIN mice compared to WT-AIN mice, while the TG-BF group showed decreased values compared to TG-AIN (Fig. 5B, right panel). No differences were observed in *st. oriens* (Fig. 5B, right panel). To evaluate whether the SIRT1 levels in *st. radiatum* were associated with the increased number of astrocytes in the same layer, double immunofluorescence (SIRT1 and GFAP) was applied. We observed that nucleus of GFAP+astrocytes had increased SIRT1 labeling in TG-AIN mice compared to WT-AIN and TG-BF mice. SIRT1 in the nucleus of GFAP + cells decreased in TG-BF compared to TG-AIN mice, but it did not reach WT values (Fig. 6).

We analyzed GluR-1 (an integral membrane protein belonging to the glutamate-gated ion channel family), PSD-95 (Post-synaptic density protein 95), Arc (Activity-regulated cytoskeleton-associated protein), and pre-synaptic synaptophysin protein levels in brain cortex. We observed that TG-AIN mice had more GluR-1, PSD-95, and Arc when compared to WT-AIN mice, indicating synaptic hyperactivity. Synaptophysin levels were lower in TG-BF compared to WT-AIN and TG-AIN mice ($p < 0.01$, both).

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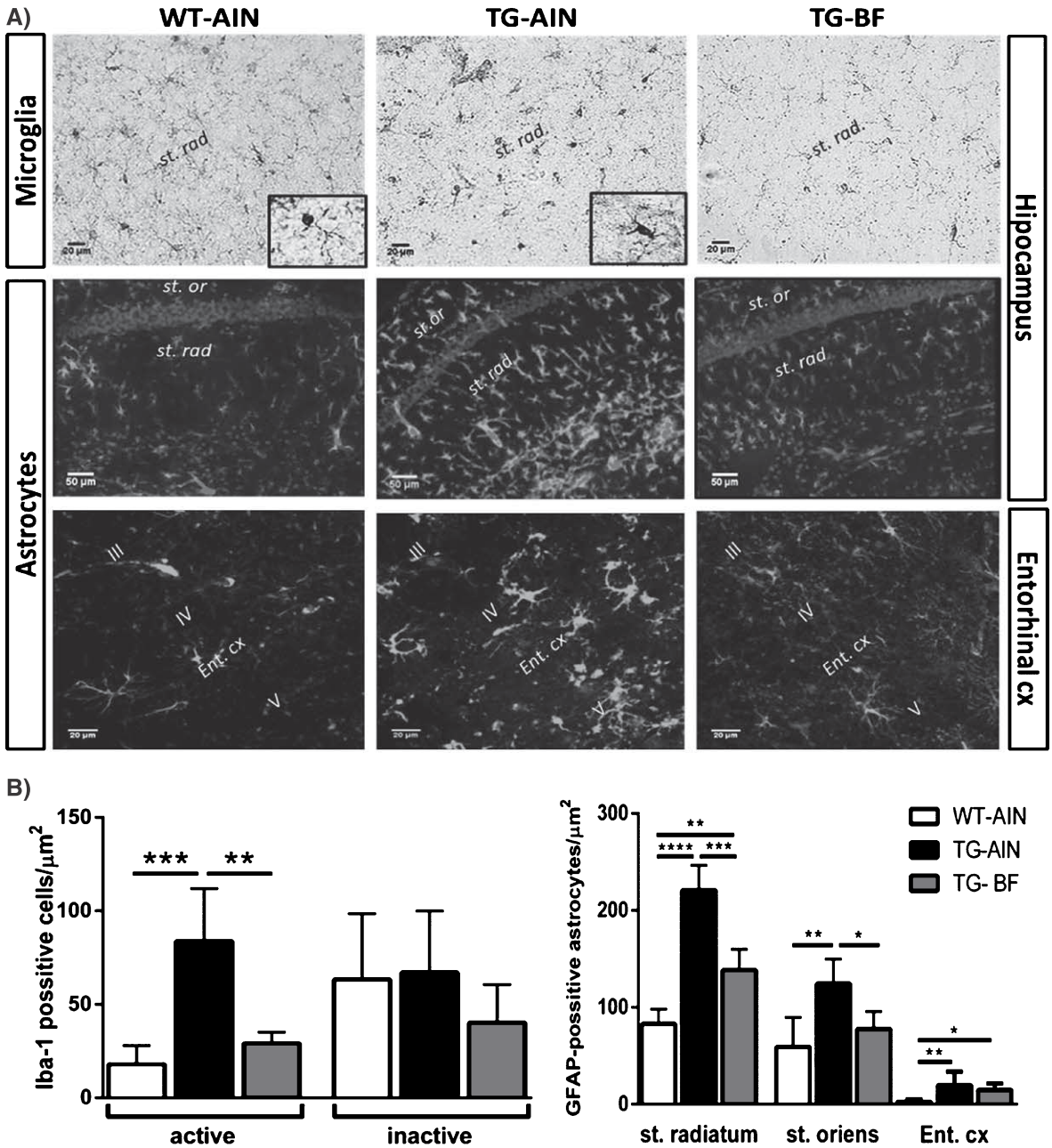


Fig. 4. Effect of BF on neuroinflammation in 3xTg-AD mice. A) Representative images of microglia labeled with iba1 in *st. radiatum* of CA1 hippocampal region (scale bar 20 µl), and reactive astrocytes labeled with GFAP in *st. radiatum, st. oriens* (scale bar 50 µl) and Ent. cx (scale bar 20 µm). Iba+cells in WT-AIN mice presented mainly an inactive phenotype (insert, WT-AIN), while in TG-AIN mice, abundant activated microglia was detected (insert, TG-AIN). B) Left panel: Quantification of microglia cells in *st. radiatum* showed an increased number of activated microglia in TG-AIN compared to WT-AIN mice, while TG-BF mice showed similar values as controls ($p < 0.001$ and $p < 0.01$, respectively). Number of inactive microglia did not showed differences between groups. Right panel: GFAP + astrocytes increased in *st. radiatum, st. oriens* and Ent. cx in TG-AIN compared to WT-AIN mice ($p < 0.0001$, $p < 0.01$, $p < 0.01$, respectively), while TG-BF mice had a reduced number of astrocytes in *st. radiatum* and *st. oriens* ($p < 0.001$ and $p < 0.05$). Note that more active astrocytes were observed in *st. radiatum* compared to *st. oriens* in TG-AIN mice. In Ent cx there was a slight recovery in TG-BF compared to TG-AIN as number of astrocytes was not so abundant compared to WT-AIN ($p < 0.05$), but it did not reach significance. Data represents mean ± SD. $N = 5$ for each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

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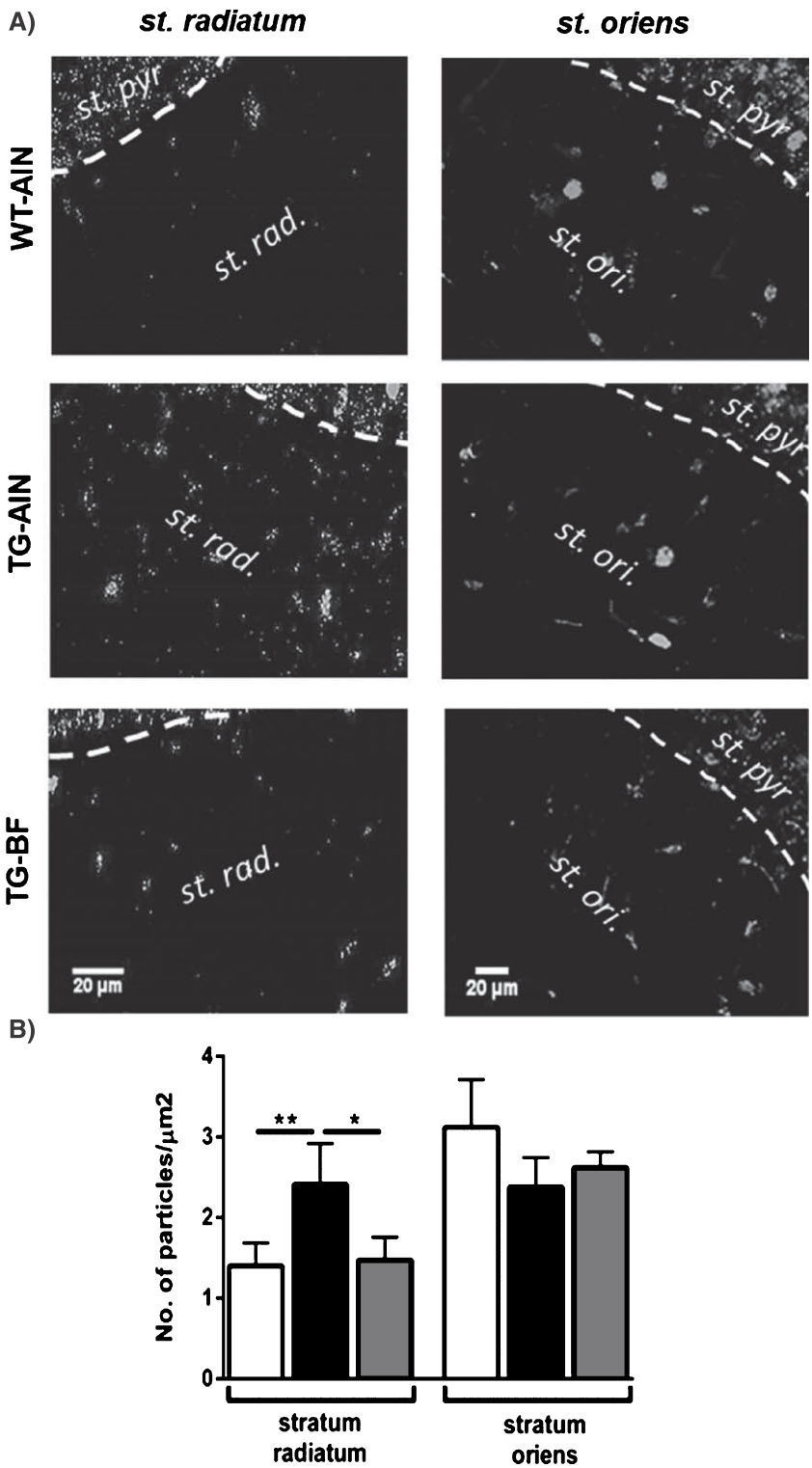


Fig. 5. Effect of bioactive food on hippocampal SIRT1 were region specific. A) Representative images of SIRT1 labeling in st. *radiatum* and *st. oriens*. Scale bar 20 µm. B) Quantification of SIRT labeling in both *strata*. In *st. radiatum,* TG-AIN mice had a greater number of SIRT1 particles compared to WT-AIN ($p < 0.01$) and TG-BF ($p < 0.05$). No differences between groups were observed in *st. oriens*. Data are expressed as the mean ± S.D. $N = 5$ per group. *$p < 0.05$, **$p < 0.01$

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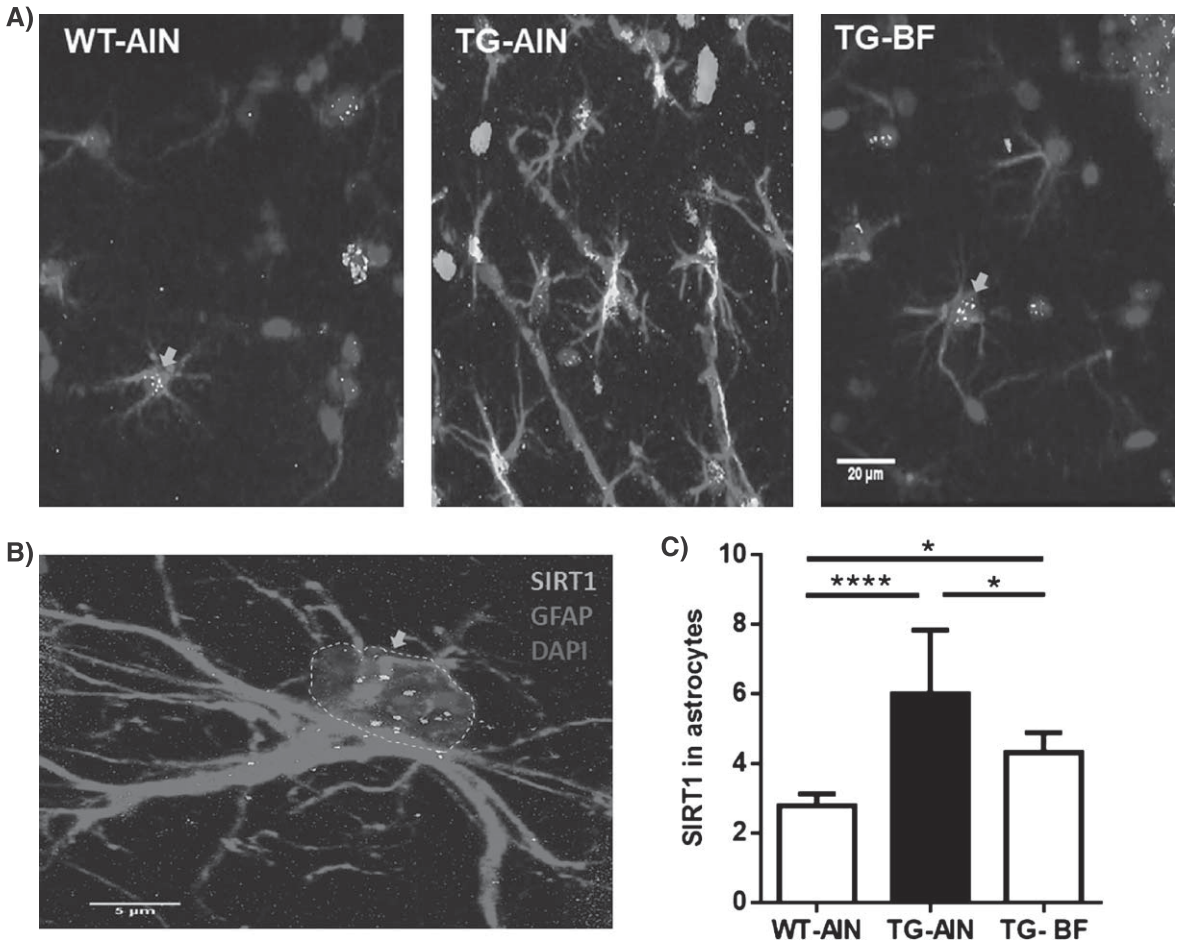


Fig. 6. Effect of bioactive food on SIRT1 in GFAP+ astrocytes. A) Representative images of GFAP+ astrocytes (red) located in *str. radiatum* of CA1 hippocampal region co-labeled with SIRT1 (green), and counterstained with DAPI (blue). Scale bar 20 µm. B) Magnification of an astrocyte where nucleus was selected to analyze SIRT1 particle intensity. Scale bar 5 µm. C) Quantification of SIRT1 in nucleus of astrocytes indicates that TG-AIN mice had more SIRT1 than WT-AIN ($p < 0.0001$) and TG-BF mice ($p < 0.05$). Moreover, TG-BF mice showed increase SIRT1 compared to WT-AIN ($p < 0.05$). Data are expressed as the mean ± SD. $N = 5$ per group. *$p < 0.05$, ****$p < 0.0001$.

TG-BF mice showed significant decreases in all those synaptic proteins (GluR-1, PSD-95, Arc and synaptophysin) compared to TG-AIN mice (Fig. 7A, B).

We also evaluated GSK-3β and p-GSK-3β protein content in the brain cortex by western blot. We observed that BF ingestion induced a significant increase in p-GSK-3β compared to WT-AIN and TG-AIN, with no changes in total GSK-3β. Therefore, the ratio p-GSK-3β/GSK-3β increased in TG-BF mice compared to the other groups (Fig. 8A). We also observed a decrease in p-AMPK in TG-BF compared to WT-AIN and TG-AIN, while the total AMPK increased in TG-AIN compared to WT-AIN. Therefore, the ratio pAMPK/AMPK decreased in TG-AIN and TG-BF compared to WT-AIN (Fig. 8B).

Gut microbiota

We analyzed the microbiota composition and diversity in feces of TG and WT mice. The alpha diversity was estimated by observed Shannon indexes. The WT-AIN group showed the highest diversity followed by TG-BF, whereas the TG-AIN group had the lowest bacterial diversity (Fig. 9A). Clustering the bacterial communities using principal component analysis (PCoA) revealed that GM in TG-AIN and TG-BF was different from WT-AIN (Fig. 9B).

At the phylum level, TG-AIN showed increased abundance of Bacteroidedetes ($p < 0.01$) and Firmicutes ($p < 0.01$), but a decreased abundance

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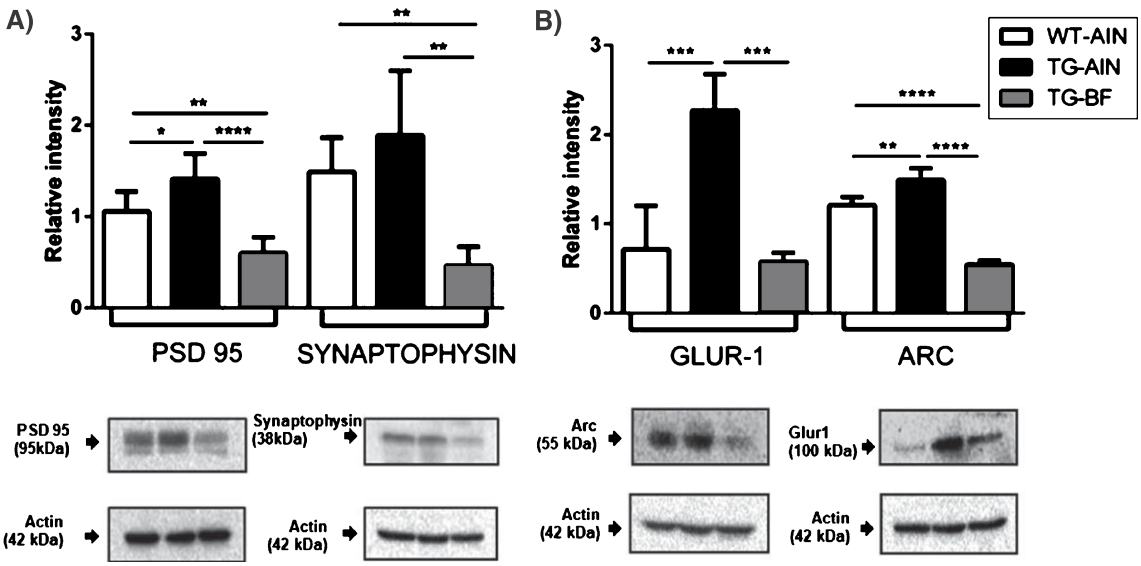


Fig. 7. Immunoblots of synaptic proteins. PSD95, synaptophysin, GluR-1, and Arc protein levels were evaluated in brain cortex. A) PSD95 levels were higher in TG-AIN compared to WT-AIN and TG-BF mice ($p < 0.05$ and $p < 0.0001$, respectively), while in TG-BF mice PSD95 levels were even lower than WT-AIN ($p < 0.01$). Synaptophysin levels were lower in TG-BF compared to WT-AIN and TG-AIN mice ($p < 0.01$, both). B) GluR-1 levels were higher in TG-AIN compared to WT-AIN and TG-BF ($p < 0.001$, both), while content of Arc protein was higher in TG-AIN mice compared to WT-AIN ($p < 0.01$) and TG-BF ($p < 0.0001$), but in TG-BF there were lower values compared to WT-AIN ($p < 0.0001$). Images below graphs are representative blots for each protein done eight (PSD95), four (Arc and GluR-1) or five (synaptophysin) times, in pool samples. Data represents mean ± S.D. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$.

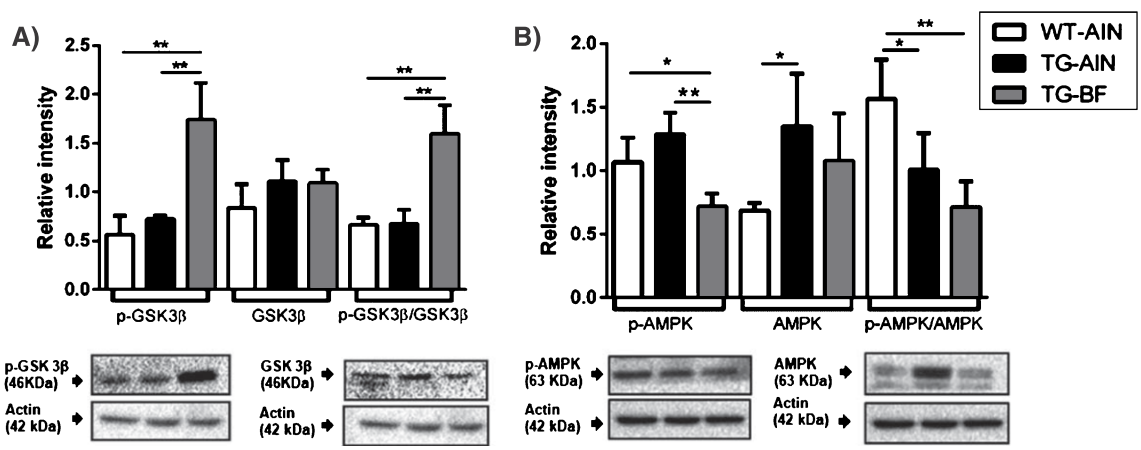


Fig. 8. Protein levels of pGSK-3β/GSK-3β and pAMPK/AMPK in **brain** cortex. A) Due to an increase level of pGSK-3β in TG-BF mice compared to the other two groups (both, $p < 0.01$), there was increase in the ratio p-GSK-3β/GSK-3β in TG-BF mice compared to the other groups (both, $p < 0.01$). B) p-AMPK was decreased in TG-BF compared to WT-AIN ($p < 0.05$) and TG-AIN ($p < 0.01$) mice. AMPK increased in TG-AIN compared to WT-AIN ($p < 0.05$). Thus, ratio pAMPK/AMPK decreased in TG-AIN and TG-BF compared to WT-AIN ($p < 0.05$ and $p < 0.01$ respectively). Blots were normalized with α-actin. Images below graphs are representative blots for each protein done by quintuplicate (GSK3) or triplicate (AMPK) in pool samples. Data represents mean ± S.D. *$p < 0.05$; **$p < 0.01$.

of Cyanobacteria ($p < 0.0001$), Deferibacteres ($p < 0.0001$), Proteobacteria ($p < 0.01$), TM7($p < 0.0001$), Tenericutes ($p < 0.0001$), Verrucomicrobia ($p < 0.0001$) compared to WT-AIN mice (Fig. 10A).

After ingestion of BF, TG mice presented an increased relative abundance of Actinobacteria ($p < 0.05$), Bacteroidedetes ($p < 0.01$), but decreased abundance of Cyanobacteria ($p < 0.0001$), Deferibacteres ($p < 0.0001$), Proteobacteria ($p < 0.001$), TM7

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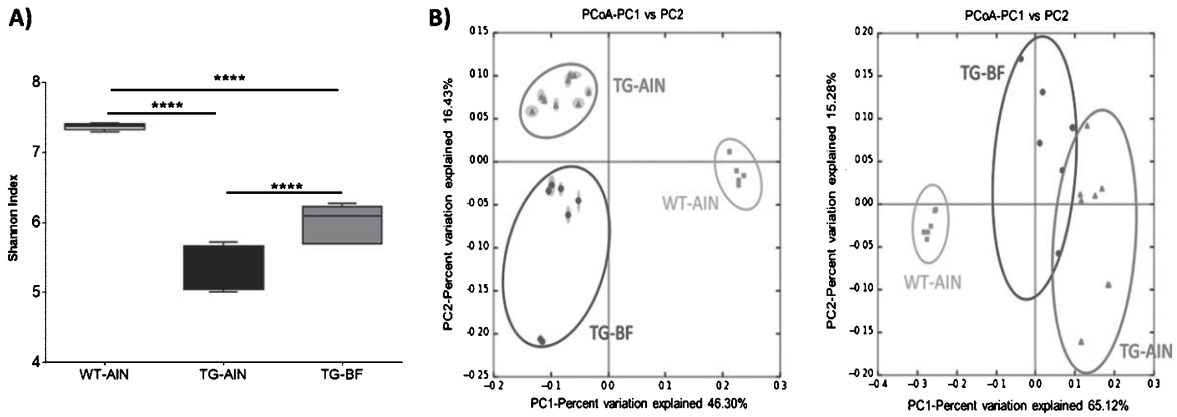


Fig. 9. Gut microbiota modifications. A) Alpha diversity by Shannon Index in the three groups studied showed statistical differences between WT-AIN and TG-AIN mice, and between TG-AIN and TG-BF mice (all, $p < 0.0001$). B) Principal components analysis (PCoA) of unweighted (left) and weighted variables (right) by ANOSIM: unweighted: $R = 0.8836$, $p < 0.001$; weighted: $R = 0.8326$, $p = 0.001$. WT-AIN, $n = 6$; TG-AIN $n = 7$; TG-BF, $n = 7$.

($p < 0.0001$), Tenericutes ($p < 0.0001$), Verrucomicrobia ($p < 0.0001$) compared to WT-AIN mice (Fig. 10A), and increase abundance of Tenericutes ($p < 0.05$) and Verrucomicrobia ($p < 0.0001$) compared to TG-AIN mice (Fig. 10A). Accordingly, there were microbial alterations at the class, order, family, and genus level (Supplementary Figures 2–5, Supplementary Table 1). At species level, there were important phenotype differences as TG-AIN presented more relative abundance of *Prevotella copri, Lactobacillus ruminis, Streptococcus anginosus, Actinobacillus parahaemolyticus*, and *Haemophilus parainfluenzae* compared to WT-AIN mice. Ingestion of BF reduced the abundance of all those bacteria in TG mice. Furthermore, BF ingestion increased the relative abundance of *Bacteroides uniformis, Faecalibacterium prausnitzii*, and *Akkermansia miciniphila* in TG mice compared to TG-AIN group (see Fig. 10B and Table 3).

Those GM alterations in TG-mice, prompted us to evaluate the levels of LPS in plasma samples. In addition, due to the dramatic increase in *Prevotella copri* in TG-AIN mice, bacteria known as propionate producers, were evaluated propionate content and its SCFA receptor (FFAR3) in brain tissue.

Propionate level and FFAR3 in brain

The PFC is an area related to T-maze working memory performance, and we have observed working memory impairment in TG mice. Therefore, we evaluated the levels of propionate in this brain region. Propionate content was larger in TG-AIN mice compared to WT-AIN and TG-BF mice in PFC (Fig. 11A). We also quantified SCFA receptor FFAR3/GPR41 in the cortex. FFAR3 has a high affinity for propionate and was more abundant in TG-AIN mice compared to WT-AIN mice, whereas BF ingestion decreased those values in TG mice (Fig. 11B).

Lipopolysaccharide levels in plasma

LPS levels in plasma were larger in TG-AIN compared to WT-AIN mice ($p < 0.01$), while BF ingestion led to a lower LPS concentration in TG mice ($p < 0.05$) (Fig. 11C).

Levels of short-chain fatty acids in feces of TG at different ages

Acetate, butyrate, and propionate are the most abundant SCFAs produced by GM [59]. We observed an increase in propionate producing bacteria in TG-AIN mice compared to WT-AIN mice, an effect accompanied by increased propionate in brain tissue. Propionate is a potent neurotoxic agent. Then, we sought to evaluate whether propionate levels increased in an age-dependent manner according to the progression of the pathology. SCFAs levels (acetate, butyrate, and propionate) were measured in feces from WT and TG mice along the ontogeny (3-, 6-, and 11-month-old). In WT mice, total SCFA concentration decreased at 6 and 11 months of age in comparison to 3 months of age (both, $p < 0.05$), whereas in TG mice the total SCFA concentration had a age delay, as it decreased only in 11-month-old mice compared to 3- and 6-month-old mice (both, $p < 0.01$). Regarding acetate

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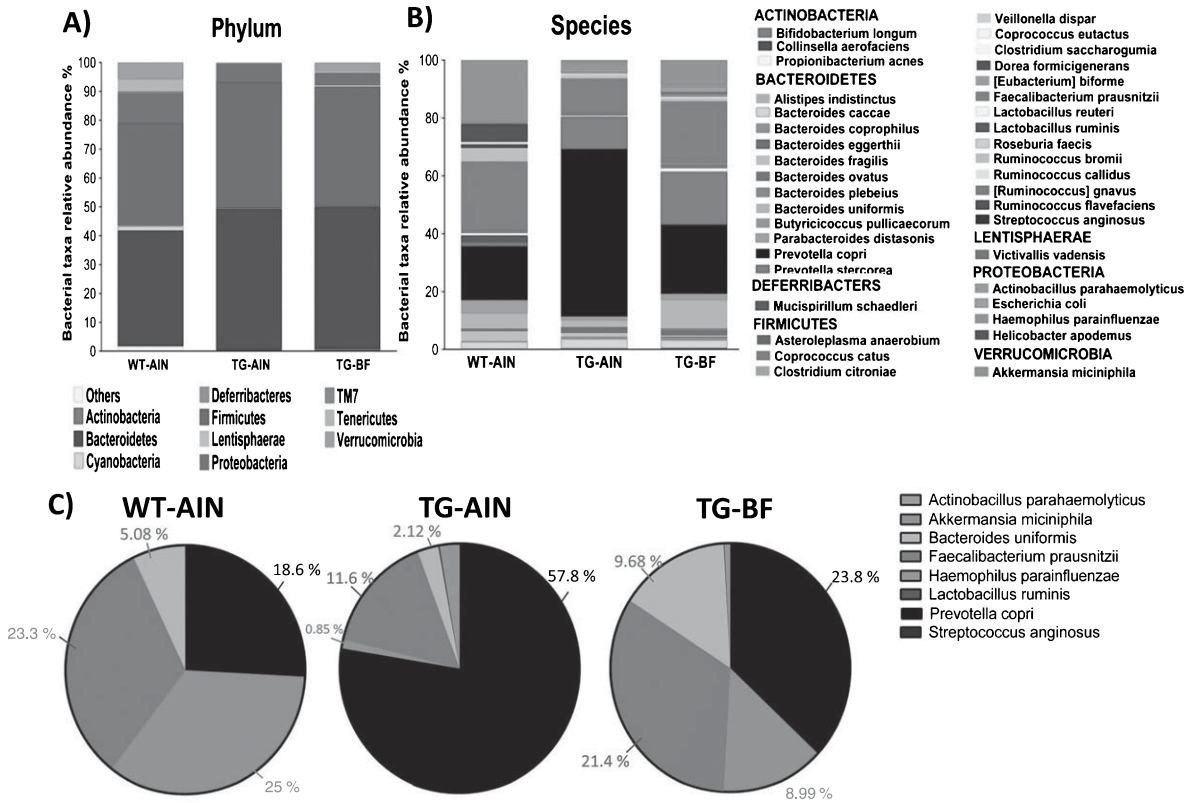


Fig. 10. Gut microbiota modifications in transgenic mice, and its restoration by BF ingestion. (A) Relative abundance of bacteria at the phylum level in all the groups (significant differences are described in the main text). (B) Relative abundance of bacteria at the species levels. C) Pie chart of main bacterial species that showed significant changes between WT-AIN and TG-AIN compared to TG-BF mice. Relative abundances (%) are represented in the figure according to the color code of each species. Statistical differences can be found in Table 3. WT-AIN, $n = 6$; TG-AIN $n = 7$; TG-BF, $n = 7$.

concentration in WT, we observed an increase in 11-month-old mice compared to the 6-month-old group ($p < 0.05$). No changes in acetate concentration were observed in TG mice. Butyrate in WT mice decreased at 6 and 11 months of age in comparison to 3-month-old animals ($p < 0.05$ and $p < 0.01$, respectively). Notably, in TG mice, the decrease in butyrate was not as early as in WT, since differences were observed between 11- and 6-month-old mice versus the 3-month-old group ($p < 0.05$ and $p < 0.01$, respectively). Regarding the genotype effect, we observed that propionate concentration increased in TG compared to WT mice at 6 months of age ($p < 0.05$) (Table 4).

DISCUSSION

In this study, we demonstrated that the ingestion of BF diminished the main pathological markers of AD, amyloid aggregates and hyperphosphorylation of tau, in TG female mice. BF also diminished neuroinflammation and synaptic and metabolic alterations, and improved working memory in TG-BF compared to TG-AIN mice, effects associated with the reduction in pro-inflammatory gut bacteria and their products.

We used female 3xTg-AD mice as recent evidence indicates that women are at greater risk for developing the disease [60], and female TG mice have more severe pathology compared to males [61–63]. Therefore, we aimed to evaluate the effects of BF on the more vulnerable gender. We concluded seven months of dietary treatment when female TG were 9 months old, an age where they show neurogenic and neuroplastic deficits resulting in cognitive decline [63–66]. However, at 9 months of age, the pathological hallmarks and cognitive impairment were moderately present in our 3xTg-AD strain [67]. Therefore, we assessed other markers which appear since early stages of the disease.

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Table 3
Taxonomic classification of pyrosequences from bacterial communities at the species level

Species	WT-AIN	TG-AIN	TG-BF
Propionibacterium acnes	0.050 ± 0.024	0.0167 ± 0.005	0.006 ± 0.002
Bifidobacterium longum	0	0.05 ± 0.017	$0.0904 \pm 0.030^{*}$
Collinsella aerofaciens	0	0.275 ± 0.1718	0.321 ± 0.101
Bacteroides caccae	2.596 ± 0.157	3.274 ± 1.507	2.825 ± 0.747
Bacteroides coprophilus	0.088 ± 0.032	0.761 ± 0.710	0.922 ± 0.833
Bacteroides eggerthii	0.105 ± 0.0118	0.013 ± 0.001	0.230 ± 0.171
Bacteroides fragilis	3.425 ± 0.187	$1.285 \pm 0.566^{\#\#}$	$0.413 \pm 0.168^{***}$
Bacteroides ovatus	0.582 ± 0.070	1.767 ± 0.555	1.750 ± 0.318
Bacteroides plebeius	0.473 ± 0.059	0.409 ± 0.355	0.777 ± 0.750
Bacteroides uniformis	5.085 ± 0.367	2.122 ± 0.670	$9.681 \pm 1.380^{++++,**}$
Parabacteroides distasonis	4.642 ± 0.230	$1.407 \pm 0.645^{\#\#}$	$2.238 \pm 0.599^{+}$
Prevotella copri	18.66 ± 0.567	$57.88 \pm 3.157^{\#\#\#\#}$	$23.86 \pm 2.791^{****}$
Prevotella stercorea	1.107 ± 0.0209	10.95 ± 1.199	$18.13 \pm 5.677^{++}$
Alistipes indistinctus	0.015 ± 0.0012	0.068 ± 0.0422	0.129 ± 0.060
Mucispirillum schaedleri	2.572 ± 0.256	$0.001 \pm 0.003^{\#\#\#\#}$	$0.001 \pm 0.003^{++++}$
Lactobacillus reuteri	0.457 ± 0.108	$0.047 \pm 0.050^{\#\#\#\#}$	$0.015 \pm 0.006^{++++}$
Lactobacillus ruminis	0	$0.091 \pm 0.014^{\#\#\#}$	$0.030 \pm 0.011^{**}$
Streptococcus anginosus	0	$0.034 \pm 0.007^{\#\#}$	$0.014 \pm 0.006^{*}$
Clostridium citroniae	0	0.018 ± 0.007	0.017 ± 0.005
Coprococcus catus	0.016 ± 0.010	$0.01 \pm 0.021^{\#}$	0.086 ± 0.021
Coprococcus eutactus	0.39 ± 0.048	0.37 ± 0.126	1.158 ± 0.392
Dorea formicigenerans	0.014 ± 0.009	$0.175 \pm 0.015^{\#\#\#}$	$0.166 \pm 0.040^{++}$
Roseburia faecis	0	$0.033 \pm 0.009^{\#}$	0.0104 ± 0.003
[*Ruminococcus*] *gnavus*	1.335 ± 0.172	0.864 ± 0.323	1.054 ± 0.344
Butyricicoccus pullicaecorum	0.031 ± 0.013	0.067 ± 0.025	0.445 ± 0.211
Faecalibacterium prausnitzii	23.35 ± 0.177	$11.59 \pm 1.337^{\#\#}$	$21.42 \pm 3.404^{**}$
Ruminococcus bromii	4.358 ± 0.358	$0.885 \pm 0.387^{\#\#\#\#}$	$0.966 \pm 0.444^{++++}$
Ruminococcus callidus	0.269 ± 0.059	$0.036 \pm 0.006^{\#\#}$	0.132 ± 0.072
Ruminococcus flavefaciens	1.220 ± 0.107	$0.001 \pm 0.004^{\#\#\#\#}$	$0.007 \pm 0.002^{++++}$
Veillonella dispar	0.393 ± 0.032	0.824 ± 0.211	0.591 ± 0.163
Asteroleplasma anaerobium	0.006 ± 0.007	0.051 ± 0.007	0.330 ± 0.316
Clostridium saccharogumia	0.503 ± 0.092	$0.001 \pm 0.001^{\#\#\#\#}$	$0.007 \pm 0.003^{++++}$
[*Eubacterium*] *biforme*	0.034 ± 0.012	$0.295 \pm 0.044^{\#}$	$0.369 \pm 0.090^{++}$
Victivallis vadensis	0.0136 ± 0.008	0.002 ± 0.001	0.870 ± 0.580
Helicobacter apodemus	6.051 ± 0.337	$0.008 \pm 0.002^{\#\#\#\#}$	$0.008 \pm 0.003^{++++}$
Escherichia coli	0.368 ± 0.048	1.333 ± 0.73	1.836 ± 1.129
Actinobacillus parahaemolyticus	0	$0.208 \pm 0.021^{\#\#\#\#}$	$0.069 \pm 0.023^{***}$
Haemophilus parainfluenzae	0.074 ± 0.017	$1.810 \pm 0.271^{\#\#\#\#}$	$0.457 \pm 0.122^{***}$
Akkermansia miciniphila	25.05 ± 1.073	$0.850 \pm 0.305^{\#\#\#\#}$	$8.998 \pm 0.688^{****,++}$

Significances between WT-AIN vs TG-AIN are reported with hashes (#); WT-AIN vs TG-BF are reported with crosses (+), TG-AIN vs TG-BF are reported asterisks (*). Data represents mean ± S.E.M. one symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$; four symbols, $p < 0.0001$. WT-AIN, $n = 6$; TG-AIN $n = 7$; TG-BF, $n = 7$.

Mitochondrial dysfunction and enhanced oxidative stress are factors leading to AD pathology [68, 69]. Increased production of reactive oxygen species is associated with age- and disease-dependent loss of mitochondrial function and reduced antioxidant defense system [70]. Lipid peroxidation refers to the process in which lipids are attacked by reactive oxygen species [68]. The BF used in this study showed a stronger antioxidant activity versus AIN93 as assessed by the ORAC method (Table 1). Furthermore, in TG mouse cortex we detected higher MDA level, a lipid peroxidation product, but BF ingestion reduced MDA levels to WT values (Supplementary Figure 1). Thus, the beneficial effects of BF may be partially mediated by its potent anti-oxidant effect.

Neuroinflammation can both be a cause, and a consequence of chronic oxidative stress, and the abnormal production of pro-inflammatory cytokines and reactive oxygen and nitrogen species by active glia cells can cause synaptic dysfunction [70]. Synaptic hyperexcitability and metabolic alterations have been reported in young 3xTg-AD model [71–73]. Astrocytes and neurons interact actively, with astrocytes supporting neuronal metabolic requirements and clearing the excess glutamate (Glu) from extracellular space to prevent neuronal excitotoxicity [74]. In 3xTg-AD mice there is strong astrogliosis [75] and

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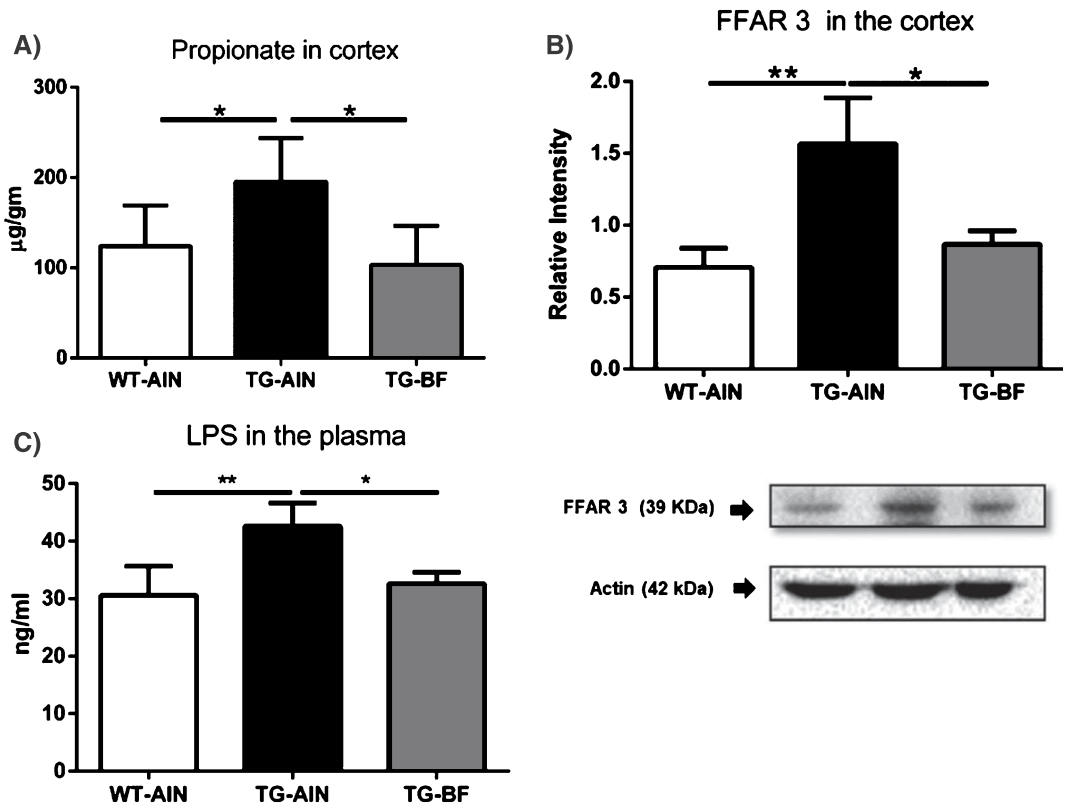


Fig. 11. Bacterial products in brain and plasma samples. A) Propionate levels in PFC were higher in TG-AIN compared to WT-AIN mice ($p < 0.05$) and ingestion of BF reduced those levels ($p < 0.05$). WT-AIN, $n = 7$; TG-AIN, $n = 6$; TG-BF, $n = 7$. B) FFAR3 receptor levels were higher in TG-AIN mice compared to WT-AIN and TG-BF mice ($p < 0.01$ and $p < 0.05$, respectively). C) LPS plasma levels were higher in TG-AIN compared to WT-AIN and TG-BF mice ($p < 0.01$ and $p < 0.05$, respectively). WT-AIN, $n = 4$; TG-AIN, $n = 3$; TG-BF, $n = 5$. Images below graphs are representative blots for each protein done by quadruplicated in pool samples. Data represents mean ± S.D. $*p < 0.05$; $**p < 0.01$.

at 7 months of age they present an impaired astrocytic support system to fulfill the neuronal metabolic demands [76]. Glu is the predominant excitatory neurotransmitter in adult mammalian brain, and in excess it can cause neuronal hyperexcitability [77]. When astrocytes are chronically activated they have impairment in Glu uptake, as the glutamate-glutamine (Glu-Gln) shuttle becomes compromised [78]. This impaired Glu-Gln shuttle results in increased Glu levels in the synaptic cleft [79], related to hyperactivity of neighboring neurons [80, 81]. Different TG mice strains [53, 82], including 3xTg-AD [83], are characterized by pro-convulsive seizures and neuronal circuit hyperexcitability since early stages of the disease. Increased neuronal firing [72, 80], and faster release of Glu into the synaptic cleft [76] is associated with increase in Arc and PSD95 post-synaptic proteins in young TG mice [53, 82]. In our present study, we also observed increased number of active astrocytes, an event accompanied by enhanced Arc and PSD95 levels in cortex of TG-AIN compared to WT-AIN mice. These data may indicate a state of neuronal over-excitation in TG mice, probably caused by impaired astrocytic function. Importantly, ingestion of BF was able to abate those synaptic alterations in hippocampus and cortex.

AD is also characterized by an early hypometabolic brain phenotype, resulting in an impaired brain bioenergetics [84, 85]. SIRT1 is consider a regulator of mitochondria biogenesis [86] by regulating peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α), that represents an upstream inducer of genes of mitochondrial metabolism [87]. We measured SIRT1 immunoreactivity in different regions of the hippocampus, and observed *stratum*-specific alteration in TG mice. There was a decreased SIRT1 immunoreactivity in *pyramidal layer* (data not shown), and an increased SIRT1 immunoreactivity in *st. radiatum* but not in *st. oriens*. In the former one, there were a greater

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Table 4
Levels of short-chain fatty acids in feces of TG at different ages

GENOTYPE	ACETATE (μg/gm)		PROPIONATE (μg/gm)		BUTYRATE (μg/gm)		TOTAL SCFA (μg/gm)	
	WT	TG	WT	TG	WT	TG	WT	TG
11 months	42010 ± 2546^{a}	38019 ± 1723	13153 ± 2252	18176 ± 2481	$14447 \pm 2246^{b,c}$	13014 ± 1646^{b}	58954 ± 7321^{b}	67054 ± 4139^{b}
6 months	25487 ± 2703^{b}	26329 ± 2862	12617 ± 4305	$32602 \pm 5448^{*}$	25810 ± 10376^{b}	37989 ± 5396^{a}	78941 ± 28372^{b}	164245 ± 16849^{a}
3 months	36009 ± 4069^{b}	37705 ± 5457	24661 ± 12851	29740 ± 5248	58617 ± 8349^{a}	32125 ± 6289^{a}	185533 ± 19064^{a}	172838 ± 29217^{a}

In WT mice, total SCFA concentration decreased at 6 and 11 months of age in comparison to 3 months of age (both $a > b$, $p < 0.05$), whereas in TG mice the total SCFA concentration decreased at 11 months of age compared to 3- and 6-month-old mice (both $a > b$, $p < 0.01$). Acetate concentration increased in WT mice at 11 months of age compared to 6 months of age ($a > b$, $p < 0.05$). Butyrate decreased in WT mice at 6 and 11months in comparison to 3 months ($a > b$, $p < 0.05$, and $a > c$, $p < 0.01$). In TG mice, butyrate decreased at 11 months in comparison to 3 months and 6 months ($a > b$, 3 months versus 11 months, by $p < 0.05$; 6 months versus 11 months, $p < 0.01$). Regarding genotype effect, we observed that propionate concentration increased in TG compared to WT mice at 6 months of age ($^{*}p < 0.05$). WT, $n = 3$ each age; TG, $n = 4$ each age. Data represents mean ± S.EM.

number of active astrocytes in TG-AIN compared to WT-AIN mice. We assessed the content of SIRT1 in those astrocytes, and found that in TG-AIN mice active astrocytes present increased SIRT1 levels compared to WT-AIN. SIRT1 has been mostly associated with beneficial effects, as improved cognition and neuronal plasticity [88]; however, less is known about its role in astrocytes. Previous reports indicate that promoting mitochondrial biogenesis—rather than glycolysis—in astrocytes helps to protect them from $A\beta_{1\text{-}42}$ toxic effects [89]. Moreover, upregulation of SIRT1 attenuates oxidative stress in astrocytes via upregulation of superoxide dismutase 2 and catalase [90], whereas LPS and interferon-γ induced activation of astrocytes results in increased mitochondrial biogenesis [91]. In our current study we detected significant increases of LPS in plasma of TG-AIN mice, while BF inclusion reduced LPS levels. This body of data may suggest that enhanced SIRT1 reflects a preferential oxidative metabolism in astrocytes in order to protect them from harmful agents (i.e., Aβ, LPS), while BF was able to abate those alterations.

We also measured GSK3β protein levels. GSK3β regulates mitochondrial energy metabolism in neurons and in glia. Activation of GSK3β also promotes tau hyperphosphorylation, neurofibrillary tangles, and amyloid plaques [92, 93]. Whereas, GSK3β inhibition (pGSK3β form) attenuates the production of pro-inflammatory cytokines (IL-1β and TNF-α) and augments the production of anti-inflammatory cytokines (IL-10) *in vitro* [94]. Here, we observed that p-GSK3β levels were dramatically increased in TG-BF mice after ingestion of BF. This enhanced p-GSK3β could be associated with reduced tau hyperphosphorylation in hippocampus and cortex, reduced neuroinflammation and oxidative damage (MDA) in TG-BF mice.

The BF used in our study was composed of nopal, soy protein, chia seed, and turmeric. We have used a combination of several foods because it has been shown that it offer a better cognitive outcome in AD patients in comparison to individual formulations [95], as they create a synergistic effect [96, 97]. Nopal (*Opuntia ficus*) is a potent antioxidant food able to increase anti-oxidant activity in plasma of healthy subjects [98]. In addition, nopal cladodes are rich in soluble and insoluble fiber [14], fermentation substrates for GM. We have reported that nopal ingestion not only reduced neuroinflammation but was able to restore GM dysbiosis in obese rats [15]. Thus, nopal can be considered a BF with potent

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modulatory GM effects. We also included soy (*Glycine max*). Soy has the ability to reduce homocysteine levels [99], associated with decrease neuroinflammation [100]. Soy also contains polyunsaturated fatty acids and isoflavones with neuroprotective actions, such as genistein [101]. Genistein is isoflavon present in soy, and we have recently demonstrate that genistein supplementation reduces microglia activation and improves cognition in obese mice [102]. BF also contained chia seed (*Salvia hispanica*), a seed rich in fiber and minerals, and constitutes a source of n-3 fatty acids [103]. It also contains various flavonoids that offer anti-inflammatory actions [104]. Finally, we included turmeric (*Curcuma long*) whose main components are curcuminoids. Turmeric has shown important beneficial effects in animal models of AD by inhibiting the generation and aggregation of Aβ [105, 106]. Curcumin administration cause an inhibition of GSK3β and reduces Aβ-induced and tau phosphorylation [107], improved mitochondrial metabolism and reduced oxidative stress [108]. Therefore, a combination of these foods may have impact AD hallmarks by its individual components, but also by a synergistic effect under different mechanism.

Several clinical studies have highlighted the impact of a healthy diet against the onset of dementia [109–114]. However, it is not clear how the ingestion of certain foods can damper the onset of brain pathologies. GM composition is highly dependent on diet [15, 115] and recent data demonstrates an important role of GM on AD pathology [23, 41, 42]. GM can influence host health [116] or alter behavior [117]. It is important to highlight the presence of pro-inflammatory bacteria and neurotoxins derived from bacterial membranes as LPS, in AD brains [18, 22, 118, 119]. Remarkably, in this study we also observed an increase in the relative abundance of pro-inflammatory bacteria (*H. parainfluenza, L. ruminus, S. anginosus, H. parainfluenza, A. parahaemolyticus, P. copri*) and decrease in anti-inflammatory related bacteria (*A. muciniphila, B. uniformis, F. prausnitzii*) in feces from TG-AIN compared to WT-AIN mice.

L. ruminis has stimulatory effects on the secretion of tumor necrosis factor (TNFα) [120], while *S. anginosus* cause brain abscesses, meningitis, cerebral venous system thrombophlebitis, intracranial arteritis, and inflammation [121]. *Actinobacillus* species are Gram-negative bacteria responsible for several disease conditions of animals [122] and *H. parainfluenzae* is considered as a gut pathogen associated with irritable bowel syndrome [123] and abundantly present in autistic children [124]. Furthermore, *H. parainfluenzae* has been reported to cause meningitis [125]. *P. copri* is a well-known propionate producing bacteria [126, 127]. Propionate is considered a potent neurotoxic agent that generates neuroinflammation [128–130]. It is known that genetic diseases that affect the function of the enzyme propionyl-CoA carboxylase (Propionic Acidemia), are characterized by high levels of propionate and high incidence of dementia [131]. Moreover, intraventricular infusions of propionate increased reactive astrogliosis and activate microglia [132], while the incubation of propionate in cultured astrocytes leads to morphological changes and phosphorylation of GFAP [128]. We observed higher propionate concentration in the brain tissue of TG-AIN compared to that of WT-AIN mice. This was consistent with the increase in the level of FFAR3/GPR41 in brain tissue, a receptor with a high affinity for propionate [133, 134]. Due to the dramatic increase in the relative abundance of *P. copri* in fecal samples and the propionate levels in brain of TG-AIN mice, we decided to determine if propionate was associated with the course of AD pathology. We measured SCFAs levels in fecal samples from 3-, 6-, and 9-month-old TG and WT mice. Overall, the total SCFA concentration decreased with aging in both WT and TG mice; however, this reduction was less pronounced in TG mice. At 6 months of age, propionate levels were higher in TG compared to WT mice. It has been reported that several bacterial fermentation products, such as acetate and propionate were higher in plasma and saliva of AD patients [135, 136], supporting our current results.

The GM analysis also indicated that TG-AIN presented a reduced relative abundance of beneficial bacteria, such as *A. muciniphila, B. uniformis*, and *F. prausnitzii*. The role of *Akkermansia* on the gut barrier has been reported, as its presence is associated with increased mucus layer thickness alleviating metabolic endotoxemia (systemic inflammation caused by bacterial associated components, such as LPS) [137]. Therefore, reductions in *A. muciniphila* in TG-AIN mice may lead to increased gut barrier leakage and free passage of LPS, whereas *A. muciniphila* relative abundance increased after BF ingestion, that could help to restore membrane permeability and reducing LPS passage to blood circulation. *F. prausnitzii´s* anti-inflammatory actions has been associated with the expression of a potent Microbial Anti-inflammatory Molecule (MAM) [138, 139]. Therefore, all this data

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may indicate that TG-AIN mice lives under a predominant pro-inflammatory microbiome, supporting recent hypothesis of an infectious aetiology for AD [32–34, 140]. Remarkably, BF supplementation reduces the relative abundance of pro-inflammatory bacteria while increases the anti-inflammatory ones.

It is important to highlight that SCFAs are recognized metabolic substrates for colonocytes [141, 142], but also for glial cells, specifically astrocytes [143]. In astrocytes, propionate is taken up and metabolized by oxidative phosphorylation [143] increasing mitochondrial function [144]. We can propose that pro-inflammatory bacteria released substances causes on the one hand, LPS-induced immune activation of glia cells. On the other hand, uptake of propionate by astrocytes as energy source induces SIRT1 increase; both events, can be associated with an impaired Glu-Gln shuttle, resulting in synaptic dysfunction and memory impairments in TG mice. All those alterations were restored in TG mice after ingestion of BF during 7 months. Based on the present data, we propose that a dietary intervention at an early stage of AD pathology is an effective strategy to abate GM dysbiosis with pro-inflammatory profile, associated with amyloid pathology, metabolic, and synaptic alteration, resulting in a better cognitive outcome.

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SUPPLEMENTARY MATERIAL

Bioactive Food Abates Metabolic and Synaptic Alterations by Modulation of Gut Microbiota in a Mouse Model of Alzheimer's Disease

Supplementary Table 1
Taxonomic classification of pyrosequences from bacterial communities at the Genus level

GENUS	WT-AIN	TG-AIN	TG-BF
Propionibacterium	0.0107 ± 0.005	0.01 ± 0.002	0.002 ± 0.001
Bifidobacterium	0	0.289 ± 0.034	0.736 ± 0.297*
Collinsella	0	0.170 ± 0.109	0.161 ± 0.050
Slackia	0	0.013 ± 0.006	0.018 ± 0.012
Bacteroides	21.37 ± 0.648	12.57 ± 1.227**	21.86 ± 2.206***
Prevotella	8.69 ± 0.684	42.53 ± 1.775****	23.00 ± 3.709****, **
AF12	1.153 ± 0.112	0.010 ± 0.001****	0.003 ± 0.002****
Alistipes	0.004 ± 0.002	0.040 ± 0.026	0.059 ± 0.03
Rikenella	0.115 ± 0.018	0.0002 ± 0.0002****	0.001 ± 0.0008****
Butyricimonas	0.121 ± 0.009	0.529 ± 0.115*	0.371 ± 0.106
Odoribacter	3.255 ± 0.121	0.726 ± 0.127*	1.921 ± 1.106
Paraprevotella	1.095 ± 0.030	0.961 ± 0.377	1.886 ± 0.686
[Prevotella]	9.166 ± 0.126	2.099 ± 0.447****	1.940 ± 0.832****
Mucispirillum	0.647 ± 0.075	0.006 ± 0.002****	0.0007 ± 0.0007****
Granulicatella	0	0.013 ± 0.003*	0.003 ± 0.002
Enterococcus	0.023 ± 0.009	0.02 ± 0.018	0.035 ± 0.021
Lactobacillus	0.889 ± 0.126	0.148 ± 0.011****	0.043 ± 0.011****
Streptococcus	0.034 ± 0.010	1.433 ± 0.245****	0.354 ± 0.070***
Turicibacter	0.063 ± 0.011	0.0002 ± 0.0002****	0****
Clostridium	0.147 ± 0.013	0.072 ± 0.029	0.109 ± 0.026
SMB53	0.383 ± 0.032	0.062 ± 0.022****	0.022 ± 0.02****
Dehalobacterium	0.631 ± 0.047	0.006 ± 0.002****	0.002 ± 0.001****
Anaerostipes	0.016 ± 0.003	0.036 ± 0.020	0.083 ± 0.03
Blautia	0.104 ± 0.008	0.660 ± 0.290	0.431 ± 0.145
Clostridium	0	0.010 ± 0.004	0.007 ± 0.002
Coprococcus	0.809 ± 0.052	0.927 ± 0.108	1.531 ± 0.420
Dorea	0.044 ± 0.005	0.423 ± 0.038***	0.333 ± 0.093**
Lachnobacterium	0.141 ± 0.028	5.710 ± 0.341****	1.99 ± 0.298****, **
Lachnospira	0.206 ± 0.02	6.8 ± 0.340***	4.661 ± 1.631*
Roseburia	0.612 ± 0.020	4.593 ± 0.312***	5.321 ± 1.03***
[Ruminococcus]	0.481 ± 0.055	1.516 ± 0.271**	0.752 ± 0.153*
Rc4-4	0.343 ± 0.043	0.002 ± 0.001****	0.003 ± 0.001****
Anaerotruncus	0.056 ± 0.009	0****	0****
Butyricicoccus	0.008 ± 0.003	0.040 ± 0.016	0.187 ± 0.080*
Faecalibacterium	4.810 ± 0.053	3.706 ± 0.561	7.999 ± 0.531****, **
Oscillospira	10.92 ± 0.243	1.915 ± 0.303****	4.583 ± 0.738**, ****
Ruminococcus	4.502 ± 0.135	1.033 ± 0.265****	2.381 ± 0.406*, ***
Dialister	0.123 ± 0.023	1.094 ± 0.314*	0.590 ± 0.149
Megasphaera	0.785 ± 0.131	0.001 ± 0.0005****	0****
Phascolarctobacterium	1.457 ± 0.083	0.737 ± 0.464	3.971 ± 1.010**, *
Veillonella	0.098 ± 0.008	0.492 ± 0.130*	0.288 ± 0.084
Allobaculum	0.112 ± 0.019	0.001 ± 0.0006****	0.001 ± 0.001****
Asteroleplasma	0.002 ± 0.002	0.030 ± 0.005	0.129 ± 0.123
Catenibacterium	0.01 ± 0.008	0.112 ± 0.013***	0.047 ± 0.019*
Clostridium	0.126 ± 0.023	0.0007 ± 0.0004****	0.003 ± 0.001253****
Holdemania	0.019 ± 0.005	0.011 ± 0.005	0.001 ± 0.002
[Eubacterium]	0.009 ± 0.003	0.175 ± 0.029**	0.182 ± 0.05**
Victivallis	0.003 ± 0.002	0.001 ± 0.0004	0.431 ± 0.311
Sutterella	5.560 ± 0.265	2.758 ± 0.336****	2.083 ± 0.386****
Burkholderia	0.005 ± 0.002	0.025 ± 0.009	0.026 ± 0.011
Bilophila	0.050 ± 0.012	0.028 ± 0.005	0.078 ± 0.011**
Desulfovibrio	0.088 ± 0.014	0.015 ± 0.008	0.134 ± 0.07

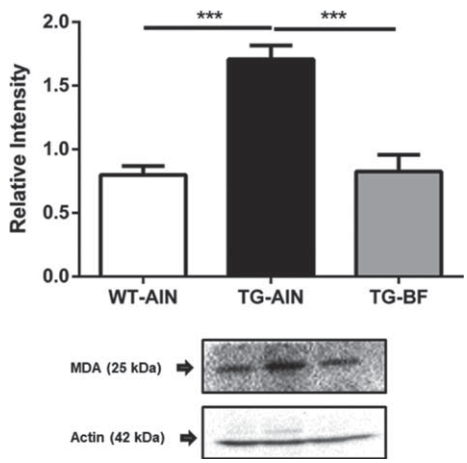
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Supplementary Table 1
(*Continued*)

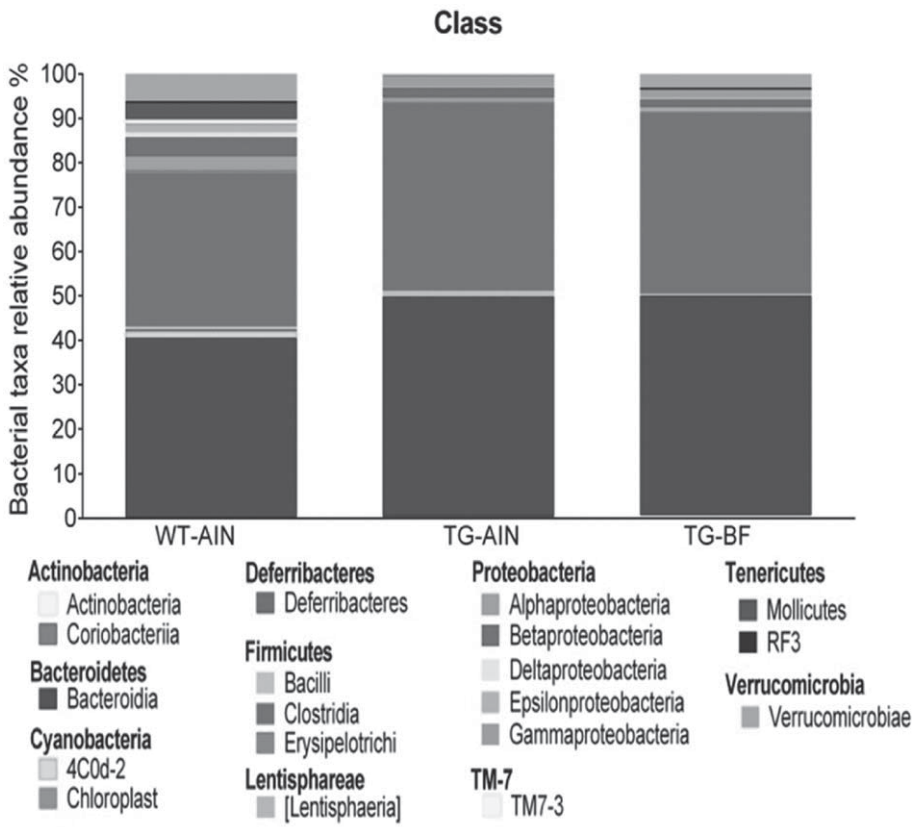
GENUS	WT-AIN	TG-AIN	TG-BF
Helicobacter	2.585 ± 0.091	0.025 ± 0.005****	0.008 ± 0.003****
Succinivibrio	0.144 ± 0.015	0.135 ± 0.134	0
Escherichia	0.09 ± 0.010	1.597 ± 0.501	2.060 ± 1.031
Klebsiella	0.073 ± 0.011	0.043 ± 0.008	0.339 ± 0.179
Actinobacillus	0	0.123 ± 0.014****	0.031 ± 0.010****
Haemophilus	0.018 ± 0.004	1.073 ± 0.170****	0.206 ± 0.057***
Pseudomonas	0.375 ± 0.044	0.027 ± 0.009****	0.024 ± 0.009****
Anaeroplasma	5.452 ± 0.219	0.008 ± 0.002****	0.003 ± 0.002****
Akkermansia	8.438 ± 0.561	0.49 ± 0.162****	3.501 ± 0.274****.****

Statistical differences between groups: WT-AIN versus TG-AIN are reported in black, WT-AIN versus TG-BF are reported in red, TG-AIN versus TG-BF are reported in green. Data represents mean ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. WT-AIN, $n = 6$; TG-AIN $n = 7$; TG-BF, $n = 7$.



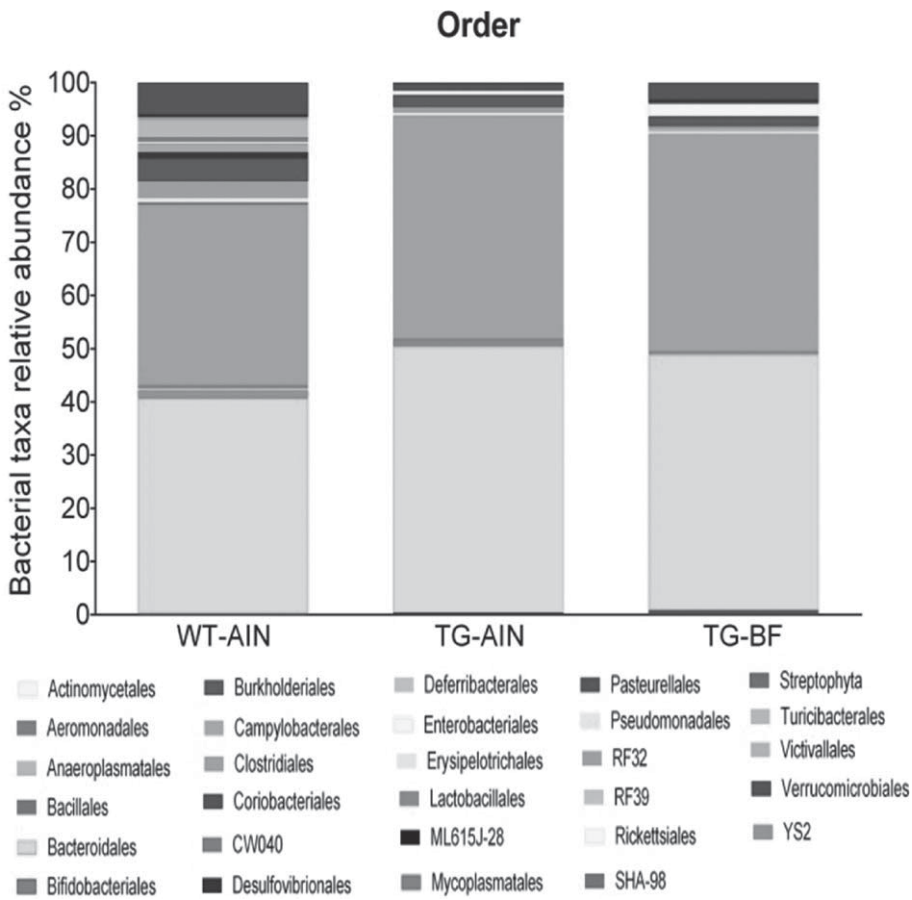
Supplementary Figure 1. Protein levels of malondialdehyde (MDA) in brain cortex. MDA levels were higher in TG-AIN compared to WT-AIN ($p < 0.001$) and TG-BF mice ($p < 0.001$. Images below graphs are representative blots for each protein done five times, in pool samples. Data represents mean ± S.D. ***$p < 0.001$.

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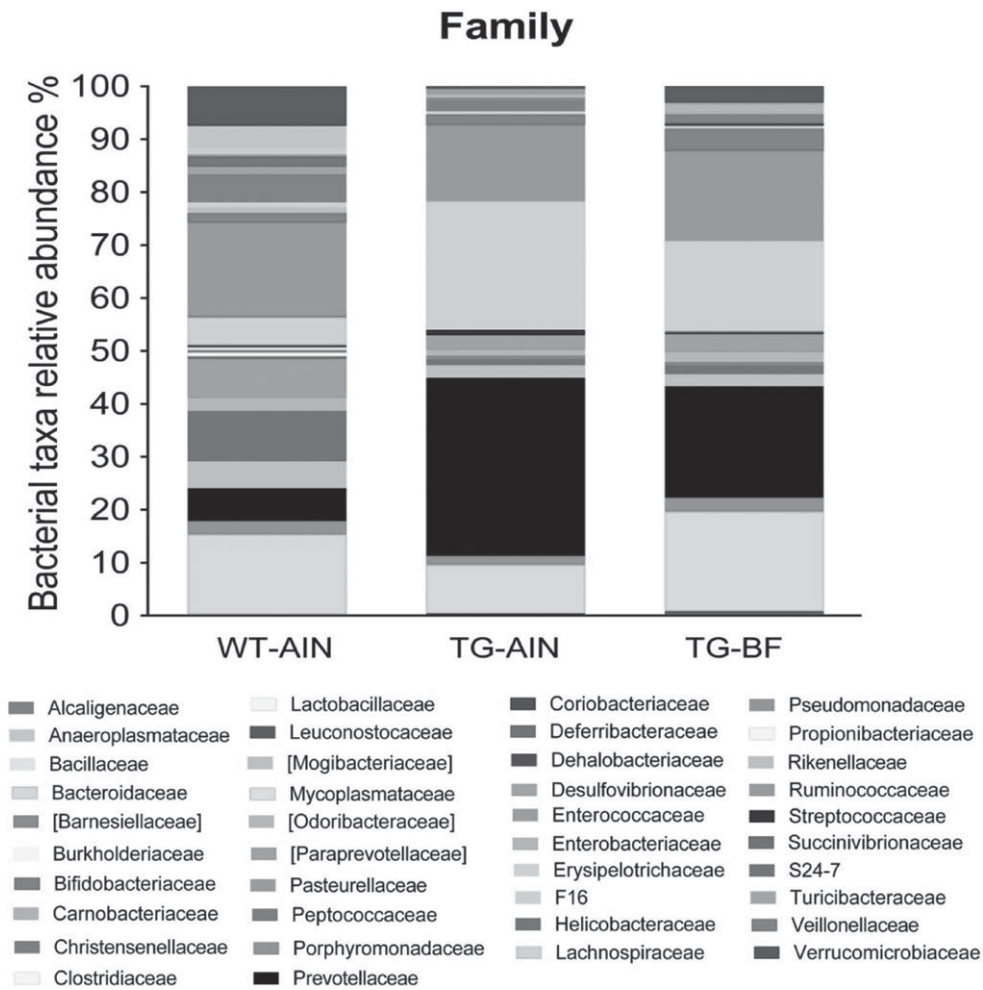
Supplementary Figure 2. Bacterial relative abundance at the class level in WT-AIN, TG-AIN, and TG-BF mice.

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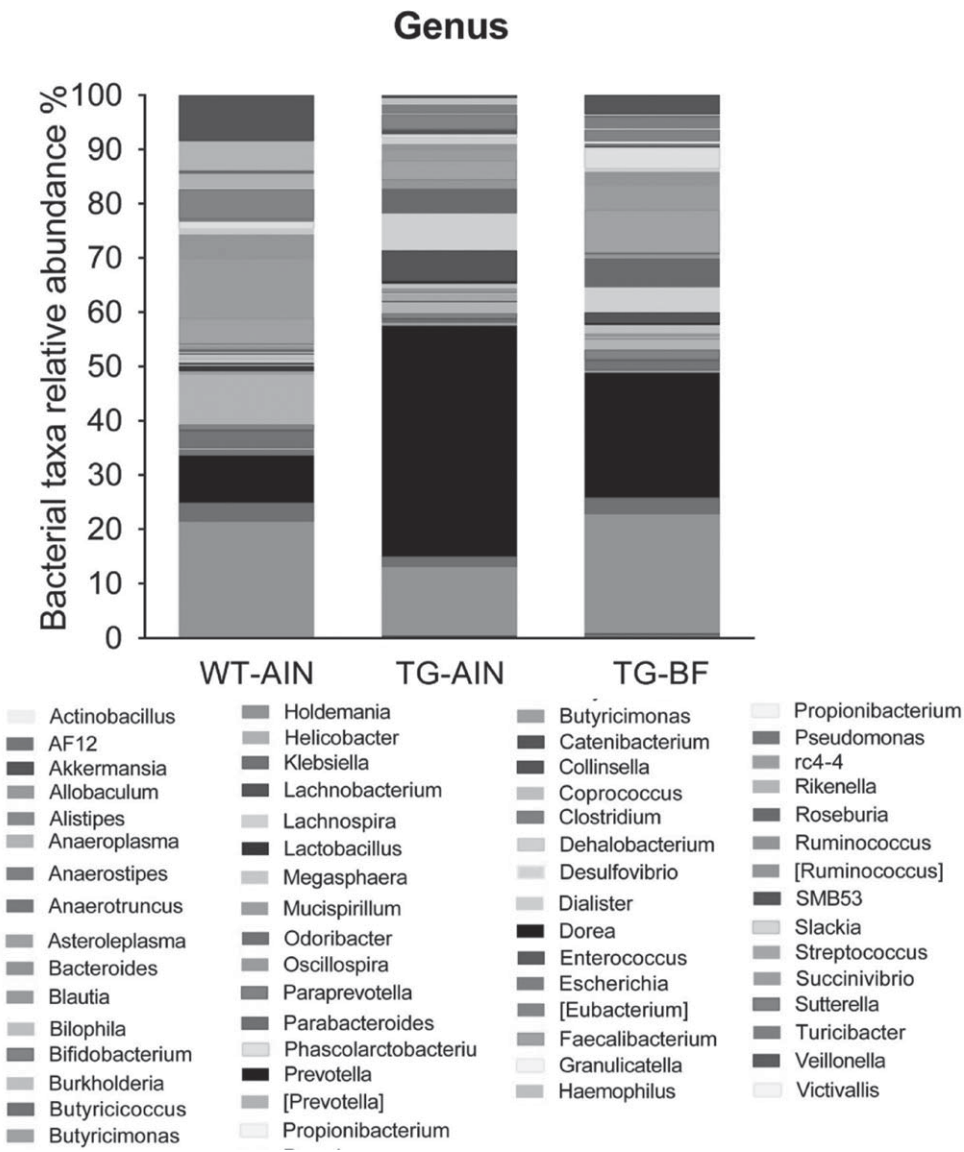
Supplementary Figure 3. Bacterial relative abundance at the order level in WT-AIN, TG-AIN, and mice.

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Supplementary Figure 4. Bacterial relative abundance at the family level in WT-AIN, TG-AIN, and TG-BF mice.

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Supplementary Figure 5. Bacterial relative abundance at the genus level in WT-AIN, TG-AIN, and TG-BF mice. Values and statistical differences are shown in Supplementary Table 1.

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Geographical Distribution and Diversity of Gut Microbial NADH:Ubiquinone Oxidoreductase Sequence Associated with Alzheimer's Disease

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Abstract. Earlier we reported induction of neurotoxicity and neurodegeneration by tryptophan metabolites that link the metabolic alterations to Alzheimer's disease (AD). Tryptophan is a product of the Shikimate pathway (SP). Human cells lack SP, which is found in human gut bacteria exclusively using SP to produce aromatic amino acids (AAA). This study is a firs attempt toward gene-targeted analysis of human gut microbiota in AD fecal samples. The oligonucleotide primers newly-designed for this work target SP-AAA in environmental bacteria associated with human activity. Using polymerase chain reaction (PCR), we found unique gut bacterial sequence in most AD patients (18 of 20), albeit rarely in controls (1 of 13). Cloning and sequencing AD-associated PCR products (ADPP) enables identificatio of Na(+)-transporting NADH: Ubiquinone reductase (NQR) in *Clostridium* sp. The ADPP of unrelated AD patients possess near identical sequences. NQR substrate, ubiquinone is a SP product and human neuroprotectant. A defici in ubiquinone has been determined in a number of neuromuscular and neurodegenerative disorders. Antibacterial therapy prompted an ADPP reduction in an ADPP-positive control person who was later diagnosed with AD-dementia. We explored the gut microbiome databases and uncovered a sequence similarity (up to 97%) between ADPP and some healthy individuals from different geographical locations. Importantly, our main findin of the significan difference in the gut microbial genotypes between the AD and control human populations is a breakthrough.

Keywords: Alzheimer's disease, bacterial enzyme sequence, environmental stool samples, human gut microbiota, PCR testing

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease with a yet unknown cause. Consequently, monitoring causative factors of AD as well as other neurodegenerative diseases is not possible. Such monitoring could both estimate AD risk and direct the treatment to blocking or eliminating causative factor(s). We previously reported that tryptamine and tryptophanol, the immediate metabolites of tryptophan (trp), an essential aromatic amino acid (AAA), induce cell death and neurodegeneration in cells and animals [1–3]. Trp is a product of metabolic shikimate pathway (SP), a seven-step metabolic route used by

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bacteria, fungi, algae, some protozoan parasites, and plants for the biosynthesis of AAA (phenylalanine, tyrosine, and tryptophan). SP is not found in human and animals, which require AAA to be obtained from bacteria or plants. The immediate decarboxylation of tryptophan results in the synthesis of tryptamine. Tryptamine upregulates at 4.1-fold the gene encoding amyloid β precursor-like protein 2 (Aplp2), which has been implicated in AD pathogenesis [4]. The tryptophan microbiota metabolites: indole-3-acetate, indole-3-aldehyde, indole, and tryptamine are aryl hydrocarbon receptor/xenobiotic-response elements (AhR-XRE) ligands [5]. The SP-AAA metabolism signifie an important role in AD pathogenesis. In the Netherlands, at the early stage of probable AD, the blood plasma total tryptophan levels were signifi cantly reduced [6]. Studies conducted in Sweden [7], USA [8–10], Wales/United Kingdom [11, 12], Austria [13], and China [14] demonstrate reduced levels of tryptophan and other AAA in AD. Metabolomics identifie alterations in blood and cerebrospinal flui tryptophan metabolites and decreases in the plasma levels of tryptophan-containing tripeptides in AD [9, 10]. Higher plasma anthranilic acid levels were associated with greater risk of dementia [15]. Anthranilic acid biosynthesized from chorismic acid is the precursor to tryptophan. Although human cells lack SP and tryptophan biosynthesis pathway, the human-associated microorganisms, particularly gut microbiome has SP and AAA biosynthesis. The organization of trp genes within operons and the regulatory mechanisms used to control trp operon expression vary greatly—from whole-pathway operons to completely dispersed genes [16, 17]. Organisms are harmed and altered through damage to SP [18]. Altogether these finding warrant our interest in human cells-gut bacteria interactome that link the SP-dependent metabolites to neurodegeneration, albeit the data on the SP and related metabolic pathways are scarce for the human gut microbiota. Reports on the human gut microbiome in AD are not yet available.

We created algorithm parameters and database consisting of sequences for environmental bacteria to design the nucleotide primers targeting the SP-related enzymes in PCR analysis. Here, by means of PCR with the usage of DNA isolated from stool samples (SS) of AD patients, we revealed the nucleotide sequence with a significan similarity to conserved domain of bacterial NQR enzyme that catalyzes conversion of its substrate ubiquinone (Q), a SP product [19] into ubiquinol. It was observed that [G-14C] shikimate was incorporated into both menaquinone and ubiquinone by *E. coli*, thus providing the firs evidence for the involvement of the SP in the Q synthesis [20]. We found that genes coding NQR subunits locate close to genes related to SP thus organizing putative gene operon in some bacteria. NQR presents mainly in pathogenic and marine bacteria [21]. Of special interest is a physiological role of the NQR substrate Q, which is neuroprotective for human cells [22, 23]. Q is a substrate of human NADH-Ubiquinone oxidoreductase (mitochondrial enzyme Complex I or CI) in the respiratory chain of mitochondria functions as a redox driven proton pump complex [24]. The early synaptic mitochondria perturbations were demonstrated in AD [25]. In our study, the mitochondrial neuropathology observed in the tryptamine-induced model of neurodegeneration [3]. Q, an important component of the electron transfer system, is constituted of a quinone structure and a side chain isoprenoid. The side chain length of Q differs between microorganisms, and this difference has been used for taxonomic study [26]. Reportedly, Q10 is a main Q form circulated in human blood, while most bacteria synthesize Q8 [27–29]. Q10 is produced by human cells from tyrosine. In children plasma, Q10 was detected at 0.15–1.32 mg/l and Q9 at 0.04–0.06 mg/l [30]. The source of Q9 is not clear, but origin from gut microbiota cannot be ruled out. We hypothesize here that bacterial Q8, a close analog of human Q10, can be an effective CI inhibitor. Such potential CI inhibition and/or disassembly by Q8 can derail mitochondrial functions in human. The importance of the length of the side chain of Q for *Saccharomyces cerevisiae* was examined by producing Q with isoprenoid chain lengths between 5 and 10 [31]. The original species of Q (in the case of *S. cerevisiae* Q6) had maximum functionality. However, the other species of Q could replace Q6. The authors concluded that a broad spectrum of different Q species is biologically functional in yeast cells, although cells seem to display a preference for their own particular Q type. Q10 defici has been determined in neurodegenerative and neuromuscular diseases [32–34] and is associated with increased risk of disabling dementia [35]. No data available on Q8 in human bodily fluids Q, a key molecule for cellular bioenergetics that is conserved from proteobacteria to humans, appeared in an ancestral proteobacterium more than 2 billion years ago [36]. Q biosynthesis has been studied only in a few model organisms. NQR is a key component of the respiratory chain of pathogenic bacteria [37].

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MATERIALS AND METHODS

Collection of stool samples (SS)

The non-invasive environmental SS from 20 clinically diagnosed AD patients and 5 cognitively normal individuals were collected from pumper/diaper (a unit used for the collection of stool samples) at the Bidwell Care Center, Connecticut, USA (BCC). The control SS were also collected from the cognitively normal volunteers living at the private residents in California and Florida (USA). The SS from AD patients (11 females and 9 males, 58 to 89-year old) and 13 control SS from: 1) Florida (58-year-old female and a companion dog); 2) California (6 samples, 19–89 years old); 3) BCC, Connecticut (5 samples, 44–65 years old) were used in this study. SS were freshly collected at home or at the BCC, as environmental cotton swabs in 2-ml plastic tubes. Two or more SS were collected for each individual. The SS were transported at room temperature, stored frozen at –80°C upon receipt and until DNA extraction and purification DNA was extracted directly from SS or from stool culture. The list of SS is included in Supplementary Table 1. The SS of a healthy companion dog used in this study because many diseases of the dog have a human counterpart, and notably AD [38]. The study protocol was approved by the Institutional Review Board of Expert Biomed, Inc. (FL, USA).

Statistical analysis

Statistical analyses were performed with Prism 7.02 software (GraphPad, San Diego, CA). Data are expressed as mean ± standard deviation (SD). The groups were compared using unpaired two tailed *t* test. p-values < 0.05 were considered significant

Methods for DNA extraction from SS, DNA extraction from bacteria, design of nucleotide primers for PCR, polymerase chain reaction, cloning and sequencing PCR products are included in the Supplementary Material.

RESULTS AND DISCUSSION

PCR test and DNA sequencing of cloned PCR products

A single set of nucleotide primers selected out of several designed sets of forward/reverse primers (Supplementary Figures 1–3) enabled us to reveal the AD-associated PCR products (ADPP) in 90% of tested AD patients (Fig. 1 and Supplementary Table 1) and 10% of control individuals (Fig. 2). The expected PCR product size of 249 base pairs (bp) is close to the observed 230 bp PCR product/s determined by comparison with a DNA ladder (a molecular weight marker). Figures 1–3 of the typical PCR test include the positive and negative samples resolved in 3% agarose gel that stained with 0.01% ethidium bromide. The DNA sequences of the cloned 230-bp PCR products from different AD patients (Supplementary Figure 2) were identical to each other in inserts with correct and opposite orientations. The sequence analysis revealed significan similarity between ADPP and conserved domain of Rnf-Nqr protein family. The Rnf-Nqr subunit is a family of integral membrane proteins including nitrogen

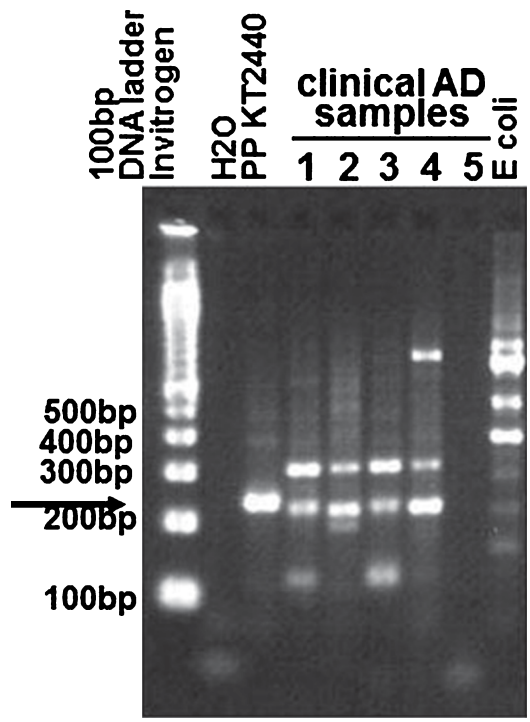


Fig. 1. A representative 3% agarose gel stained with 0.01% (v/v) ethidium bromide. PCR products obtained after amplificatio of DNA (50 ng per reaction) extracted from stool specimens of AD patients (SS1 to SS5, lanes 1 to 5, respectively) using the KTHR-F and KTHR-R primer pair. The control bacterial DNA (1 ng per PCR reaction) was isolated from *Pseudomonas putida* (PP) KT2440 and *E. coli*. DNA markers are the left lane. PCR conducted in 20 μl, the 5 μl loaded per lane. Markers (100 bp DNA Ladder, Invitrogen) loaded at 7 μl per lane (1 μg/μl). Note, the specifi PCR product was undetectable with DNA extracted from the same SS of AD patients 1–5 and the usage of control sets of primers (Supplementary Figure 3 and Materials and Methods).

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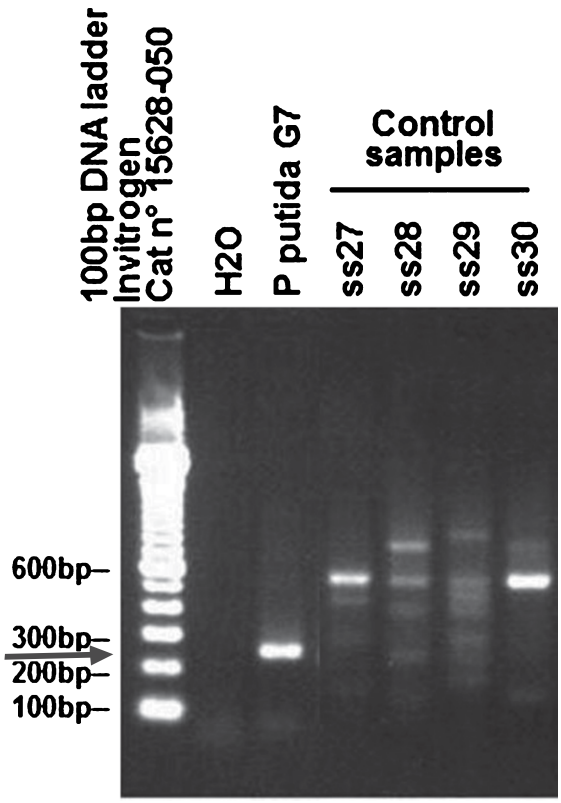


Fig. 2. A representative 3% agarose gel electrophoresis of PCR products obtained after amplificatio of DNA extracted from control human stool samples (SS27 to SS30) using the KTHR-F and KTHR-R primer pair. The gel was stained with 0.01% (v/v) ethidium bromide. DNA was extracted from SS of donors with no prior diagnosis of AD. DNA markers: the left lane (Invitrogen, 100 bp ladder Cat No 15628-050). DNA extracted from *Pseudomonas putida* (P. putida) G7 used as a positive standard control. Arrows indicate a position/size of the expected PCR product.

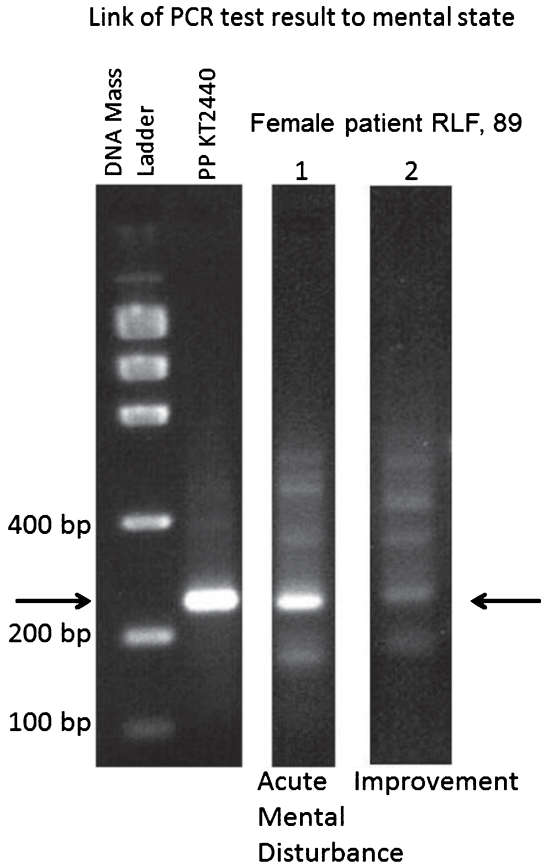


Fig. 3. The 3% agarose gel electrophoresis of PCR products for analysis of DNA extracted from stool of case RLF with no prior diagnosis of AD: comparison of DNA specimens of the periods of active mental disturbance and mental state improvement with synthetic primers KTHR-F and KTHR-R. DNA mass ladder (Life Technologies) used for estimating the mass (quantity) of unknown DNA samples by 0.01% (v/v) ethidium bromide staining. The Low DNA Mass Ladder is composed of an equimolar mixture of six blunt-ended DNA fragments of 2000, 1200, 800, 400, 200, and 100 bp. Electrophoresis of 4 μL of the Low DNA Mass Ladder results in bands containing 200, 120, 80, 40, 20, and 10 ng of DNA, respectively.

fixatio (rnf) proteins and Na+-translocating NADH: Ubiquinone oxidoreductase (Na+-NQR) subunits. Both NQR and electron complex RNF are multisubunit membrane proteins. It was postulated that evolution of the Na+-NQR complex involved a functional divergence from its RNF homolog, following the duplication of the rnf operon [39]. We found that in some human gut bacteria, an apparently single gene operon includes both rnf and nqr genes. A closely-related complex, RsxABCDGE, reduces the 2Fe-2S-containing superoxide sensor SoxR, active as a transcription factor when oxidized. This family of putative NADH oxidoreductase complexes exists in many of the same bacterial species as the related NQR. We found that gene for an enzyme that supplies octaprenyl diphosphate, the precursor for the side chain of the isoprenoid quinones – ubiquinone and menaquinone – heptaprenyl diphosphate synthase component (HDS) locates in close proximity to the rnf-nqr genes probably in the same gene operon like in human gut *Eubacterium* sp. (GenBank: CBIL010000031.1). Another flankin gene, XRE (xenobiotic-response elements) family transcriptional regulator, locates upstream of the rnf-nqr genes in this bacteria. Therefore, we developed a method wherein the designed nucleotide primers produce PCR products of ∼230 bp in agreement with expected estimation of 249 bp, and the additional product of 300 bp in approximately 85–95% of AD samples and 10% of control samples (Fig. 4). The

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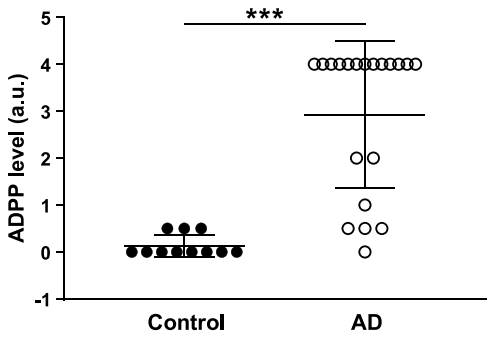


Fig. 4. ADPP abundance in stool samples from AD patients and control individuals. PCR products generated with KTHR-F and KTHR-R nucleotide primers were analyzed by 3% agarose gel electrophoresis following by ethidium bromide (0.01%, v/v) staining. A relative intensity of the 230 bp ADPP band was scored using an arbitrary (conventional) scale (a.u.) from 0 to 4 (Supplementary Table 1). ***$p < 0.001$. In accordance with the quantitative DNA markers (Fig. 3 and Supplementary Figure 2A), the 1 a.u. corresponds to ~20 ng of DNA.

supplementary 800 bp PCR product was detected in 20% of AD samples. A semi-quantitative score in arbitrary (conventional) units (a.u.) was used to evaluate the ADPP abundance in SS. The intensity of the 230 bp ADPP band on agarose gel (Supplementary Table 1) was graded from 0 (negative sample) to 4 (maximal observed level). Statistical analysis demonstrated significantl higher ADPP levels in the SS of AD patients compared to control individuals (2.925 ± 1.567 a.u. versus 0.136 ± 0.234 a.u., respectively; $p < 0.001$, unpaired t test). In accordance with the quantitation of the DNA markers (Fig. 3 and Supplementary Figure 2A), 1 a.u. corresponds to ~20 ng of DNA. Thus, the significan difference between the two groups supports the hypothesis of an essential role of gut microbiota in AD. The tested dog (Supplementary Table 1, SS13), a companion pet of a control individual (Supplementary Table 1, SS12), showed a weakly positive PCR response but no signs of mental and cognitive impairments. The analysis of the companion dog sample (SS13) might shed light on the transmission source for the agent leading to the PCR weak positivity in SS12. In the present study, this dog sample is used as a negative control. However, the risk for human persons to get infection from their pets, the possible carriers of AD-associated bacteria, remains to be more extensively tested. In spite of the small sample size, the statistically significan difference between the two groups of cognitively normal and AD gut microbial populations is a breakthrough finding

In the control DNA isolated from SS of female RLF, 89, living in a private residence, California, USA, we revealed ADPP positivity. RLF had no clinically diagnosed AD but had the symptoms of acute mental disturbance with balance impairment and auditory deficit characterized by fluctuatin hearing levels at the time of the firs SS collection (October, 2010). The PCR product for RLF was similar to that revealed for AD patients. A second biosample of RLF (February, 2011) was collected following antibacterial treatment with Avelox (400 mg/day) prescribed due to pneumonia (Supplementary Table 1). The PCR product was barely detectable after the treatment with Avelox (Fig. 3, lane 2, Fig. 4). The mental status of RLF improved following the antibacterial therapy. In a 3-year follow-up observation, RLF had been diagnosed with dementia. In 2017, RLF has late stage AD-type dementia and does not recognize family members. The samples from RLF were provided with informed consent given by RLF's son (his sample SS16 showed no ADPP). RLF's sister (SS17) who lives in different location (one year younger than RLF) showed no ADPP in SS and no mental problems during the observation period 2010–2017. In the case of RLF, the appearance of ADPP correlates with the acute mental disturbance that can be suggested as an early sign of dementia that was later diagnosed. The antibacterial treatment resulted in significan reduction of ADPP that correlated with a prolonged mental improvement (Fig. 3). Note, that in May 2016, the FDA issued an Avelox (Fluoroquinolone antibiotic) warning, indicating that the drug is too dangerous to be used for many common types of infections. The young 19-year-old male, MAF (Supplementary Table 1, SS14), is a grandson of RLF (SS15, SS32, SS33). The analysis of the family members of RLF (Supplementary Table 1)—sister (SS17), son (SS16), and grandson (SS14)—might shed light on the nature of the RLF test positivity. Our data demonstrate that only one of 4 tested family members showed PCR positivity with a consequent development of AD-type dementia. The other 3 family members were negative and showed no signs of cognitive impairment. The analysis of the NCBI databases revealed human sequences similar to AD-associated PCR sequence in young individuals (Table 1). We tested SS collected in different locations and different vectors for cloning and sequencing to exclude effects of putative nosocomial infection, contamination in the research laboratory, or errors in cloning/sequencing PCR products.

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Table 1
Percent of nucleotide (NT) and amino acid (AA) sequence identity/similarity between AD-associated PCR product (ADPP) and human gut microbiome databases from different geographical locations (cutoff is from 86% to 97% in amino acid sequences in National Center for Biotechnology Information (NCBI) Blast alignment

Country	NT identity %	AA identity %	Health status	gender	age	Bacteria	References
MO, USA	87	97	obese	female	26	Cl	[40, 41]
Canada-1	89	97	antibiotic	female	35		[45]
Canada-2	89	97	antibiotic	male	23		[45]
Denmark-1a	71	87	obese	female	49	Cl	[46, 47]
Denmark-1b	71	86	obese	female	49	Ru	[46, 47]
Spain-1a	70	88	Overweight, UC	female	36	Ru	[46, 47]
Spain-1b	76	86	Overweight, UC	female	36	Ru	[46, 47]
Spain-2	73	87	UC	female	30	Eu	[46, 47]
Japan-1 (spouse)	72	87	healthy	female	36		[50]
Japan-2 (spouse)	73	87	healthy	male	37		[50]
Japan-3 (single)	73	87	healthy	female	28		[50]

Cl, *Clostridium* sp.; Ru, *Ruminococcus* sp.; Eu, *Eubacterium* sp.; a and b, the same individual tested twice; NT, nucleotides; AA, amino acids; UC, ulcerative colitis.

Comparison of ADPP sequence with human gut microbiome NCBI databases

USA study

The 76 amino acids deduced from a single open reading frame (ORF) of ADPP shows 97% identity (Table 1) to the sequence deduced from a single ORF of ∼30-kilobase (kb) DNA-fragment from whole genome shotgun (WGS) of uncultured unclassifie bacteria *Clostridium* sp. from a SS of a 26-year-old obese identical co-twin (Missouri, USA). This USA human gut metagenome presents sequenced fecal microbial communities of 154 individuals including adult female monozygotic and dizygotic twin pairs concordant for leanness or obesity, and their mothers [40, 41]. Note, that metagenomic data acquired by deep sequencing of human stool collections is immensely complex, lacks apparent structure, and is typically dominated by unknown species. A large proportion (73%) of the MetaGenomic Species displays no sequence similarity to any previously sequenced organism. The human gut databases include unsupervised structuring of a highly complex series of metagenomic samples into biological entities, including a global analysis of the genetic interdependencies between bacteria, plasmids, phages, and genetic islands in the human distal gut. In some human gut bacteria containing fragment of high sequence similarity to ADPP, the rnf-ngr gene operon appears to include the gene coding HDS that supplies octaprenyl diphosphate, the precursor for the side chain of the isoprenoid quinones ubiquinone, and menaquinone. Hereupon, the hypothesis that we suggested in the Introduction that bacterial Q8 can compete with human Q10 for the binding with human Q-related enzyme(s) and affect enzymatic activity of human mitochondrial complex I (CI) is plausible. In addition, we revealed that at least 12 contigs comprising HDS present in different uncultured gut bacteria (*Clostridium* sp., *Ruminococcus* sp., *Eubacterium* sp., *Collinsella* sp.) in a US co-twin with the ADPP nucleotide-like sequence of a high identity (E value 6e-69) versus 4 contigs in uncultured *Ruminococcus* sp. of a co-twin with a lower similarity/identity to ADPP (E value 6e-24). The potential role of CI misassembly in CI deficiencies AD, and Parkinson's disease is suggested [42]. Reduced level of CI subunits in multiple zones of postmortem AD brains was reported [43]. Mammalian CI is built of 45 subunits. Fourteen "core" subunits are conserved from bacteria [44]. The Q-binding site lies at the interface of the core subunits – hydrophilic 49 kDa/PSST and membrane ND1/ND3 subunits.

Canadian study

The bacterial DNA sequence of the *Clostridium* sp. revealed in the USA study is near identical to DNA fragments (contigs) of ∼4 kb and ∼2 kb (99% identity, 100% cover, E value 0.0) isolated from SS of 3 healthy adults of Canadian human gut metagenome study. Bacterial fragments containing sequence near identical (97% identity in amino acid sequences) to ADPP marker were revealed in two healthy young adults from a Canadian human gut metagenome study after treatment with antibiotic Cefprozil (Table 1). This study included 24 healthy volunteers [45]. SS collected before antibiotic exposure, at the end of the treatment (7-day course), and three months later were analyzed using shotgun metagenomic sequencing. In 5 of 10 individuals, the amino acid sequence

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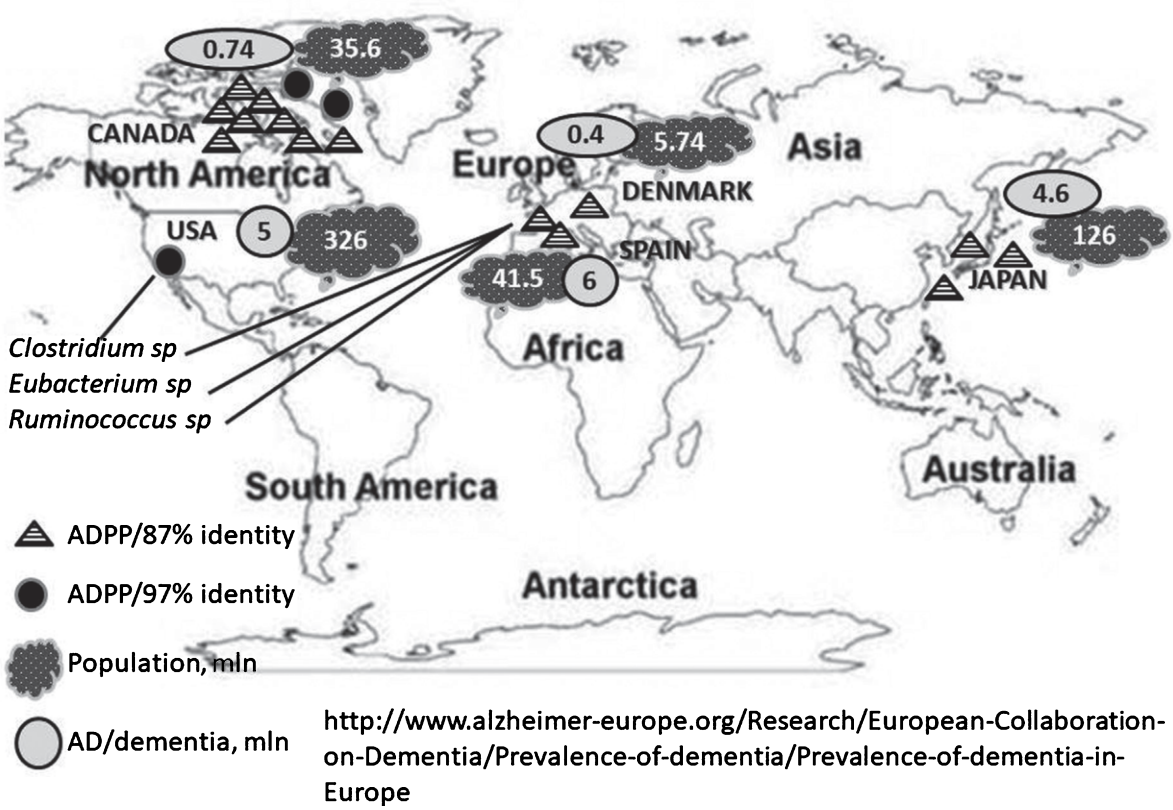


Fig. 5. Geographical distribution of gut microbial ADPP variants in the world population. The total populations and AD prevalence in different countries are indicated in millions (mln).

similar (85–97%) to ADPP appeared following treatment with antibiotic Cefprozil. Hence, we conclude that bacteria comprising ADPP sequence are hidden in many samples. The hidden bacteria can be uncovered following removal of antibiotic-sensitive bacteria from human gut. By other words, the Canadian study supports our suggestion that ADPP-minus bacteria can successfully compete with ADPP-plus bacteria in human gut microbiome. Note, in Canada with a 35.6-million population, over 747,000 individuals are living with AD or another dementia (http://www.alz.org/ca/dementia-alzheimers-canada.asp).

European study

Comparison of human gut metagenomes (Table 1) using the best-BLAST-hit against ADPP (E-value cutoff of 9e-42, minimum 92% coverage, 86% identity) revealed a maximum 87% identity (E-value 1e-42, 93% coverage) in amino acid sequences between the ADPP and 5 different SS of 3 females (Spanish, 36-year old, overweight with ulcerative colitis (UC); Spanish, 30-year old with UC, and Danish, 49-year old healthy overweight/obese) in the European study [46, 47]. This study includes 396 SS from 177 Danish and 141 Spanish individuals [46]; 124 samples were sequenced previously [47]. Spanish study included 13 individuals with Crohn's disease and 69 with UC; 77 of the Spanish individuals were sampled twice with, on average, 6 months between the samplings. For two Spanish females with UC, appearance/disappearance of ADPP sequence in WGS sequence database correlated with weight gain/loss between the samplings, respectively. The Danish samples include healthy individuals ranging in body mass index 18–42. Study of prevalence of AD and other dementing disorders in Pamplona, Spain (Fig. 5) revealed 194 subjects affected by dementia and 119 had AD in the total population 1,127 [48]. The prevalence rate was 7.1%, including the very mildly demented in Denmark [49].

Japanese study

The 87% of amino acid sequence identity (E-value 1e-42, 93% coverage) occurs between ADPP and 3 adults (one individual and a family of married couple)

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of the Japanese study [50]. This study included 13 healthy individuals. In Japan with a 126.6 million-population, more than 4.6 million people are living with dementia (http://www.alz.org/jp/dementia-alzheimers-japan.asp). The findin of fecal bacterial ADPP in the family (Table 1) supports the infectious nature of the environmental insult (Fig. 5).

Conclusions

This study is a firs attempt toward gene-targeted analysis of human gut microbiota in AD fecal samples. The oligonucleotide primers newly-designed for this work target the environmental bacteria associated with human activity (Supplementary Figure 4). The results of the follow-up case suggest ADPP as an AD predictor. A human gut metagenome database comparative analysis and geographical distribution of the NQR-ADPP sequence is explored. We analyzed samples of our own collection and also NCBI human gut microbiota databases of the studies conducted in different geographical locations (Fig. 5). We found that human gut nucleotide/protein sequences with a high percent identity to ADPP present in USA and Canada and with a lower similarity in Europe (Denmark and Spain) and Japan. The ADPP sequence can be a part of bacterial chromosome, plasmid, or a mobile bacterial element. This prediction is in agreement with the earlier report indicating that some plasmids or plasmid families are present in the gut microbiomes of geographically isolated human hosts with a broad global distribution (America, Japan, and Europe), and are potentially unique to the human gut microbiome [51]. To create any testable model for the quantitative information on the parameters of the substrate (product) binding (release) for possible interaction between human and gut microbial NQR enzymatic activities is needed. Here the current state of knowledge of bacterial NQR conserved protein domain associated with AD is briefl summarized and discussed. Our data support an important role of microbe-host interactions in AD. The NQR bacterial sequence can be used in the population studies as a new marker for monitoring AD risk and as a new target for anti-bacterial therapy.

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Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-0764r2).

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SUPPLEMENTARY MATERIAL

Geographical Distribution and Diversity of Gut Microbial NADH: Ubiquinone Oxidoreductase Sequence Associated with Alzheimer's Disease

MATERIALS AND METHODS

DNA extraction from stool samples

The 1.4 ml of buffer ASL (Qiagen GmbH, Hilden, Germany) was added to each frozen stool sample. Samples were vortexed continuously for 1 min or until the stool sample is thoroughly homogenized. Thereafter, 0.8 g of acid-washed glass beads ($\leq$106 μm in diameter; Sigma-Aldrich, St. Louis, MO) was added to each sample. Samples were then agitated in a MagNA Lyser Instrument (Roche Instrument Center, Rotkreuz, Switzerland) at 6,500 rpm, for 60s. This step was repeated two more times. Lysates were heated at 95°C for 5 min, vortexed and centrifuged 1 min at 13,000 x g. Each supernatant was transferred to a new centrifuge tube. 360 μl of thus obtained supernatant was underwent a DNA extraction procedure, while the remaining part was frozen and kept at –80°C. Genomic DNA was isolated using phenol/chloroform/isoamyl alcohol extraction method. The 40 μl of 20 mg/ml of proteinase K (Qiagen) and 400 μl of AL buffer (Qiagen) were added to 360 μl of supernatant obtained at the previous step, and samples were incubated 10 min at 70°C. Samples were extracted with an equal volume of the UltraPure Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Life Technologies, Carlsbad, California) and centrifuged 10 min at 13,000 x g. DNA was precipitated from the aqueous layers by the addition of ½ vol of 7.5 M ammonium acetate and 2 vol of 100% ethanol and collected by centrifugation for 15 min at 20,000 x g. DNA pellets were washed twice with 70% ethanol, air dried, and re-suspended in 50 to 100 μl of DNase, RNase free sterile water. Nucleic acid concentration and quality was accessed by determining the UV absorption spectra across the range 220 nm to 320 nm using the NanoVue spectrofotometer (GE Healthcare, Uppsala, Sweden).

DNA extraction from bacteria

The strains of environmental bacteria with sequenced genomes were used for the isolation of standard bacterial DNA to control PCR test. The bacteria strains used in this study are: *Pseudomonas putida* (*P. putida*) KT2440 and *Pseudomonas* sp. strain CF600, the gifts from Dr. Victoria Shingler, professor at the Department of Molecular Biology, Umeå University, Sweden; *P. putida* G7 from Professor Milton Schroth of the University of California Berkeley in Berkeley, CA kindly provided by Dr. Robert Zdor of Andrews University, MI, USA; *Escherichia coli* (E. coli) strain BL21 (DE3) was purchased from Novagen (Merck, Danvers, Massachusetts). Immediately upon receipt of stub cultures, bacteria were plated on a Luria-Bertani (LB) agar plate (Sigma-Aldrich, St Louis, MO) by plate streaking, to isolate single colonies. The plate was incubated overnight at 37°C. A single colony from the overnight LB agar culture was inoculated into 5 ml of liquid LB medium (Sigma-Aldrich) and incubated overnight with vigorous shaking (250 rpm) at 37°. Next morning, 1 ml of thus obtained overnight culture was inoculated into 100 ml of liquid LB medium and incubated with vigorous shaking at 37°C during the day, until the OD600 reaches 1.4-1.6. The bacteria were pelleted by centrifugation at 1,500 x g for 15 min. Bacterial pellet was resuspended in 1.8 ml of ATL buffer (Qiagen) plus 0.2 ml of proteinase K solution (Qiagen) and incubated at 56°C for 30 min followed by the incubation at 95°C for 10 min. Bacterial DNA was isolated using phenol/chloroform/isoamyl alcohol extraction method, as described above.

Design of nucleotide primers for PCR

We designed the sets of nucleotide primers for PCR using algorithms developed in Expert Biomed Inc. and Stop Alzheimers Corp. For programming algorithms, we composed a database that includes nucleotide sequences of the bacterial species of soil and marine environments previously associated with human activities. The sets of forward (F) and reverse (R) PCR primers used in this study include the oligonucleotide primers designed to target a) the structure of enzymatic catalytic centers of bacterial metabolic enzymes and b) sequences outside of the enzymatic catalytic centers. The following primers used in this study as controls: PP16R forward, 5'-CTACGGGAGGCAGCAGTGG-3'; PP16R reverse, 5'-GTAACGTCAAAACAGCAAAGT-3'; KTADSP forward, 5'-CTCAACAAGGCGAACCTTTC-3', KTADSP reverse, 5'-CCAGACCGGTACTGAGGAAG-3'; RUAD forward, 5'-AACTGGACCGATTTTGCATC-3'; RUAD reverse, 5'-GGCATCACCTTTTCCTCAAA-3'. Synthesis of primers has

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been ordered at Life Technologies (Carlsbad, California) and conducted using a highly automated, computer-controlled system and standard cyanoethyl phosphoramidite chemistry. Oligonucleotides were purified using a desalting procedure.

Polymerase chain reaction

DNA samples (50 ng per reaction) were subjected as templates to PCR using Light Cycler 1.5 Instrument (Roche Diagnostics), the kit LightCycler FastStart DNA Master SYBR Green I and 250 nM primers. The amplification protocol contained the following steps: 1) pre-incubation for activation of FastStart DNA polymerase and denaturation of the DNA, 95°C for 10 min (1 cycle); 2) amplification of the target DNA, 95°C for 10s, 60°C for 10s, 72°C for 10s (35 cycles); 3) incremental increase of the temperature (0.1°C/s) from 65°C to 95°C to obtain the dissociation profiles. PCR products were analyzed using 3% agarose gel electrophoresis. Bands were visualized using the ethidium bromide staining and images were recorded using a Herolab gel documentation system (Wiesloch, Germany) equipped with a Herolab E.A.S.Y. 429K digital camera. Values of PCR products are given as arbitrary (conventional) units (a.u.) from 0 to 4 (Supplementary Table 1, Supplementary Material, and Fig. 4) where a.u. derives from the size and optical density of PCR products. The graphic display of laboratory data (Fig. 4) used as central to the evolving computerized patient record.

Cloning and sequencing PCR products

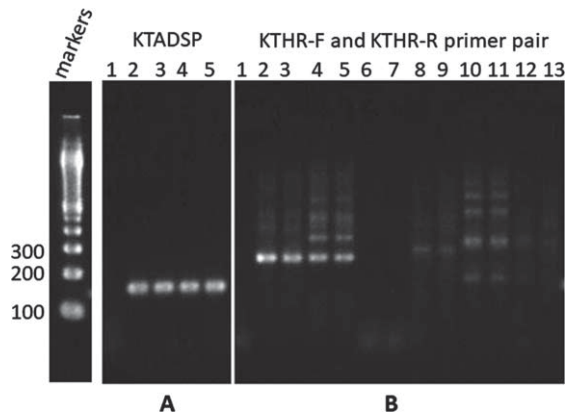
PCR products were subcloned into a SMART-HC Kan vector using a CloneSmart Cloning kit (Lucigen Corporation, Middleton, Wisconsin) according to the manufacturer's instructions. The heat-inactivated CloneSmart Ligation reaction was heat shock transfected into Lucigen's E. cloni 10G Chemically Competent Cells. The transformed cells were spread on the LB medium agar plates containing kanamycin; selected bacterial clones were further purified using agar LB culture medium with kanamycin. Plasmid DNA was isolated using a Qiagen HiSpeed plasmid Midi Kit and sequenced. For sequencing of the cloned AD-associated PCR products, we utilized the SMART-HC Kan (GeneBank accession # AF532107) cloning vectors with Kanamycin resistance cassette. The forward and reverse nucleotide primers SL1 (SL1:5'–CAG TCC AGT TAC GCT GGA GTC–3') and SR2 (5'–GGT CAG GTA TGA TTT AAA TGG TCA GT–3') were used for sequencing of sub-cloned PCR inserts. Each AD patient is represented by two independently cloned PCR products, sequenced in both orientations. DNA sequencing was conducted at the Sequencing Core Facility of University of Miami (Miami, Florida, USA).

Evaluation of Positive, Negative, Weakly Positive, and Unclear PCR Results

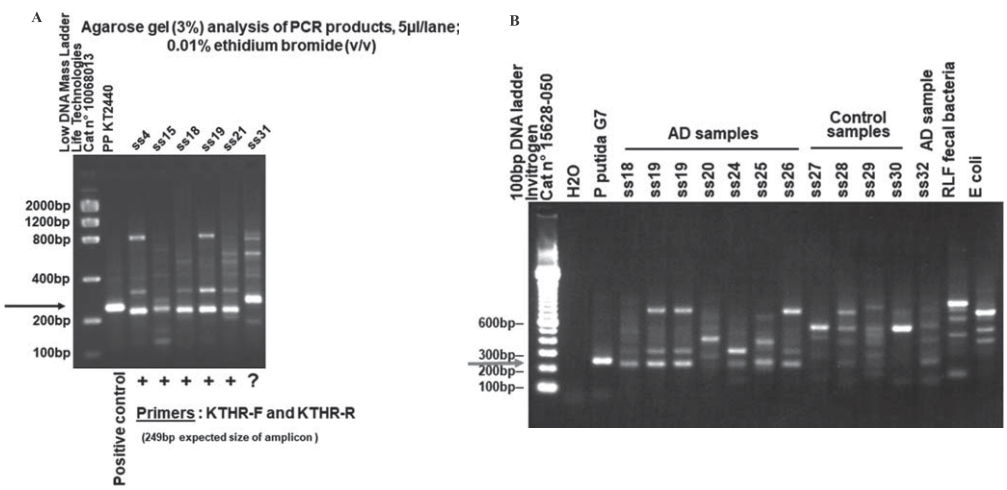
The strongly positive 4+ results (Supplementary Table 1) based on the optical density of both ∼250 and 300 bp PCR products specific for KTHR primers were detected in DNA extracted from AD stool specimens (Fig. 1, lanes 1-4). The positive 3+ results based on the optical density of ∼250 bp PCR product specific for KTHR primers were detected for DNA probes extracted from stool specimens of volunteer RLF with symptoms of acute mental disturbance (Fig. 3).

The positive 2+ results based on the optical density of ∼300 bp PCR products specific for KTHR primers were detected in DNA extracted from AD stool specimens. The weak positive 1+; refers to ∼250 bp PCR product (Fig. 3, RLF improvement of mental state/remission). The "negative or weak positive not clear results" (Fig. 2, SS28 and SS29) may indicate the presence of gut opportunistic bacteria in non-active phase of growth (3 out of 11 control specimens and 3 out of 20 AD patients). The clear negative PCR result such as that for DNA specimen #5 from stool of AD patient may be caused by: 1) bad quality of DNA probe; 2) usage of antibiotics by patient prior testing; 3) absence or low level of bacteria of interest in gut; 4) mutation/s in gene sites specific for KTHR primers.

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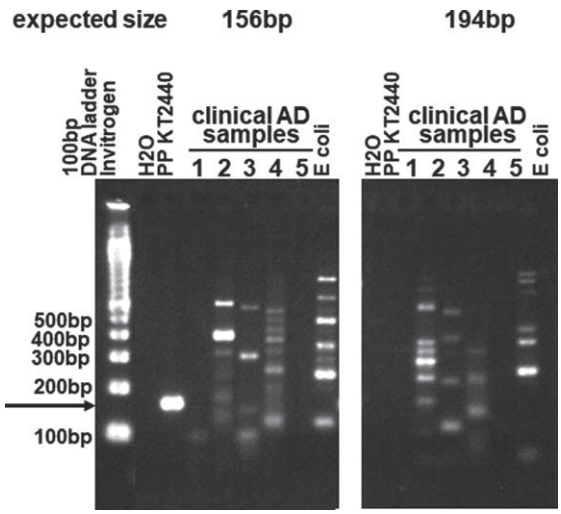


Supplementary Figure 1. PCR test development. The agarose gel electrophoresis of PCR products for analysis of DNA from different *P. putida* strains, human genomic DNA (gDNA) extracted from peripheral blood mononuclear cells of healthy donors and mouse DNA extracted from liver using two sets of nucleotide primers KTADSP-F, KTADSP-R (A) and KTHR-F, KTHR-R (B). DNA markers (Invitrogen, as in Fig. 1) show nucleotide size equal to base pairs (left). Staining of the 3% agarose gel was conducted with 0.01% (v/v) ethidium bromide. Lanes: A1, B1 - negative control (water). A2, A3 - gDNA from P. putida KT2440, 1 ng and 100 pg/reaction, respectively. A4, A5 - gDNA from P. putida CF600, 1 ng and 100 pg/reaction, respectively. B2, B3 - gDNA from P. putida KT2440, 10 ng and 1 ng/reaction, respectively. B4, B5 - gDNA from P. putida CF600, 10 ng and 1 ng/reaction, respectively. B6, B7 - human gDNA, 10 ng/reaction; B8, B9 - human gDNA, 1 ng/reaction. B10, B11 - mouse gDNA, 10 ng/reaction; B12, B13 – mouse gDNA, 1 ng/reaction.

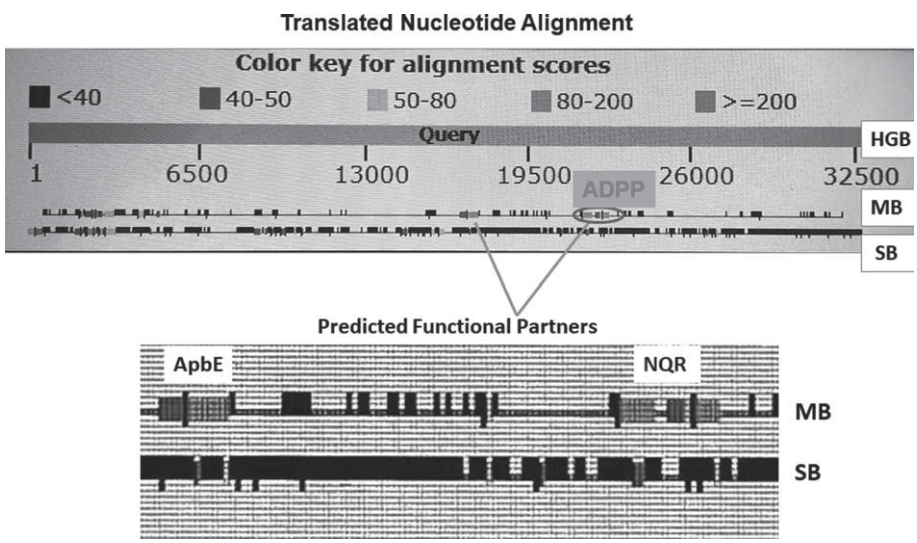


Supplementary Figure 2. PCR products with the usage of the primer's set KTHR visualized by staining of 3% agarose gel with 0.01% (v/v) ethidium bromide. A) The stool samples (SS) are from AD patients of BCC (SS4, SS18, SS19, SS21); positive donor RLF (SS15) from the private residence in CA; a non-AD resident of BCC (SS31). The Low DNA Mass Ladder (LDML) used for estimating the size and quantity of unknown DNA samples by ethidium bromide staining. The LDML is composed of an equimolar mixture of six blunt-ended DNA fragments of 2000 bp to 100 bp (left lane). Electrophoresis of 4 µL of LDML results in bands containing 200, 120, 80, 40, 20, and 10 ng of DNA, respectively. B) The Invitrogen DNA ladder used as the DNA size markers (left lane). The control and AD SS are from BCC. The RLF fecal bacteria were isolated from SS under aerobic conditions following antibiotic Avelox treatment, at the period of remission.

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Supplementary Figure 3. PCR of DNA from SS of AD patients (1-5) using nucleotide primers KTADSP and RUAD. PCR was conducted with nucleotide primers KTADSP forward and KTADSP reverse (expected size of PCR products – 156 bp; RUAD forward and RUAD reverse with expected PCR product - 194 bp. No unique PCR products specific for AD with usage of these primers were detected. Note, the SS #5 is totally negative with both sets of primers and also with KTHR-F and KTHR-R pair, the primers used in the Fig.1. The PCR products are visualized on 3% agarose gel stained with 0.01% (v/v) ethidium bromide. Left lane show the marker DNA ladder with 100 bp steps (Invitrogen).



Supplementary Figure 4. Sequence comparison between human gut bacteria possessing ADPP sequence and environmental bacteria using basic local alignment search tool (BLAST). Translated BLAST similarity between HGB – human gut bacteria (*Clostridium* sp.) and MB – marine bacteria (*Silicibacter* sp.) or SB – soil bacteria (*Pseudomonas* sp.). The ApbE genes cluster with genes for Na+-NQR and other flavoproteins in bacterial genomes.

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Supplementary Table 1
Sample List. The results of PCR assay of DNA extracted from stool specimens (ss) of AD patients and donors with primers KTHR-F and KTHR-R: analysis of ∼249 bp and ∼300 bp PCR products. Each PCR result represents duplicate or triplicate testing of the same sample

SAMPLE LIST						
n°	Name	Origin	Gender	Age	Diagnosis	PCR result
1	ss1	#1 care facility	M	70	AD	4+
2	ss2	#2 care facility	F	80	AD	4+
3	ss3	#3 care facility	M	71	AD	4+
4	ss4	#4 care facility	M	68	AD	4+
5	ss5	#5 care facility	M	75	AD	negative
6	ss6	#6 care facility	F	71	AD	4+
7	ss7	#7 care facility	F	80	AD	2+
8	ss8	#8 care facility	F	80	AD	1+
9	ss9	#9 care facility	M	71	AD	4+
10	ss10	#10 care facility	F	76	AD	2+
11	ss11	#11 care facility	F	86	AD	**pos/neg*
12	ss12	#1 donor Miami	F	58	Control	*pos/neg*
13	ss13	#2 donor Miami dog	F	5	Control	negative
14	ss14	#3 donor MAF CA	M	19	Control	negative
15	ss15	#4 donor RLF CA	F	89	**AMD/diabetes	3+
16	ss16	#5 donor AMF CA	M	61	Control	negative
17	ss17	#6 donor SLF CA	F	88	Control	negative
18	ss18	#12 care facility	F	76	AD	4+
19	ss19	#13 care facility	F	84	AD	4+
20	ss20	#14 care facility	F	71	AD	*pos/neg*
21	ss21	#15 care facility	F	84	AD	4+
22	ss22	#16 care facility	M	89	AD	4+
23	ss23	#17 care facility	M	80	AD	*pos/neg*
24	ss24	#18 care facility	M	58	AD	4+
25	ss25	#19 care facility	M	78	AD	4+
26	ss26	#20 care facility	F	70	AD	4+
27	ss27	#101 care facility	F	44	Crtl/Bronchitis	negative
28	ss28	#102 care facility	F	65	Crtl/Hip replecemen	*pos/neg*
29	ss29	#103 care facility	M	53	Crtl/BPH	*pos/neg*
30	ss30	#104 care facility	F	50	Crtl/MI-s/p-CAB6	negative
31	ss31	#105 care facility	M	52	Crtl/CVA@R-side	negative
32	ss32	#4 donor RLF CA	F	89	improvement	1+
33	ss33	#4 donor bacteria	F	89	improvement	negative

*negative or weak positive result not clear. **AMD acute mental disturbance. Positive results from 1+ (weakly positive) to 4+ (strongly positive).

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Bile Acids as Key Modulators of the Brain-Gut-Microbiota Axis in Alzheimer's Disease

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Abstract. Recently, the concept of the brain-gut-microbiota (BGM) axis disturbances in the pathogenesis of Alzheimer's disease (AD) has been receiving growing attention. At the same time, accumulating data revealing complex interplay between bile acids (BAs), gut microbiota, and host metabolism have shed new light on a potential impact of BAs on the BGM axis. The crosstalk between BAs and gut microbiota is based on reciprocal interactions since microbiota determines BA metabolism, while BAs affect gut microbiota composition. Secondary BAs as microbe-derived neuroactive molecules may affect each of three main routes through which interactions within the BGM axis occur including neural, immune, and neuroendocrine pathways. BAs participate in the regulation of multiple gut-derived molecule release since their receptors are expressed on various cells. The presence of BAs and their receptors in the brain implies a direct effect of BAs on the regulation of neurological functions. Experimental and clinical data confirm that disturbances in BA signaling are present in the course of AD. Disturbed ratio of primary to secondary BAs as well as alterations in BA concertation in serum and brain samples have been reported. An age-related shift in the gut microbiota composition associated with its decreased diversity and stability observed in AD patients may significantly affect BA metabolism and signaling. Given recent evidence on BA neuroprotective and anti-inflammatory effects, new therapeutic targets have been explored including gut microbiota modulation by probiotics and dietary interventions, ursodeoxycholic acid supplementation, and use of BA receptor agonists.

Keywords: Alzheimer's disease, bile acids, brain-gut-microbiota axis

INTRODUCTION

Recently, the concept of the brain-gut-microbiota (BGM) axis disturbances in the pathogenesis of Alzheimer's disease (AD) has been receiving growing attention [1, 2]. Accumulating data indicate that amyloid-β deposits constituting the characteristic feature of AD may occur at different levels of the axis including both the central and enteric nervous systems [2]. Importantly, gut microbiota is also the source of significant amount of amyloids [3]. Moreover, numerous alterations in the gut microbiota composition and function in AD patients have been reported [1–6]. The mechanisms involved in regulation of the BGM axis through which gut microbes can communicate to the brain include neural, immune, and neuroendocrine pathways [7, 8]. Additionally, microbial metabolites such as serotonin, dopamine, short-chain fatty acids, as well as secondary bile acids (BAs) may serve as direct neurotransmitters or neuromodulators [8, 9].

Over recent years there has been also growing understanding of the complex role of BAs in regulating host physiology and host-microbiota interactions

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[10–15]. In the past, BAs as naturally acting detergents were considered to play a cardinal role in the digestion, micelle formation, and absorption of fat and fat-soluble vitamins. Currently, they are recognized as important signaling molecules actively participating in the regulation of numerous metabolic processes and significantly affecting gastrointestinal motor, sensory and secretory functions, as well as intestinal barrier permeability and regulation of inflammatory response [16]. Given close interactions between BAs and gut microbiota, they emerge as potential modulators of the BGM axis that may have direct impact on neurodegeneration. In fact, numerous experimental and clinical studies confirm the BA involvement in the pathogenesis of AD [17–23]. This review is aimed at highlighting most recent progress in the field with respect to the BA impact on neural, immune, and neuroendocrine interactions within the BGM axis.

BILE ACID SYNTHESIS, METABOLISM, AND ENTEROHEPATIC CIRCULATION

While cholesterol metabolism is thought to play a pivotal role in the pathogenesis of AD, BAs are the products of cholesterol clearance [24]. Importantly, many cholesterol metabolism-related genes (e.g., *ABCA7*, *ABCG1*, *SORLI*, *BINI*, *CLU*, and *PICALM*) are among the main AD susceptibility loci identified by genome-wide association studies [25, 26]. Three different pathways of cholesterol clearance may be distinguished (reviewed in detail in [24]). The classic pathway, which is the main pathway of BA synthesis in the liver, begins with the conversion of cholesterol to 7α-hydroxycholesterol by CYP7A1, which is subsequently hydroxylated by sterol 12α-hydroxylase (CYP8B1) or sterol 27-hydroxylase (CYP27A1) to form the primary BAs: cholic acid (CA) and chenodeoxycholic acids (CDCA), respectively [27, 28]. The alternative BA synthesis pathway is mediated by CYP27A1, which unlike CYP7A1, is expressed in the mitochondria of most cells [28]. The second (acidic) pathway is responsible for about 5–10% of BA synthesis in humans, and about 25% in rodents [28]. The alternative pathway produces regulatory oxysterols [16]. The third pathway, called the neural one, is responsible for the majority of cholesterol turnover in the central nervous system (CNS) [29]. The brain clears excess cholesterol via CYP46A1 that catalyzes the conversion of cholesterol to 24S-hydroxycholesterol, which being more polar than cholesterol can more easily pass the blood-brain barrier (BBB) to exit the brain and pass into the bloodstream [28]. It has been shown that in AD patients, there is significantly decreased expression of cholesterol-24 hydroxylase in neurons [30].

Primary BAs (CA and CDCA) after conjugation in the liver with glycine or taurine are secreted into the bile and transported to the gut lumen when they are metabolized by bacteria. Gut microbiota initiates bile acid metabolism via a critical first step catalyzed by bile salt hydrolases (BSHs) [31]. These enzymes hydrolyze and deconjugate glycine or taurine from the sterol core of the primary BAs. Deconjugated BAs can subsequently undergo a variety of microbiota-encoded transformations (i.e., 7 α-dehydroxylation, dehydrogenation, and epimerization) that generate secondary BAs, which have widespread effects on the host and resident microbiota [31–33]. CA is converter to deoxycholic acid (DCA), while CDCA is converted to lithocholic acid (LCA) and ursodeoxycholic acids (UDCA) [31, 33].

In the terminal ileum about 95% of BAs are reabsorbed primarily through active transport by the apical sodium-dependent BA transporter (ASBT) [34]. Subsequently, they are released into the portal vein for return to the liver where they are conjugated to produce their glycine and taurine forms [35]. BAs, which are not reabsorbed in the ileum, enter the colon, and serve as substrates for microbial metabolism and undergo biotransformation to secondary BAs [36]. BAs regulate their own synthesis and transport via the nuclear farnesoid receptor (FXR). After binding to the ileal FXR, BAs induce expression of fibroblast growth factor FGF19 (FGF15 in mice), which travels via the portal bloodstream and binds to hepatocyte receptors repressing BA synthesis by inhibiting the rate-limiting enzyme CYP7A1 via negative feedback [37].

BILE ACID SIGNALING

A wide variety of BA characteristics is associated with their lipophilicity or hydrophilicity, but also with their ability to bind numerous receptors as well as activate ion channels [38]. While unconjugated BAs may be able to easily cross the plasma membrane, conjugated BAs might cross the plasma membrane using transporters. Nuclear receptors activated by BAs are the FXR, pregnane X receptor (PXR), vitamin D receptor (VDR), liver X receptor (LXR), and glucocorticoid receptor (GR). Whereas

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G protein-coupled receptors (GPCR) on cell surface activated by BAs include Takeda G-protein receptor 5 (TGR5), sphingosine-1-phosphate receptor 2 (S1PR2), muscarinic receptors (M2 and M3), and formyl peptide receptor (FPR). Moreover, BAs are able to activate ion channels such as epithelial Na+ channel (ENaC), and large-conductance Ca2+- and voltage-activated K+ (BK) channels. Noteworthy, all these nuclear and membrane receptors as well as bile acid-sensitive ion channels (BASIC) are expressed in the brain [38].

BAs vary in their ability to activate nuclear receptors and GPCRs. FXR is activated most potently by the primary BA – CDCA, while TGR5 receptor is mainly activated by secondary BAs (LCA and DCA) [12]. Additionally, UDCA (secondary BA in humans) and murine primary BAs (TαMCA and TβMCA) act as FXR antagonists. The ability to activate two major BA receptors occurs in the following order – for FXR: CDCA > LCA = DCA > CA and for TGR5: LCA > DCA > CDCA > CA [16, 39]. FXR is involved in BA synthesis, transport, and enterohepatic circulation as well as multiple glucose and lipid metabolic processes [40]. FXR is also a suppressor of inflammation in the liver and intestines and enhances epithelial barrier properties [41]. TGR5 being expressed on neuroendocrine cells induces glucagon-like peptide (GLP-1) release and plays an important role in glucose homeostasis [40, 42]. Moreover, TGR5 is also expressed in brown adipose tissue, muscles, and immune cells [43]. BA signaling through membrane-bound or nuclear receptors as well as neuromodulatory effects of BAs are accurately summarized in a graphic form in the review by McMillin and DeMorrow [27].

INTESTINAL CROSSTALK BETWEEN BILE ACIDS AND GUT MICROBIOTA

The crosstalk between BAs and gut microbiota is based on reciprocal interactions since microbiota determines BA metabolism, while BAs affect gut microbiota composition [12, 44, 45]. Gut microbes directly involved in BA biotransformation control the composition of BA pool, which in turn has a profound impact on the size of BA pool [11]. Comparing germ-free rodents to rodents with typical microbiota significant differences in the BA pool size have been confirmed [46]. Gut microbiota is also able to modulate BA synthesis impacting the enzyme reactions. For example, antibiotic treatment associated with microbiota depletion inhibits the expression of CYP7A1 [47]. The expression of CYP7A1 and CYP27A1 was also found to be reduced in germ-free mice compared to normal mice [48]. Changes in the BA pool composition associated with altered ratio of primary to secondary BAs may directly affect their signaling due to different affinities to their various receptors [16, 27, 39].

On the other hand, BAs participate in the regulation of the gut microbiota composition by direct antimicrobial effects and indirect effects through FXR-induced antimicrobial peptide synthesis in the small intestine [49, 50]. In general, a decreased BA level in the gut lumen favors gram-negative bacteria containing lipopolysaccharides (LPS) in their outer membranes, whereas an increase in BA concentration appears to favor gram-positive bacteria such as Firmicutes that are able to turn primary BAs via 7α-hydroxylation to more toxic secondary BAs [11]. In a mouse model, biliary duct ligation resulted in overgrowth of aerobic and anaerobic bacteria in the ileum and cecum, but that overgrowth could be blocked by the FXR agonist [50]. Another clinically relevant example of BA impact on the gut microbiota ecosystem is infection with *Clostridioides difficile*. It has been shown that in a healthy intestinal environment microbiota-derived secondary BAs inhibit the growth of this pathogen. However, antibiotic-induced dysbiosis may result in a rapid shift in the BA pool leading to decreased secondary BA level that promotes the growth of *C. difficile* [48].

Clostridia are among a narrow group of commensal bacteria able to perform 7α-dehydroxylation constituting a crucial reaction in secondary BA origination. Noteworthy, these spore-forming bacteria have also important impact on tryptophan metabolism in the gastrointestinal tract [51]. Golubeva et al. [52] have suggested some association between microbiota-related alterations in tryptophan metabolism and inadequate bacterial conversion of BAs that are both likely to contribute to intestinal dysmotility and impaired gut barrier function in a mouse model of autism.

Another interesting aspect of the connection between human and microbiota BA metabolism is related to probiotic BSH-containing strains like *Lactobacillus* and *Bifidobacterium* that not only participate in deconjugation of BAs but may also accumulate primary and secondary BAs in their cytoplasm either spontaneously or following intracellular BA deconjugation [53, 54]. Accumulation of primary BAs in the cytoplasm of probiotic bacteria results

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in their reduced conversion to secondary BAs in the colon which may have some implications in chronic inflammation [53, 55].

BILE ACIDS IN THE BRAIN

The presence of twenty BAs and their receptors has been demonstrated in the brain [38, 56]. Among them are unconjugated BAs (CA, CDC, DCA, and UDCA) and conjugated BAs (GDCA, TCA, TDCA, and TUDCA) which are related to brain physiology and pathophysiology [57]. Mano et al. [56] found that CDCA composes about 95% of total BA composition with CA and DCA being about 2-3% in the rat brain. It has been also suggested that since BA level in the brain under normal conditions is approximately 10-fold higher than circulating level, they may be actually synthesized locally [56]. It is consistent with the observation that BAs may serve as potent neuroactive steroids [58]. In fact, CDCA was found to be about 10 times more abundant than the prototypical neurosteroid pregnenolone [56]. However, BAs can be also transported into the brain from the peripheral circulation. While conjugated BAs require transporters to cross the BBB, unconjugated and lipophilic BAs can cross the BBB by passive diffusion [38, 59]. In addition, BAs in the bloodstream may affect the BBB permeability as it has been shown that phosphorylation of occludin—one of the tight junction proteins—may be increased by CDCA and DCA via ras-related C3 botulinum toxin substrate 1 (RAC1) [60]. On the other hand, UDCA and its glycine-conjugated form (GUDCA) exert a protective effect on brain endothelial cells by reducing apoptosis [61, 62].

Noteworthy, both amino acids, glycine and taurine, conjugated with BAs in the liver have neurotransmitter properties [63, 64]. Glycine is an inhibitory neurotransmitter in the CNS serving as a co-agonist along with glutamate for N-methyl-D-aspartate (NMDA) receptors [63]. Taurine demonstrates functions of a neurotransmitter, neuromodulator, and neuroprotectant, and acts as a trophic factor in the CNS development. The neurotransmitter properties of taurine are illustrated by its ability to elicit neuronal hyperpolarization [64].

The role of BAs as neurosteroids is related to their affinity to receptors for several neurotransmitters [38, 58]. TCA, GDCA, and TDCA activate muscarinic acetylcholine receptors (M_2 and M_3) that are known to play an important role in cognitive function, memory, and learning [65]. Moreover, CDCA, DCA, and CA block both γ-aminobutyric acid type A ($GABA_A$) receptors and NMDA receptors [66]. The NMDA receptor activation is also important for learning and memory [67], while $GABA_A$ activation leads to the inhibition of neurotransmission [68]. All these observations imply an important role of BAs in the regulation of neurological functions. Additionally, there are accumulating data supporting neuroprotective effect of some BAs such as TUDCA [38]. Their neuroprotective action may be associated with inhibition of amyloid-β accumulation, mitochondrial damage, and apoptosis. It has been also suggested that TUDCA may induce neurogenesis in adults, but the mechanism of that action remains to be elucidated [38].

INTERACTIONS BETWEEN BILE ACIDS AND THE BRAIN-GUT-MICROBIOTA AXIS

Disturbances along the BGM axis may significantly contribute to the pathogenesis of AD [1, 4, 5]. Alterations in the gut microbiota composition induce increased permeability of the gut barrier and immune activation leading to systemic inflammation, which, in turn, may impair the BBB and promote neuroinflammation, neural injury, and ultimately neurodegeneration associated with the formation of amyloid-β plaques and neurofibrillary tangles [1, 69–71]. Bacterial amyloids through molecular mimicry may elicit cross-seeding of misfolding and cause priming of the immune system, consequently enhancing immune response to endogenous production of neuronal amyloid in the brain [72]. Recognition of bacterial amyloid by the human TLR2 receptor (toll-like receptor 2) results in the release of pro-inflammatory cytokines such as Il-1β and Il-6 produced by activated bone marrow macrophages as well as Il-11 and Il-17A produced by activated T-lymphocytes [73]. These cytokines are able to cross the BBB and induce the production of reactive oxygen species, activation of the TLR2/1 and NF-κB signaling pathways, which are directly related to neuroinflammation and neurodegeneration [74, 75]. Also bacterial LPS can bind with microglial cell receptors (TLR2, TLR4, and/or CD14) [76, 77]. In the brain of animals exposed to curli-producing bacteria enhanced microgliosis and astrogliosis together with increased expression of TLR2, Il-6, and TNF were found [31]. In addition, microglia activation further impairs phagocytosis leading to the accumulation of $A\beta_{42}$ [78, 79].

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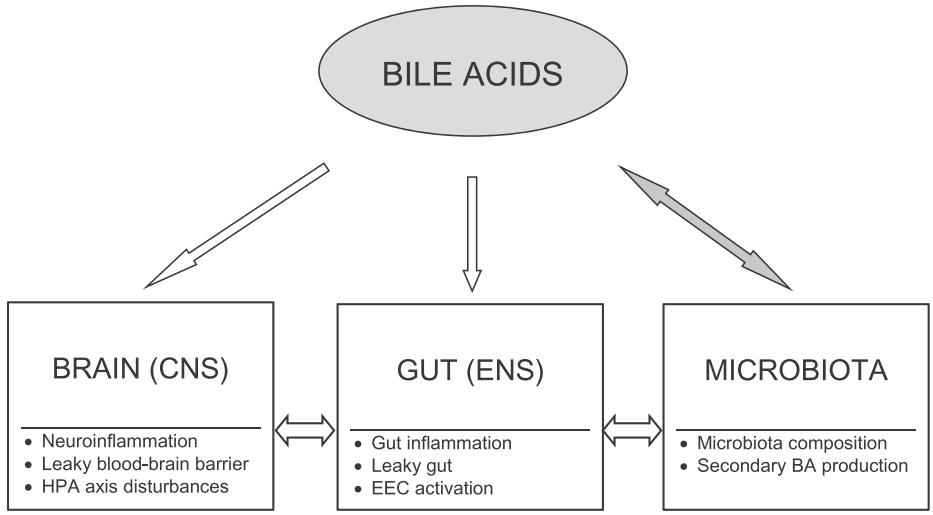


Fig. 1. Bile acid modulatory effects on the brain-gut-microbiota axis in Alzheimer's disease. Bile acids (BAs) may act as modulators of each level of the brain-gut-microbiota axis. There are bidirectional interactions between gut microbiota and BAs. The BA pool composition depends on gut microbiota producing secondary BAs, whereas BAs significantly influence microbiota composition. The primary to secondary BA ratio determines their signaling due to diversified binding affinities of BAs to various receptors. BAs may modify activation of both peripheral and central neural and immune cells as well as affect gut barrier and blood-brain barrier permeability. Moreover, BAs through their receptors exert neuroendocrine effects via enteroendocrine cells (EEC) and the hypothalamic-pituitary-adrenal (HPA) axis. CNS, central nervous system; ENS, enteric nervous system.

Recently, BAs possessing neuroactive properties have been emerging as a direct communication channel between the gut microbiome and the brain, since gut microbiota is directly involved in BA synthesis and metabolism affecting their pool composition and properties [80]. That, in turn, may affect BAs ability to bind and signal through their receptors.

The contribution of gut microbiota to the pathogenesis of AD is well depicted in animal models as well as clinical studies (reviewed in detail in [1]) [1, 5, 6, 70, 81]. Regarding interactions between BAs and gut microbiota, an important observation in AD patients concerns a decrease in *Clostridium* and *Bifidobacterium* [81]. Both genera have BSHs which allow BAs to become deconjugated and less toxic to the microbiota. Since BSHs are the only enzymes responsible for the pivotal deconjugation reaction, their activity serves as a gatekeeper to subsequent BA transformations [82]. BA deconjugation enables also subsequent metabolism of BAs via 7α-dehydroxylation (*Clostridium spp*.) to secondary BAs which act as ligands for FXR [21]. The BA pool, influenced by microbial BSH activity, shapes in turn the microbiome composition and function [83]. The significant association between increased gut microbiota-derived secondary BAs both conjugated and unconjugated and changes in AD markers such as amyloid and tau proteins have been recently reported in AD patients by Nho et al. [19]. In that study serum-based BA metabolites were investigated and several BA ratios that reflect BA conversion by gut microbiota were assessed. Among numerous reported alterations in BA profiles, higher GDCA:CA levels were associated with greater global cortical amyloid deposition, while two conjugated secondary BA (GLCA and TLCA) were correlated with higher p-tau concertation in the cerebrospinal fluid (CSF) [19]. This observation supports the role of the gut microbiota transformation of BAs as a potential contributing factor in the pathogenesis and/or progression of AD.

Moreover, a growing body of evidence indicates that BAs may serve as important modulators of each of three main routes through which interactions within the BGM axis occur including neural, immune, and neuroendocrine pathways that will be discussed below (Fig. 1). A wide spectrum of the BA effects may be exerted via their receptors found throughout the body. Among BA target cells are neurons, immune cells, and enteroendocrine cells [21].

Neural pathways

BA signaling via neural pathways may occur directly (via BA receptors present in the brain and enteric nervous system) or indirectly (via the FXR-

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FGF15/19 axis). The most studied BA receptors are found in the brain [84]. The function of FXR and TGR5 in the brain is still unclear, but it has been reported that FXR knockout mice showed disturbed neurotransmitter system and altered neurobehavior [85]. The results from studies with FXR knockout mice confirmed changes in serotoninergic as well as glutamatergic, GABAergic, norepinephrinergic neurotransmission in either the hippocampus or cerebellum [85]. Additionally, TGR5 being expressed on neurons, glial and microglial cells can be activated by numerous neurosteroids [86, 87]. In neurodegenerative processes such as AD, BA receptors may significantly affect decline in cognitive function by affinity to muscarinic receptors as well as $GABA_A$ and NMDA receptors [66].

Among other BA receptors expressed in the brain, special attention in the context of AD is focused on pregnane X receptor (PXR), vitamin D receptor (VDR), and glucocorticoid receptor (GR) [38]. PXR as xenobiotic nuclear receptor activating cytochrome P450 can dispose toxins, for example at the BBB [88]. Vitamin D in the brain can act as neurosteroid and its beneficial effect in AD has been confirmed [89, 90]. The GR activation in the hypothalamus by BAs may suppress the hypothalamic-pituitary-adrenal (HPA) axis, which is the key arm of neuroendocrine interactions along the BGM axis [91]. Interestingly, UDCA can bind to and translocate GR [91].

Moreover, TGR5 receptors have been also identified in the enteric nervous system where their activation result in increased colonic motility, which may lead to BA-induced diarrhea [92]. Noteworthy, BA effects on the gastrointestinal tract may show regional differences between various segments of the tract, since for example activation of TGR5 on gastric smooth muscle results in delayed gastric emptying [92].

The indirect neural signaling pathway of BAs to the CNS involves FGF15/19. FGF19 can cross the BBB and bind to FGFRs that are also expressed in the brain, specifically in the hypothalamus, medial habenular nucleus and dorsal vagal complex [93]. FGF19 binds to FGFR4 but a more solid bond is created when β-Klotho, the FGF19 cofactor, is bound to FGF19-FGFR4 complex [61, 94].

Immune pathways

Although amyloid-β deposition and tau pathology have been considered as the main features of AD, accumulating data show that disturbances in immune responses resulting in neuroinflammation considerably contribute to AD pathogenesis and may be even a trigger of the pathology [95–97]. The effect of BA action is determined by various factors such as concentration of BAs, their physicochemical properties, interactions with the gut microbiota and target cells [98]. Activation of BA receptors in immune cells results in several regulatory functions that are mainly inhibitory in nature [99]. Recent findings have been pointing at a crucial role of FXR in the modulation of inflammatory response and intestinal barrier integrity [41, 100]. The results of both *in vivo* and *in vitro* studies have demonstrated that on the one hand the activation of FXR exerts anti-inflammatory effect by reducing the production of pro-inflammatory cytokines such as TNF-α, IFN-γ, interleukin-1β (IL-1β), IL-2, and IL-6 [101]. On the other hand, inflammation reduces FXR expression [102]. It has been suggested that the reduced FXR transcriptional activity upon TNF-α/IL-1β stimulation is mediated by NF-κB [41]. In fact, FXR and TGR5 knockout mice develop spontaneous intestinal inflammation [103, 104]. While in a mouse model of ulcerative colitis it has been demonstrated that experimental colitis may disturb BA synthesis by the negative feedback signaling within the FXR-FGF19 axis [105].

Additionally, both nuclear and membrane BA receptors are highly represented in immune cells such as intestinal and liver macrophages, dendritic cells, and natural killer T cells [99]. Activation of these receptors shifts the macrophage phenotype toward anti-inflammatory one (M2) with an upregulation of Il-10 and a downregulation of the pro-inflammatory cytokines including Il-6 and IFN-γ. In turn, Il-10 increases the production of Treg cells reducing the recruitment of monocytes from blood to the colon. The TGR5 signaling involves cAMP-protein kinase A (PKA)-mediated inhibition of NF-κB, and FXR signaling leads to repression of NF-κB-responsive elements. BA stimulation of both pathways blunt NF-κB-dependent gene expression. Regarding action on dendritic cells, BAs downregulate the production of TNF-α and IL-12 [99].

Another important aspect of the BGM interactions and immune response concerns the integrity of intestinal and blood-brain barriers that may also be affected by BAs [106]. As mentioned above, BAs increase phosphorylation of occludin that may result in disruption of these barriers [60]. Additionally, TGR5 activated by BAs can activate the cJun NH_2-terminal kinase (JNK) pathway lead-

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ing to degradation of tight junction proteins [107, 108]. Intestinal barrier integrity is also regulated by an interplay between gut symbionts and PXR through a pathway which involves luminal sensing and signaling by TLR4 [109]. Direct interactions between gut microbiota, gut immune system activation, and intestinal permeability constitute a critically important element of the BGM axis [1]. In fact, increased biomarkers of gut permeability and inflammation in AD has been recently reported [110]. An age-related shift in the gut microbiota composition associated with its decreased diversity and stability observed in AD patients may significantly affect BA metabolism and signaling [4, 5]. Additionally, dysbiosis-related intestinal inflammation and increased gut permeability are associated with an increase in proinflammatory cytokines and bacteria-derived products in the circulation resulting in systemic inflammation. Subsequently, due to concomitant BBB impairment, systemic inflammation may induce neuroinflammation observed in AD [1].

Interesting findings concern sex-dependent differences in aging-associated inflammation and dysregulation of systemic BA homeostasis [111–114]. Recently, sexual dimorphisms in human immune system aging have been reported pointing to higher innate and pro-inflammatory activity and lower adaptive activity in older men compared to older women [115]. The rate of BA synthesis and BA pool composition are also sexually dimorphic [116]. For example, in animal studies female mice have a larger total BA pool than males. Moreover, females excrete less fecal BA and catabolize less cholesterol via BA production than males [117]. Additionally, age-related hormonal changes are implicated in the differential production of BA in females [118]. Ma et al. [119] demonstrated that aging in mice has a greater impact on changing hepatic BA profiles in females, but gut BA profiles in males. Moreover, gut microbiota remodeling by co-housing with younger mice partially rescued the above changes in old mice [119]. Even more importantly, BAs and FXR as well as gut microbiota alterations are implicated in the sex-related differences in metabolic disorders, many of which constitute risk factors for AD [120]. Given the role of BA homeostasis disturbances in neurodegeneration, those sex-dependent differences in BA profiles, especially in the elderly, could potentially explain female predominance in AD. Indeed, recently, Varma et al. [23] have suggested that cholesterol catabolism and BA synthesis may impact dementia progression through sex-specific effects on signaling pathways in the brain.

Neuroendocrine pathways

BAs as neurosteroids may exert a direct impact on the hypothalamus and the HPA axis. Suppression of the HPA axis has been shown to occur during cholestatic liver injury [69]. Furthermore, McMillin et al. [91] have demonstrated that in a model of cholestasis, serum BAs gain entry into the brain via a leaky BBB and that hypothalamic BA content is increased.

Currently, another neuroendocrine pathway in which BAs may be involved is emerging as BA receptors are expressed on enteroendocrine cells (EECs) forming the diffuse neuroendocrine system [61, 121, 122]. These interactions may have direct clinical implications in AD. A particular group of EECs, L-cells, responsible for the production of glucagon-like peptide-1 (GLP-1), are predominantly located in the terminal ileum and the colon [123]. Secondary BAs are powerful stimulants of GLP-1 release through the activation of TGR5 receptors located at the basolateral membrane of L-cells [124]. Additionally, L-cells may release GLP-1 in response to other endocrine or neural signals, not involving BA-induced TGR5 activation [125]. Although BA receptors have not been found on the vagus nerve so far, vagal afferent fibers express GLP-1 receptors and are therefore responsive to local GLP-1 level [126]. The nodose ganglion projects to the nucleus of the solitary tract (NST in the hindbrain is connected with other brain regions including the hypothalamus) [126, 127]. That points at the vagal-brainstem-hypothalamic pathway for the signal transmission of GLP-1 from the gastrointestinal tract to the CNS, which may be indirectly impacted by BAs [61].

Lately, GLP-1 and connections between its release and BA signaling are attracting attention as a possible link between metabolic and brain impairment [128, 129]. GLP-1 not only plays a key regulatory role in glucose metabolism stimulating insulin secretion, inhibiting glucagon secretion, decreasing food intake, reducing appetite, and inducing weight reduction, but affects also neurological and cognitive functions [129]. Hyperinsulinemia and insulin resistance, which are known as pathophysiological features of type 2 diabetes and obesity, have also been demonstrated as risk factors for AD [130]. Importantly, GLP-1 receptor agonists are used in the treatment for diabetes and obesity [131]. Moreover, modulation of GLP-1 activity can influence amyloid-β aggregation in AD and GLP-1 receptor agonists reduce hippocampal neurodegeneration [132]. There

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is also growing evidence on neuroprotective effects of these agonists in animal models of neurodegenerative diseases, regardless of diabetes [128]. Physiological consequences of BA impact on the TGR5-dependent release of GLP-1 in patients with obesity and/or on high fat diet remain to be elucidated. Nonetheless, it has been recently highlighted that BA signaling mediated by gut microbiota may contribute to obesity susceptibility [40]. Therefore, modulation of BAs could be a promising therapeutic target in obesity and obesity-related diseases including AD.

It has been also shown that secondary BAs acting through TGR5 may activate intestinal stem cells and epithelial regeneration [133] as well as induce L-cell differentiation and serotonin signaling [134]. Of note, serotonin in 95% stored in the EECs, is a key mediator of the neuroendocrine pathway within the BGM axis [135, 136].

BILE ACID DISTURBANCES IN EXPERIMENTAL AND CLINICAL SAMPLES OF ALZHEIMER'S DISEASE

Substantial BA perturbations have been reported in AD. Pan et al. [17] performed metabolomic profiling of BAs in both experimental and clinical samples of AD. Quantification of BAs in brain tissue extract and plasma was performed using a BA kit enabling simultaneous evaluation of 22 BAs. Human brain samples in that study were obtained from postmortem confirmed AD cases, while mouse brain samples from APP/PS1 male mice. Pan et al. [17] found higher plasma CA level in APP/PS1 mice compared to wild type mice. Additionally, in the brains of APP/PS1 mice higher LCA and lower tauromuricholic (TMCA), β-muricholic, and Ω-muricholic acids as well as lower TCA and TUDCA were demonstrated [17]. Intriguingly, CDCA was not detected in mouse brain [17], contrary to an earlier study performed in rats in which CDCA level in rat brain was approximately 30 times higher than those in the serum [56]. The beneficial or detrimental effects of BAs are related to their hydrophobicity or hydrophilicity. For example, hydrophobic BAs, like CDCA can damage biological membranes [137], while hydrophilic BAs, like UDCA and TUDCA, are able to inhibit apoptosis stabilizing mitochondrial membrane [138, 139].

In the above-mentioned study, Pan et al. [17] reported the following disturbances in BA profiles in clinical samples. In AD patients compared to age-matched control subjects lower plasma CA and lower brain TCA levels were found [17]. In contrast, the plasma level of LCA, a secondary BA known for its toxicity, was significantly higher in AD patients than in controls [140]. In another study the ratio of secondary to primary BAs (DCA/CA) in serum has been shown to be significantly higher in AD patients [18]. Higher level of microbiota-derived secondary conjugated BAs (GDCA, GLCA, and TLCA) were significantly associated with worse cognitive function [18]. Furthermore, in a follow-up study those changes were also correlated with alterations in CSF markers of the disease and with brain imaging changes [18]. Altogether, it suggests that the effects of gut-directed dysregulation of primary versus secondary BAs are not limited to the periphery but may also affect BA homeostasis and signaling in the human brain. Additionally, it supports the significance of the BGM axis interactions in the pathogenesis of AD in the context of BA disturbances [38]. Nevertheless, the casual relationship of these interactions needs to be further elucidated.

Interestingly, serum BA profile in AD has been shown to be associated with AD-related structural and functional neuroimaging as well as CSF biomarkers including amyloid-β, t-tau and p-tau [19]. For example, one conjugated primary BA (GCDCA) and two microbiota-derived conjugated secondary BAs (GLCA and TLCA) were associated with higher t-tau and p-tau values, reduced glucose metabolism, and larger structural brain atrophy [19]. Very recently, Baloni et al. [20], based on the brain metabolic network analysis using AD patient-derived postmortem transcriptomics and metabolomics, have revealed differential usage of alternative BA pathway for cholesterol clearance in AD. Additionally, metabolomics analysis of postmortem brain samples identified higher ratio of GCDCA/CA and secondary BAs like DCA, LCA, TDCA, CDCA, and GDCA in AD compared to controls suggesting the association of those BAs with cognitive decline in AD [20]. The presence of secondary BAs in the brain metabolomics strongly points at the role of gut microbiome in AD.

Major limitations in BA research are differences in BA profiles and metabolism among species making it challenging to translate data from animal models to the clinic [45]. Noteworthy, not only significant differences in BA profiles have been reported between human and rodents, but also between mice and rats. These differences may result in discrepancies observed in experimental vs clinical studies, like for example alteration in CA plasma level that was lower in AD patients but higher in a mouse model of

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AD [17]. Another example concerns UDCA which is considered to be the primary BA in bears, beavers, and mice, but the secondary, microbiota-derived BA in humans [141]. Furthermore, in mice one of the main primary BAs is muricholic acids which is not present in humans and which can act as an antagonist of FXR/FGF15 axis [142]. In humans primary BAs act as potent FXR agonists [12]. Given these discrepancies caution in interpreting translational research is required.

Other important limitations in BA research in patients with AD are related to dietary factors, concomitant disorders such as obesity and diabetes, and medications including statins commonly used in the elderly that may directly or indirectly, via alterations in microbiota composition, affect BA synthesis and metabolisms [111]. On the one hand dietary patterns have impact on BA metabolism, while on the other hand food intake is partially regulated via BA-related changes in FXR and/or TGR5 signaling modulating the secretion of gastrointestinal hormones [143]. For example, in the study comparing BA profiles in vegans versus omnivores, a higher dietary fiber and lower fat intake was correlated with lower fecal BA concentrations [144]. Significant changes in dietary patterns leading to malnutrition are common in the elderly, and the lack of fiber and healthy fat intake can induce a state of systemic low-grade chronic inflammation [145]. Malnutrition itself is a risk factor for cognitive decline [146]. Eating and swallowing problems, which are present in about 80% of AD patients [147], along with the behavioral and psychological symptoms of dementia, can strongly affect nutritional status [148]. Moreover, an association between food intake and oral health problems common in the elderly may also contribute to neurodegeneration [149].

THERAPEUTIC IMPLICATIONS AND PERSPECTIVES

Given recent evidence on BA neuroprotective and anti-inflammatory effects their therapeutic potential has been intensively studied. In particular, better understanding of a critical role of BA-microbiota interactions in the regulation of metabolism and BGM axis has directed growing interest in microbiota-based approaches to modulate BA metabolism and associated conditions including neurodegeneration [15]. Numerous reports support the beneficial effect of probiotics by enhancing intestinal epithelial integrity, protecting against barrier disruption, reducing pro-inflammatory response, and inhibiting initiation or propagation of neuroinflammation and neurodegeneration [150, 151]. Additionally, probiotic administration may increase deconjugation of BAs and their fecal exertion in association with increased BSH activity in the gut and general modification of the microbiota composition [152, 153]. While supplementation with probiotics such as *Lactobacillus* and *Bifidobacterium* has been shown to improve significantly Mini-Mental State Examination scores in AD patients [154], administration of a BSH-positive *Lactobacillus* strain remarkably also reduces cholesterol levels in subjects with hypercholesterolemia [155].

Dysbiosis and dysregulated BA synthesis are implicated in Western diet-induced systemic inflammation, microglial activation, and reduced neuroplasticity [156]. Therefore, alternative approaches to manage diseases linked to BA dysmetabolism include dietary interventions aimed at modulating BA metabolism through balancing the microbiota [157]. Food-based therapies affect the gut microbiota composition or may have a direct influence on neuronal functioning in both the enteric and central nervous systems [158, 159]. The MIND diet, which is a hybrid of the Mediterranean and DASH (Dietary Approaches to Stop Hypertension) diets, was designed to emphasize nutrients that have been associated with dementia prevention and to eliminate products associated with increased risk of dementia [160]. Healthy diet is characterized by a high intake of fiber, probiotics, antioxidants, and omega-3 polyunsaturated fatty acids, as well as low intake of animal-derived proteins, saturated fats, and refined sugar. Such a diet has been shown to inhibit inflammatory response, reduce insulin resistance, and decrease the risk of neurocognitive impairment and eventually the risk of AD [145, 161]. The use of prebiotics as food-compounds inducing the growth and/or activity of beneficial microorganisms is of particular interest. Among most studied prebiotics are nondigestible fibers such as inulin and fructooligosaccharides and dietary polyphenols [145]. A summary of the effects of probiotics and synbiotics (combination of pro- and prebiotics) in AD patients and animal models of AD is presented in an extensive review by Arora et al. [145]. Probiotic, prebiotic, and polyphenol-rich interventions have been reported to exert beneficial effects on fasting lipid profiles, with changes in the gut microbiota composition associated with lipid regulation [162].

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Table 1
TUDCA effects in experimental models of Alzheimer's diseases

Model/Subject	Effect	Ref.
Mouse neuroblastoma cells	↓ p53 and its downstream targets	[168]
Rat cortical neurons	↓ Translocation of pro-apoptotic Bax to the mitochondria	[173]
Rat neuronal-like PC12 cells	↓ E2F1-p53 apoptotic pathway	[174]
	↓ Caspases activation	[175]
	↓ JNK early activation	[176]
Rat neuronal-like PC12 cells and primary cortical neuron	↓ Anti-apoptotic ΔNp63 degradation	[177]
Human cerebral endothelial cells	↓ Apoptosis induced by the vasculotropic E22Q mutant of Aβ (AβE22Q)	[178]
	↓ Cytochrome *c* release from mitochondria	
	↓ Bax translocation	
APOE4 macrophages with ApoE4-induced ER stress	↑ Efferocytosis	[179]
	↓ Cell death	
	↓ LPS- and oxidized LDL-induced apoptosis	
APP/PS1 mice	↓ mEPSCs	[180]
	↑ Number of dendritic spines	
	↑ Postsynaptic marker PSD-95	
	↓ Synaptic loss	[181]
	↓ Aβ plaque accumulation	[172, 182]
	↑ Spatial, recognition, and contextual memory	
	↓ Activation of astrocytes and microglia	[172]
	↑ Immunoreactivity of MAP2 used as a marker of neuronal integrity	
	↓ *APOE* gene expression	[172]
	↓ Amyloidogenic AβPP processing pathway	[172, 181]
	↓ $A\beta_{41}$ and $A\beta_{42}$ levels	

ApoE, apolipoprotein E; AβPP, amyloid-β protein precursor; Bax, Bcl-2-associated X protein; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP2, microtubule-associated protein 2; mEPSCs, miniature excitatory postsynaptic currents; PS1, presenilin 1; PSD-95, postsynaptic density protein 95.

Additionally, the effect of fecal microbiota transplantation (FMT) is being investigated in many animal models exploring pathogenetic mechanisms of neurodegenerative disorders [163]. Recently, the first case report on rapid improvement in AD symptoms following FMT for *C. difficile* infection has been published [164]. Intriguingly, the main FMT action might be related to restoring secondary BA synthesis [165] that would confirm the role of BA in the modulation of BGM interactions in AD.

Other options to modulate BA signaling include supplementation of neuroprotective BAs such as UDCA or TUDCA [166, 167]. Most central effects of TUDCA are dependent on the activation of TGR5, S1PR2, and α5β1-Integrin, which have already been identified in the brain [167]. Indeed, TUDCA has been shown to increase cell survival and inhibit neuronal apoptosis in a number of experimental models of neurodegenerative diseases such as AD, Huntington's disease, and Parkinson's disease [168, 169]. Furthermore, in animal model of acute neuroinflammation TUDCA exerts "triple" anti-inflammatory effect including reduction in glial cell activation, microglial cell migratory capacity, and expression of chemoattractants and vascular adhesion proteins [170]. TUDCA also reduced the accumulation of amyloid-β in the hippocampus and frontal cortex as well as rescued memory deficits in APP/PS1 transgenic mice [171, 172]. The TUDCA effects in experimental models of AD are summarized in Table 1. Although there is still no strong evidence on benefits in a clinical setting there are several ongoing registered trials with TUDCA and UDCA in AD, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and progressive multiple sclerosis [167].

A promising new direction of BA research would be to target BA receptors and indirect pathways of BA signaling to the brain mediated by FXR-FGF19 and TGR5-GLP-1 axes [61, 183]. FXR agonists targeting the gut-liver axis may reduce gut inflammation and preserve the intestinal barrier [41] as well as improve metabolic disorders associated with increased risk for AD [184]. Furthermore, GLP-1 receptor agonists have been reported to exert therapeutic effect in diabetes, obesity, and cognitive decline [128].

BA co-metabolism of both host and gut microbiota, and the diversified binding affinities to BA receptors among primary, secondary, free, and conjugated BAs result in the immense complexity of BA signaling

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[47, 119]. The advent of new analytical tools and strategies such as metabolomics and metagenomics is expected to bring a rapid progress in gaining insight in these complex BA-gut microbiota-host interactions [47]. It should enable to elucidate not only therapeutic but also diagnostic potential of BAs, which may serve as disease biomarkers. Noteworthy, intensive efforts focusing on unraveling BA signaling in AD should take into account multiple confounding factors such as gut dysbiosis, diet, sex, age, concomitant disorders, and medications. Most importantly, the causal relationship between BA disturbances and the AD pathology needs to be verified.

CONCLUSIONS

Given a direct reciprocal crosstalk between gut microbiota and BAs, they are emerging as key signaling molecules within the BGM axis. Moreover, since bacteria-derived secondary BAs can not only affect the composition and function of gut microbiota but also can modulate host physiology, they have been recognized as interkingdom signaling molecules [11, 45]. BAs have even been called a communication channel tuning the BGM interactions [80]. The observed disturbances in BA homeostasis in experimental and clinical samples of AD support the association between BA and the AD pathology as well as the significance of the BGM axis [32]. It indicates some potential therapeutic targets including gut microbiota modification, UDCA supplementation or administration of BA receptor agonists. The application of multi-omics techniques in unraveling the molecular mechanisms that underly BA-gut microbiota-host interactions should help to provide the basis for interventional studies.

DISCLOSURE STATEMENT

The author's disclosure is available online (https://www.j-alz.com/manuscript-disclosures/21-0608r1).

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Interaction Between Diet and Microbiota in the Pathophysiology of Alzheimer's Disease: Focus on Polyphenols and Dietary Fibers

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Abstract. Animal studies increasingly indicate that the gut microbiota composition and function can be involved in the pathophysiology and progression of Alzheimer's disease (AD) at multiple levels. However, few studies have investigated this putative gut-brain axis in human beings, and none of them considered diet as a determinant of intestinal microbiota composition. Epidemiological studies highlight that a high intake of fruit and vegetables, such as that typical of the Mediterranean diet, can modulate AD progression. Thus, nutritional interventions are being increasingly studied as a possible non-pharmacological strategy to slow down the progression of AD. In particular, polyphenols and fibers represent the nutritional compounds with the higher potential of counterbalancing the pathophysiological mechanisms of dementia due to their antioxidant, anti-inflammatory, and anti-apoptotic properties. These actions are mediated by the gut microbiota, that can transform polyphenols and fibers into biologically active compounds including, among others, phenyl-γ-valerolactones, urolithins, butyrate, and other short-chain fatty acids. In this review, the complex mechanisms linking nutrition, gut microbiota composition, and pathophysiology of cognitive decline in AD are discussed, with a particular focus on the role of polyphenols and fibers. The gaps between pre-clinical and clinical studies are particularly emphasized, as well as the urgent need for studies comprehensively evaluating the link between nutrition, microbiome, and clinical aspects of AD.

Keywords: Aging, butyrate, cognitive impairment, short-chain fatty acids

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INTRODUCTION: ALZHEIMER'S DISEASE AND DIET

A large body of evidence linking dietary patterns with cognitive function in aging has emerged from population-based studies in recent years [1, 2]. Namely, in older community-dwellers higher adherence to Mediterranean-style diet has been associated with better cognitive performance [3, 4], lower frequency of subjective cognitive complaints [5], and lower prevalence and incidence of mild cognitive impairment (MCI) and dementia [6, 7]. Host-related genetic factors, ethnicity, and gender can modulate these associations, but do not substantially modify the assumption that a healthy dietary pattern has a positive influence on the pathophysiology of Alzheimer's disease (AD) and other types of dementia [8, 9].

The Mediterranean diet is a dietary model which has been widely studied for the pivotal role in the maintenance of the health status and the prevention of several disease risk factors [10], among which neurological disorders [11]. The daily and abundant consumption of plant-based food groups, such as fruit, vegetables, cereals, and pulses, leads to the intake of several nutrients that exhibit biologically activities on homocysteine metabolism (B-group vitamins), oxidative stress (vitamin C, vitamin E), neural inflammation, degeneration, and plasticity (vitamin D, n-3 fatty acids) [12, 13]. Such foods are also a great source of non-nutrient bioactive compounds, like polyphenols, with antioxidant, anti-inflammatory, and putative neuroprotective properties [14–16]. Not only the Mediterranean diet, but also the adherence to other dietary patterns in which the intake of such nutrients and bioactives is particularly emphasized and with reduced intakes of rapidly digested carbohydrates, saturated fatty acids, and salt, like the Mediterranean-DASH for Neurodegenerative Delay (MIND) diet or the NU-AGE diet, is associated with better cognitive performance and slower age-related cognitive decline [17, 18].

The ketogenic diet, with very low carbohydrate and high lipid content, has also gained attention for its potential neuroprotective action, especially in the prodromal phases of AD [19, 20]. Despite the promising findings of *in vitro* and animal studies, mainly on the effects of ketones on the mitochondria and their cellular energy-related functions, there still is a lack of clinical studies, as well as a low quality of evidence from the few running ones, to recommend these diets for the prevention of dementia [21, 22].

Conversely, the presence of malnutrition in older age, with insufficient energy and micronutrient intake, is a well-established risk factor for the onset and progression of MCI and dementia [23, 24]. Diets including an excessive intake of animal proteins, saturated fatty acids, and rapidly digested carbohydrates, are also associated with higher risk of MCI and dementia, and with increased cerebral amyloid-β deposition [25, 26]. Interestingly, recent evidence from animal models also links excessive salt intake with progression of dementia through promotion of tau phosphorylation, a central mechanism of neurodegeneration [27].

Despite the evidence from epidemiological and preclinical studies, the effective role of nutrition in the prevention and treatment of neurodegenerative disorders is far from being fully understood. Nutritional interventions for primary or secondary prevention of AD in older subjects have shown only limited benefits, and, to date, diet has only a secondary role in the clinical management of dementia [12, 28, 29]. Apart from methodological and ethical issues making the design of specific trials in this field very challenging, there are also other elements that must be considered, among which the impact of food intake on the whole organism, by starting from the digestive tract where nutrients are metabolized and absorbed.

In fact, diet represents one of the main factors contributing to shape the intestinal microbiota composition and function. Conversely, the microbiota of each individual interacts with food compounds in a complex way, influencing the whole physiology of the host. The microbiota can also influence the pathophysiology of dementia through multiple mechanisms [30, 31], and for these reasons the relationship between diet and dementia should be absolutely unraveled by also considering the key role of the gut microbiota and its metabolic activity.

Unfortunately, the interplay between diet, microbiota composition, and cognitive function has not been comprehensively studied to date. The current state of knowledge allows to hypothesize that dietary patterns can modulate the pathophysiology of AD through mediation of the gut microbiota, and that the consumption of certain types of foods can positively modulate cognitive function only in the presence of specified microbiota composition. However, these assumptions are supported only by indirect evidence, mainly coming from preclinical and animal studies. The aim of this review is to discuss the most recent advances in this field, with a particular focus on how dietary polyphenols and fibers can influence

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the pathophysiology of AD through mediation by the intestinal microbiota.

THE GUT-BRAIN AXIS AND ALZHEIMER'S DISEASE: PATHOPHYSIOLOGICAL MECHANISMS

The term "gut-brain axis" is commonly used to describe a bidirectional channel of communication between the gastrointestinal tract and the central nervous system (CNS). More recently, this term has been extended to "microbiota-gut-brain axis", in light of the accumulating evidence obtained from germ-free animals as well as probiotics, antibiotics, and infection studies on the impact of the intestinal microbiota on this gut-brain interaction [32].

Several neural, immune, endocrine, and metabolic pathways of communication between the gut microbiota and the CNS have been proposed. Undoubtedly, one of the most important is mediated by the vagus nerve, which conveys information between the gastrointestinal tract and the CNS in both afferent and efferent direction [33]. Relatedly, several microbial-derived intermediates, including short-chain fatty acids (SCFAs) and tryptophan metabolites [34, 35], interact with enteroendocrine cells, enterochromaffin cells, and the mucosal immune system, and might propagate bottom-up signaling via vagal and/or spinal afferents [36–38]. In addition, some of these microbial-derived molecules cross the intestinal barrier, entering portal and then systemic circulation, and might even reach brain sites directly [36–38]. The microbiota can also produce or contribute to the production of several neuroactive molecules including gamma-aminobutyric acid, serotonin, norepinephrine, and dopamine [39–42]. Yet, it is unclear whether these neurotransmitters reach relevant receptors in the brain or achieve sufficient levels to induce CNS responses.

In the past decade, advances in sequencing technology, metabolomics, and neurophysiology have allowed a deeper investigation of the cross-talk between the gut and the CNS via the microbiota both in health and disease states [43, 44]. An involvement of gut microbiota in the pathogenesis of neurodegenerative diseases, including AD, has thus been hypothesized [45, 46]. The association between gut microbiota and AD might be related to the central role of inflammation in the development and progression of this neurodegenerative disorder [47]. Indeed, the gut microbiota is a source of a large amount of amyloids—though different in their primary structure from the amyloids in the CNS – lipopolysaccharides, and other toxins that may contribute to systemic inflammation and disruption of physiological barriers [48]. Bacteria and/or their products can then migrate from the gastrointestinal tract to the CNS, especially in older subjects, where gut mucosa permeability tends to increase. In the CNS, bacterial products might promote neuronal amyloid aggregation and neuro-inflammation, ultimately leading to neural injury and degeneration [44, 45, 49, 50]. For example, rats exposed to bacterial amyloids displayed increased neuronal alpha-synuclein deposition in both the gut and the brain, enhanced microgliosis and astrogliosis, and increased central levels of proinflammatory cytokines compared to rats exposed to bacteria without the ability to produce amyloids [51].

Animal models have also been useful for gaining a better understanding of the relationship between microbiota dysbiosis and AD-like pathology. For example, in a series of elegant studies using amyloid precursor protein (APP) transgenic mice, a well-established preclinical model of AD, analysis of bacterial 16S rRNA from fecal samples revealed significant changes in gut microbial composition of transgenic mice compared to wild-type counterparts [52]. Remarkably, germ-free APP transgenic mice exhibited a less severe cerebral amyloid-β pathology compared with control mice with intestinal microbiota, suggesting that the absence of microbiota may retard the progression of AD-like pathology [52]. Intriguingly, colonization of germ-free APP transgenic mice increased cerebral amyloid-β to a greater extent when the microbiota originated from conventionally-raised APP transgenic mice compared with wild-type mice [52].

Supporting these findings, another study in APP transgenic mice revealed a shift in microbial composition compared to wild-type mice, as shown by the higher abundance of *Helicobacteraceae* and *Desulfovibrionaceae* at the family level and *Odoribacter* and *Helicobacter* at the genus level, and the lower abundance of *Prevotella* [53]. Notably, AD-related histological (i.e., amyloid plaque burden) and behavioral (i.e., impaired spatial learning and memory) features were found to be correlated with the specific microbiome state of animal models [53]. A subsequent study investigated age-related changes in the microbiota of APP transgenic mice and found that AD pathology shifted gut microbiota composition during aging towards an inflammation related bacterial profile (i.e., *Proteobacteria* and *Erysipelotrichaceae)*,

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suggesting that these changes could contribute to disease progression and severity [54].

From a theoretical point of view, an abnormal microbiota composition characterized by a high abundance of proinflammatory bacteria and low abundance of anti-inflammatory bacteria may generate a systemic inflammatory response through a defective (i.e., "leaky") gut barrier. This inflammatory response may, in turn, impair the blood-brain barrier, promote neuro-inflammation, and contribute to the pathogenesis of AD [49, 50]. The fecal transplantation from transgenic mice with dementia to cognitively healthy mice induced a worsening of cognitive abilities [55], suggesting a correlation between the gut microbiota composition and cognitive dysfunction. Similarly, studies based on *Drosophila* models of dementia [56] and mouse models of stroke [57, 58] showed an association between gut microbiota dysbiosis, cognitive functions, and clinical course of the disease.

Supporting this view, animal studies have also shown that substances that are able to modify microbiota composition, such as antibiotic agents or probiotics, can modulate inflammatory responses and positively or negatively affect the progression of the disease. For example, long-term antibiotic treatment induced perturbation in gut microbial diversity and altered peripherally circulating cytokine/chemokine composition in APP transgenic mice [59]. This was associated with a reduction in amyloid plaque deposition and elevated levels of soluble amyloid-β, reduced plaque-localized gliosis, and altered microglial morphology [59]. In other experimental studies, antibiotic-induced gut dybiosis was instead associated with increased systemic inflammation and poorer cognitive performance in mice [60, 61].

Likewise, gut microbiota manipulation induced by administration of probiotics belonging to *Bifidobacterium* spp. [62–65] or *Lactobacillus* spp. [62, 63, 66, 67] in transgenic mouse models of AD resulted in improved cognitive performance and reduced markers of inflammation and neuropathology. For example, in one of these studies four-week treatment with a probiotic formulation (i.e., SLAB51, a mixture of lactic acid bacteria and bifidobacteria) in the early stage of the disease led to an increase in the abundance of anti-inflammatory *Bifidobacterium* spp. and a reduction in the abundance of proinflammatory *Campylobacterales* [62]. These changes in microbiota composition were associated with a reduction in the levels of proinflammatory cytokines and a less severe cognitive decline, due to a reduction in brain damage and reduced accumulation of amyloid-β aggregates [62]. Moreover, this probiotic regimen caused a reduction of oxidative stress, another important element involved in the pathogenesis of AD [62].

Few studies also demonstrated that the manipulation of the gut microbiota of mouse models of AD through administration of functional foods, like fructooligosaccharides, flavonoids, or tea saponins resulted in improved cognitive functions [67–70].

Despite this body of evidence, it must be remembered that the results of animal studies may not be immediately transferrable to human beings. Moreover, microbiota-gut-brain communications are bidirectional and the possibility that any changes observed in the microbiota are secondary should be always considered. For example, the activity of the hypothalamic–pituitary–adrenocortical axis and the sympathetic nervous system may be increased in AD, thereby influencing top-down signaling to the gastrointestinal tract [71, 72]. Therefore, activation of these neuroendocrine stress response systems may contribute to the change of microbiota profile, both directly via host-enteric microbiota signaling and indirectly via changes in the intestinal milieu [73].

THE GUT MICROBIOTA IN ALZHEIMER'S DISEASE: HUMAN STUDIES

In the last decade, several studies have investigated the correlations between the fecal microbiome and presence of acute or chronic illness [74–76]. Notably, several microbial taxa of the intestinal microbiota play a crucial role in maintaining host homeostasis by modulating immunological, nutritional, metabolic, and physiological functionalities, from infancy to senescence [77–80]. The alteration of this symbiotic homeostasis may be involved in the onset and progression of diseases, not involving only the gastrointestinal system [77–80]. This concept also applies to CNS pathology [81]. Remarkably, the human intestinal microbiota can influence cognitive function, and intestinal bacteria may be involved in the pathophysiology of neurodegenerative disorders, including multiple sclerosis, Parkinson's disease, and of course AD [81].

Several studies, mainly conducted with metagenomics sequencing of fecal samples, have explored the possible role of gut microbiota in the pathogenesis of dementia, particularly of AD, highlighting alteration in the intestinal microbial composition of

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patients in comparison with controls [82–89]. The findings of these studies are summarized in Table 1.

As highlighted by Vogt and colleagues [83], one of the main differences in the gut microbiota composition, determined through the use of 16S rRNA gene profiling approach, between AD patients and controls is represented by reduced bacterial richness and diversity in subjects with dementia. Moreover, a distinct bacterial profile seems to characterize the gut microbiota of AD patients, who display high abundance of species belonging to Bacteroidetes phylum, such as *Bacteroides* spp. and *Alistipes* spp., and reduced representation of members of the *Bifidobacterium* genus and taxa belonging to Actinobacteria, and Firmicutes phyla [83, 84, 89].

Remarkably, overrepresentation of *Bacteroides fragilis* in the microbiota has been associated with the development of AD, probably due to its production of lipopolysaccharide, which could contribute to systemic inflammation [90]. Two studies from China also show increased representation of bifidobacteria in fecal samples of patients with AD, suggesting that this genus may represent a microbial marker of AD [88, 89]. However, bifidobacteria were underrepresented in the fecal microbiota of patients with AD in the Vogt study [83], and the genus *Bifidobacterium* is generally associated with longevity and healthy aging [91].

In one study, the gut microbiota of AD patients was also characterized by low abundance of butyrate-producing taxa, such as members of the *Butyrivibrio*, *Eubacterium*, *Roseburia*, and *Faecalibacterium* genera [86]. Furthermore, deep metabolomic investigations based on microbiome data of AD patients compared to individuals without dementia highlighted a decrease in butyrate-coding genes in AD disease [86]. These findings might suggest that lower proportions of butyrate-producing species would contribute to the onset of the disease.

Moreover, AD patients displayed a tendency towards increase in relative abundance of bacterial species related to human diseases or able to act as opportunistic pathogens, like *Klebsiella pneumoniae*, *Bacteroides fragilis*, and *Eggerthella lenta* [48, 83, 86, 88, 92]. These species could be characteristic of a pro-inflammatory microbiota, which might contribute to the pathophysiology of AD.

Oddly, Emery et al. explored the bacterial load of frozen and fixed postmortem tissue from AD and control temporal cortex [93]. This study reported that AD brains tend to have higher bacterial load than controls, reinforcing the notion of a possible correlation between human gut microbiota and the development of AD [93].

Two recent systematic reviews and meta-analysis have considered the human studies where cognitive function was measured as one of the clinical endpoints after administration of probiotics or prebiotics to subjects suffering from dementia or MCI [94, 95]. The large majority of these studies were of poor methodological quality, with small sample size, absence of randomization of treatments and, sometimes, absence of a control group receiving standard of care treatment [94, 95]. Only five studies, with an overall number of 297 participants, had a randomized controlled design and sufficient methodological quality [96–100]. These studies showed improvement in cognitive function of participants after probiotic administration, mainly consisting in blends of bifidobacteria and lactobacilli, supporting the existence of a gut-brain axis also in human beings with AD [94–100].

However, the clinical significance of these findings, and their impact on the future management of AD is still unknown. More importantly, none of these studies conducted on human beings considered diet as a confounding variable or as a possible modulator of gut microbiota composition.

POLYPHENOLS AND GUT MICROBIOTA IN ALZHEIMER'S DISEASE

Overview of polyphenols and their gut metabolites

The consumption of foods rich in polyphenols has been associated with numerous beneficial effects on the risk and development different neurodegenerative diseases [101]. Among polyphenols, positive findings have been highlighted for chlorogenic acids (mostly present in coffee, leafy vegetables, and berries), curcumin (mainly present in turmeric), and resveratrol (a stilbene present in grapes and wine) [102], but the most robust evidence is related to flavonoids, the most abundant subclass of polyphenols [103].

Two recent surveys conducted on US adult cohorts "The Nurses' Health Study", "The Health Professionals Follow-up Study" [104] and "Framingham Offspring Cohort" [105] confirmed that the long-term intake of flavonoids significantly decreased the risks of developing AD and related dementias and subjective cognitive decline markers. Among flavonoid subclasses, the most positive findings on cognitive

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Table 1
Overview of the findings of the main human studies investigating gut microbiota composition in AD

Author, year	Country	Sample size	Age	Key findings in AD patients
Cattaneo et al., 2017 [82]	Italy	40 amyloid-positive with dementia 33 amyloid-negative with dementia 10 healthy controls	71 ± 7 (AD) 68 ± 8 (controls)	Increase of pro-inflammatory taxa, such as *Escherichia* Decrease of anti-inflammatory taxa, such as *E. rectale* Correlation between microbiota composition and circulating inflammatory cytokines
Vogt et al., 2017 [83]	US	25 patients with AD 25 age- and sex-matched controls	71 ± 7 (AD) 69 ± 8 (controls)	Reduced species richness and representation of several taxa including *Bifidobacterium* Correlation between the abundance of these taxa and cerebrospinal fluid markers of amyloid burden
Zhuang et al., 2018 [84]	China	43 patients with AD 43 age- and sex-matched controls	70 ± 9 (AD) 69 ± 9 (controls)	Different microbiota composition with increase in *Ruminococcaceae* and *Lactobacillaceae* and decrease in *Lachnospiraceae*
Liu et al., 2019 [85]	China	33 patients with AD 32 patients with amnestic MCI 32 healthy controls	75 ± 11 (AD) 70 ± 11 (MCI) 77 ± 9 (controls)	Reduced commensal diversity Decreased representation of *Lachnospiraceae* and *Ruminococcaceae* Increased representation of *Enterobacteriaceae* that negatively correlated with cognitive performance
Haran et al., 2020 [86]	US	24 nursing home residents with AD 33 nursing home residents with other types of dementia 51 nursing home residents without dementia	85 ± 8 (AD) 88 ± 8 (other dementia) 83 ± 10 (controls)	Different microbiota composition clusters among groups Increased representation of *Bacteroides, Alistipes* and *Barnesiella*, and reduced representation of *Lachnoclostidium* and butyrate-producing species
Ling et al., 2021 [88]	China	100 patients with AD 71 age- and gender-matched controls	74 ± 9 (AD) 73 ± 8 (controls)	Reduced species richness Decreased representation of eight key genera, including *Faecalibacterium* and *Roseburia* Increased representation of *Bifidobacterium*
Zhou et al., 2021 [89]	China	60 patients with AD (30 with neuropsychiatric symptoms) 32 healthy controls	73 ± 7 (AD) 71 ± 6 (controls)	Reduced species richness Decreased representation of *Eubacterium* and increased representation of several genera, including *Bifidobacterium* The abundance of several key genera was associated with symptoms and severity of AD
Xi et al., 2021 [81]	China	21 patients with AD 44 controls with normal cognition	76 ± 10 (AD) 78 ± 6 (controls)	Alteration of representation of 15 key bacterial taxa Increased representation of *Faecalibacterium* Altered predicted metabolic profile

AD, Alzheimer's disease; MCI, Mild Cognitive Impairment; US, United States.

health have been found for flavones, flavonols, and anthocyanins [102, 105]. In another survey, based on 921 older subjects from the US, the authors extrapolated intake data for single phenolic compounds, observing that high dietary flavonol intake, and particularly of kaempferol and isorhamnetin, may be protective against the development of AD [106].

Despite the *in vitro* experimental findings of the last decades confirm these molecular mechanisms, one should not forget the complexity of human physiology and, in particular, of the digestive system, and the now proven extensive catabolism of polyphenols in the lower gastro-intestinal tract has induced scientists to re-think what the real biological effectors might be. In fact, once ingested, the polyphenols present *in planta* pass through the oral cavity and stomach almost unmetabolized. Once in the small intestine, only a small amount is absorbed and able to reach the liver and, subsequently, the systemic circulation [107]. The most relevant fraction of ingested polyphenols reaches the colon and undergoes substantial modifications of the parent chemical structures through the action of bacterial enzymes. Thus, most of the biological effects of (poly)phenols are mediated by the intestinal microbiota. The generated bacterial metabolites are then absorbed and may

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undergo hepatic phase I and, most prominently, II metabolisms [108].

The result of such biochemical transformation is the release in the bloodstream of smaller phenolic compounds, including benzene diols and triols, benzaldehydes, benzoic acids, phenylacetic acids, phenylpropanoic acids, cinnamic acids, hippuric acids, and many other compounds, which might sometimes be very specifically linked to their parent phenolic structure [109]. For this reason, in the most recent years, the studies focusing on the role of (poly)phenols on health outcomes are considering, as real biological effectors, the colonic metabolites rather than the compounds that are naturally present in the food source. A recent review by Carregosa and collaborators [109] summarized in a very exhaustive manner the role of the several metabolites, categorized for the single deriving subclasses of flavonoids, on several markers of neurodegenerative diseases, among which AD, tested in very different model organisms, from fungi to humans.

Flavan-3-ols

Flavan-3-ols are among the most representative contributors of the flavonoid intake in several European countries [110–112]. A recent review from our group comprehensively described the possible colonic metabolites following flavan-3-ol ingestion, showing that phenyl-γ-valerolactones, phenylvaleric acids, and their glucuronide, sulfate, and methylated forms are the most abundant in humans [113]. In a study using different *in vivo* animal models, Angelino et al. [114] also demonstrated that one of these metabolites, namely 5-(hydroxyphenyl)-γ-valerolactone-sulfate (3′,4′ isomer), was effectively detected in brain tissues of the different animals used. Concerning the biological activity, recently our group evaluated the role of such metabolites in counteracting the formation of amyloid-β oligomers (AβO) in yeast, mammalian cell, and mouse models, at physiological concentrations [115]. Results showed that 5-(4′-hydroxyphenyl)-γ-valerolactone was able to counteract the toxicity induced by the formation of oligomers, but not fibrils [115]. The mouse model, characterized by individuals with AβO-induced memory impairment, showed a relieve of the brain tissue morphology and an improvement of the memory functions when the animals were co-exposed to the metabolite [115]. The same compound, as well as 5-(3′,4′-hydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, tested up to 5 μM, was effective in modulating cellular proteolysis via proteasome inhibition and consequent autophagy upregulation, together with the inhibition of cathepsin B activity [116]. This led to the decrease of the amount of intra- and extracellular $A\beta_{1\text{-}42}$ peptides.

Two recent studies aimed at investigating the amelioration from the AβO toxicity by using extracts rich in flavan-3-ols in cell culture models and confirmed the presence of flavan-3-ol metabolites in murine models [116, 117]. Another study evaluated the role of a lotus extract (*Nelumbo nucifera*) in the reduction of cellular deformation and apoptosis rate in cells pre-incubated with $A\beta_{25\text{-}35}$ by affecting different cascades involving brain-derived neurotrophic factor (BDNF), phosphorylation of cAMP-responsive element-binding (CREB), protein kinase B (also known as AKT), and the extracellular signal-regulated kinase (ERK) [116]. Authors evaluated the bioavailability of the extract by feeding rats with a 200 mg/kg bole dose of extract for two days, and confirmed the presence of small phenolic acids, mainly caffeic, vanillic, and *p*-hydroxyphenylacetic acids (no conjugated metabolites were measured as β-glucuronidase/sulfatase enzymes were used before analysis) [116]. Similarly, a study from the US demonstrated that different concentrations of a pine bark extract, rich in proanthocyanidins and (epi)catechin, was able to block the formation of aggregates of AβO and tau protein [117]. Authors studied the bioavailability of the extract by feeding bole doses of 200 mg/kg pine bark extract to rats for then days, identifying in plasma and brain tissues the methylated and glucuronidated conjugates of catechin and epicatechin [117].

Concerning human studies, Desideri et al. supplemented with increasing concentrations of cocoa flavan-3-ols, up to 990 mg/day for 8 weeks, older subjects with cognitive impairment, and showed a significant amelioration of the cognitive functions of participants consuming high doses of flavan-3-ols [118]. Moreover, Brickman et al. used the functional magnetic resonance imaging technique to demonstrate that healthy active older subjects consuming cocoa flavan-3-ols (900 mg/day) had an improvement of the cerebral blood flow in the body of the hippocampal circuit, primarily to the dentate gyrus and subiculum, driver of the age-related cognitive decline [119].

Different projects are now running to improve our knowledge of the role of (poly)phenol-rich foods on cognitive parameters. Among these, the "Cognitive

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Ageing, Nutrition and Neurogenesis (CANN)" trial is evaluating the effects of the supplementation of long-chain ω-3 polyunsaturated fatty acids plus 500/day of cocoa flavan-3-ols, for 12 months, on cognitive markers in older subjects from the UK and Australia with mild cognitive or subjective memory impairments [120]. The "Valerolactones and healthy Ageing: LInking Dietary factors, nutrient biomarkers, metabolic status and inflammation with cognition in older adults (VALID)" project aims to demonstrate the validity of the phenyl-γ-valerolactones as biomarker of flavan-3-ol intake and to investigate whether the consumption of foods and beverages rich in flavan-3-ols—and so the presence of specific colonic metabolites–may have a role in the preservation of the cognitive health in the Irish TUDA elderly cohort participants [121].

Ellagitannins

Pomegranate (*Punica granatum L.*) is a rich source of ellagitannins, a class of condensed tannins which are very poorly absorbed and, instead, reach the colon where colonic bacteria metabolize them to form a group of compounds named urolithins [108]. Pomegranate has been supplemented in different animal studies to evaluate putative biological effects on AD and more general cognitive markers. A Brazilian study revealed that the supplementation of pomegranate peel extract (800 mg/kg/die) for 35 days to mice infused with AβO was able to decrease amyloid plaque density and the expression of several brain inflammatory features, in addition to improving animal behavior, compared to control mice [122]. While in the latter study there is no focus on the colonic metabolites, Kujawska et al. identified quantifiable amounts of urolithin A in brain tissue of rats treated with rotenone to induce cognitive impairment and after the administration of pomegranate juice (500 mg/kg/die) for 35 days [123]. The authors demonstrated that the pomegranate juice supplementation, and putatively the presence of the microbial metabolite urolithin A, was able to decrease the expression of different oxidative, neuroinflammatory, and apoptotic markers [123]. The effect of single urolithins has been investigated both *in vitro* and through animal studies. DaSilva and collaborators evaluated the modulation of inflammation by urolithin A, B, and their methylated forms, up to 10 μM, in single LPS-stimulated microglia cells or in co-culture with neuroblastoma cells [124]. Results showed that all the compounds were able to reduce some neuroinflammatory mediators, like nitric oxide, interleukins, prostaglandins, and pro-apoptotic factors, compared to control. Finally, Gong and colleagues supplemented transgenic AD mouse models with 300 mg/kg urolithin A for 14 days and found an attenuation of neuron death through the inhibition of AβO deposition and a trigger of neurogenesis via inhibition of several markers of neuroinflammation and apoptosis [125].

Anthocyanins

Anthocyanins are the main contributors to the red/purple color of fruit and vegetables and include compounds such pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin and their glycoside conjugates. Their main colonic metabolites are small phenolic acids, as protocatechuic, gallic, vanillic, syringic, and hydroxybenzoic acids [108]. A recent and exhaustive review pointed out that the main metabolites showing *in vitro* neuroprotective effects were protocatechuic, gallic, and vanillic acids, limited for other phenolic acids [126]. The most important target of these phenolic metabolites, mostly protocatechuic and vanillic acids, were the antioxidant activities at intracellular level, so counteracting the depletion of glutathione and catalase activities due to peroxide stimulation or improving cell viability after nitro-radical increase in the cell [127, 128]. Not only antioxidant, i.e., superoxide dismutase or catalase, but also inflammation enzymes, i.e., cyclooxygenases, were also inhibited by gallic acid treatment of neuronal cells, despite in high concentrations, with consequent decrease of cytokine production [129].

Concerning immortalized cells, several studies pointed out that gallic, vanillic, and hydroxybenzoic acids are able to block the oligomerization of Aβ, destabilize pre-fibril and fibril formation, by structural interaction, by metal ion chelation, or by decreasing neuro-inflammatory and apoptotic mediators [130–132]. These findings have been also confirmed in numerous animal models, with an improving of the endogenous antioxidant enzyme activities, as well as the reduction of the presence of several pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, at brain level, by vanillic, gallic, and protocatechuic acids [126].

Flavanones

Flavanones are a flavonoid subclass mainly found in citrus fruits, and the most recurrent aglycones are

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hesperetin, naringenin, eriodictyol, isosakuranetin, and taxifolin, even if the major amounts of them are present as glycoside conjugated [133]. Several studies confirmed a low bioavailability of such compounds, with the identification and quantification of several colonic metabolites in biological fluids, such as phenylpropionic, phenylacetic, benzoic, and hydroxycarboxylic acids, and benzenetriol and benzoylglycine derivatives [134]. By considering blood oranges, mandarins, lime and lemon juice, the most representative compound in terms of amount in such fruits is hesperidin, which is the rutinoside of the hesperitin [133]. Hesperidin has been the object of several *in vitro* and *in vivo* animal studies to investigate its role as a neuroprotective compound. Concerning AD, the main results have been reported for the role of hesperidin in counteracting the oxidative stress at neuronal level, with improvement of the antioxidant endogenous mechanisms, such as counteracting glutathione depletion, and decrease of malondialdehyde and H_2O_2 accumulation, other than inhibiting some protein kinase phosphorylation leading to oxidative damage, in animal studies [135, 136]. Hesperidin at 100 mg/kg orally administered per 60 days was also found to be effective in the attenuation of the expression of amyloid-β precursors and oligomers in rats after aluminum chloride injection to mimic the damage caused by AD [137]. Several other molecular patterns involved in the boosting of neuroinflammation have been reduced in a mouse model affected by cognitive impairment when 100 and 200 mg/kg hesperidin has been supplemented for 15 days [138]. Anti-inflammatory and fibril anti-aggregative effects have also been observed in a recent study, where human neuroblastoma cells were incubated with up to 50 μM liquiritigenin, mainly found in licorice [139]. To date, no human studies have considered the putative biological effects of the flavanones or their colonic metabolites on pathophysiological and clinical markers of AD, and this represents a promising area for future research.

Isoflavones

Among the so called "phytoestrogens", isoflavones are a class of compounds mainly found in soy and its derivatives, genistein, daidzein, glycitein, formononetin, and their glycosides being the most representative compounds [108]. Studies on urine samples revealed a scarce bioavailability of such compounds, which are mainly metabolized by colonic bacteria in smaller compounds, such as equol, *O*-desmethylangolesin, and its 6'-hydroxylated form, which leads to 2-(4′-Hydroxyphenyl)propionic acid, and their conjugates [108]. There is solid evidence of neuroprotective effects of soy and soy-derived foods on several cognitive outcomes in animals and humans [140, 141]. Additionally, AD has been targeted in for studies focusing on isoflavones and, as previously detailed, the most relevant mechanisms highlighted in cell and animal models has been the counteraction of the amyloid-β deposition, oxidative damage, neuroinflammation, and apoptosis processes [142].

However, very few studies considered the colonic metabolites as real biological effectors towards the above-mentioned effects on the nervous system. In a randomized controlled trial, the administration of 100 mg/day of soy isoflavones to 65 older patients suffering from AD did not result in any significant improvement of cognitive function or dementia biomarkers after six months [143]. Researchers correctly argued that not all the individuals are equol-producers, mainly due to the gut microbiota composition and functionality, and so the missing effects of the isoflavone supplementation might be biased by the lack of consideration of this fact in the study design [143, 144]. Actually, when equol was administered to fifteen women with AD, the oxidative and inflammatory markers failed to be effectively modulated, but, clearly, further studies are needed to better understand this interesting aspect [145].

Flavonols

Flavonols are among the most ubiquitous polyphenols in fruit and vegetables, and quercetin, kaempferol, isorhamnetin, and myricetin are the principal representative aglycones, mainly conjugated in several different combinations with one or more sugar molecules [108]. Until recently, the major focus in biological fluids have been aglycones conjugated with glucuronide and/or sulfate moieties, mainly formed during the II detoxification at the upper gastro-intestinal and liver level. However, the simple fermentation of some compounds, such as quercetin, with fecal samples of healthy donors, revealed a deep rearrangement of its chemical structure, leading to the release of smaller phenolic acids, as phenylpropanoic, phenylacetic, and hydroxybenzoic acids [146]. A huge load of literature has been published regarding flavonols, and particularly quercetin, involvement in the processes leading to AD and, generally, cognitive disorders [147–148]. Results confirm a role of such compounds in the

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amelioration of oxidative status at neuronal level, inhibition of inflammatory and apoptotic key pathways, as well as amyloid-β oligomerization and deposition [147–149]. Also, for this class of compounds, colonic metabolites have not been deeply studied for their putative effects on cognitive decline markers.

Flavones

Regarding flavones, a flavonoid subclass widely present in vegetable-derived foods and beverages, the most studied compounds are apigenin and luteolin, which are usually present in foods as glycosides, which are barely absorbed along the small and large intestine and, above all, colonic bacteria seem to be unable to cleave the C-linked sugar moiety [108]. However, some data on protective effects of the *in planta* compounds towards AD-related markers, in cell and animal studies, have been reported for apigenin [150] and luteolin [151].

Gut microbiota involvement in polyphenol metabolism

As previously stated, the catabolism of phenolic compounds in the lower gastrointestinal tract is strictly dependent on the presence of certain bacteria strains, able to vastly modify the parent structures giving origin to smaller phenolic acids that are, in turn, absorbed quite efficiently. In a recent review, Cortés-Martín and collaborators summarized that, despite the many microbiological studies present in the literature, most of the microbial species responsible for specific metabolite production are still unidentified [152].

The hydrolysis of the sugar, in the case of the glycosylated polyphenols, is carried out by different species of *Lactobacillus, Bifidobacterium*, and *Enterococcus*, characterized by the activities of enzymes like glucosidases and rhamnosidases, able to cleave the *O*-sugar moiety (e.g., *Lactobacillus plantarum or Bifidobacterium pseudocatenulatum*) and, with a lower affinity, C-linkages (e.g., *Enterococcus* spp., *Eubacterium cellulosolvens*, and *Lactococcus* spp.) [152].

The elucidation of which species are able to cleave flavonoid rings is still open. Peiroten et al. summarized the main findings related to the metabolism of isoflavones by several different bacteria, identifying several species belonging to *Clostridium and Eubacterium* genera as able to transform daidzein into *O*-desmethylangolesin, and *Bifidobacterium*, *Eggerthella, Lactococcus*, and *Slackia* species as able to produce equol from daidzein [153]. A study from Germany identified two species, namely *Eggerthella lenta* and *Flavonifractor plautii,* in human fecal suspension, able to convert (–)-epicatechin and (+)-catechin into phenylpropanoic and phenylvaleric acids as well as to phenyl-γ-valerolactones [154]. Spanish researchers identified different species belonging to the *Egghertellaceae* family (*Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae*) as able to convert ellagic acid into urolithins A and B and isourolithin A [155, 156].

The inter-individual variability role in the polyphenol gut microbial metabolism

Epidemiological studies have associated the intake of fruit, vegetables, and beverages rich in polyphenols with multiple beneficial effects on the human health. However, when intervention studies looked at the real biological effectors and at cause-effect relationships, a huge variability in terms of production/not production and, sometimes, concentrations of polyphenol gut microbial metabolites in fluids and tissues was observed. The inter-individual variability in the gut microbial capacity to produce peculiar profiles of phenolic metabolites seems to be the most important factor to explain the bioavailability of phenolic compounds and, possibly, the biological response to their intake [157]. The term "metaboytpe"—metabolic phenotype—has been introduced to indicate this characteristic excretion of metabolites after intake of specific dietary compounds [158]. Specifically, the new frontiers in this field will be the identification of cluster of individuals showing a metabotype associated with a well profiled gut ecosystem and, in turn, to a specific health status or improvement of health in response to food intake.

The definition of a metabotype should primarily include qualitative data, such as absence or presence of specific metabolites, but could also derive from quantitative aspects, i.e., relative ratios of metabolites [158]. The first pioneering studies in this field have been conducted on soy isoflavones, where the excretion of the urinary metabolites equol and *O*-desmethylangolesin have been observed only in certain individuals after soy consumption [159]. Equol producers have been shown to be characterized by more favorable hormonal profiles and by a lower breast cancer risk [160].

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The research group led by Tomás-Barberán has shown that not only producers and not producers of urolithins, metabolites of dietary ellagitannins, are linked to specific enterotypes, but that producers can be further clusterized in metabotypes that are then associated to different presence of dysbiotic symptoms and also different cardiometabolic risk profiles [161]. Similarly, our group is working on the elucidation of the possible metabotypes following the ingestion of flavan-3-ol rich foods, with the preliminary findings indicating the presence of distinct groups of individuals excreting trihydroxyphenyl-γ-valerolactones, dihydroxyphenyl-γ-valerolactones, and hydroxyphenylpropionic acids [162, 163].

DIETARY FIBERS, GUT MICROBIOTA, AND SHORT-CHAIN FATTY ACIDS IN ALZHEIMER'S DISEASE

Dietary fibers include plant-based carbohydrates (non-starch polysaccharides, such as cellulose, resistant oligosaccharides, such as inulin, and resistant starch) that cannot be metabolized by human digestive enzymes [164]. Some gut bacteria are, however, able to metabolize such dietary compounds, generating SCFAs, i.e., acetate, propionate, and butyrate, as endproducts [165].

Most bacteria able to synthetize SCFAs belong to the *Ruminococcaceae* and *Lachnospiraceae* families [166, 167]. Few bacterial taxa, including *Roseburia* spp. and *Eubacterium rectale*, are able to complete the degradation of fibers to SCFAs alone [166, 167]. More frequently, there are cross-feeding interactions between taxa belonging to the same ecological niche: fibers are degraded to intermediate products by one group of bacteria, and then these products are utilized by SCFA producers [166, 167]. Alternatively, fiber degradation by one strain is essential for stimulating the growth and metabolic activity of a SCFA-producing strain [166]. The most known of these cross-feeding interactions regards *Bifidobacterium* spp. and *Faecalibacterium prausnitzii*, whose butyrate-producing capacity relies on the supply of acetate by bifidobacteria [168, 169].

SCFA synthesis by the gut microbiota is strongly influenced by the amount of fibers introduced with diet, that is able to shape the microbiota composition and enhance the SCFA metabolic pathways [170]. A recent population-based study has shown that the consumption of large amounts of fiber-rich plants is associated with increased representation of SCFA-producing genera in fecal microbiota [171]. A similar effect has also been demonstrated in an intervention study specifically focused on older subjects [172]. However, these effects do not necessarily correspond to an increase in SCFA production, due to

Table 2
Proposed physiological functions of short-chain fatty acids produced by gut microbiota in human beings

Actions on gastrointestinal function	– Increased expression of tight junctions – Reduced intestinal permeability – Increased mucus production – Improved mucus quality (glycosylation of mucins) – Energetic supply to colonocytes – Modulation of colonic motility – Possible increase of calcium absorption
Actions on gut mucosal immunity	– Modulation of mucosal inflammation – Reduction of synthesis of pro-inflammatory cytokines – Modulation of dendritic cell function – Reduction of oxidative stress by enhanced glutathione function – Inhibition of the expression of chemokines and cytokines induced by lipopolysaccharide – Promotion of conversion of naïve T-cells into FoxP3 + T_{reg} cells – Stimulation of IgA production (mediated by retinoic acid synthesis) – Induction of expression of antibacterial peptides (cathelicidins)
Actions on the host metabolism	– Modulation of body weight (butyrate) or increase in body weight (acetate) – Increased satiety and reduced energy intake – Increased energy expenditure, thermogenesis, and basal metabolic rate – Stimulation of lipid oxidation – Decreased circulating fatty acid levels – Stimulation of lipolysis – Stimulation of gluconeogenesis – Decreased fasting glucose levels – Improved insulin sensitivity – Modulation of insulin secretion
Actions on systemic inflammation	– Modulation of chronic release of pro-inflammatory cytokines (IL-6, IL-1β, TNFα) – Modulation of adipokine secretion – Reduction of eicosanoid production – Increased neutrophil apoptosis

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the complex cross-feeding interactions among bacteria and variable expression of bacterial metabolic pathways [173].

Around 90% of SCFAs produced in the gut lumen are absorbed by colonocytes or enter circulation, while the remaining 10% is excreted in feces [166]. SCFAs exert a wide range of physiological functions in the intestine and in human metabolism, which have been extensively reviewed elsewhere [166, 174] and are summarized in Table 2.

The studies investigating fecal microbiota composition in patients suffering from AD generally show a depletion SCFA-producing bacteria in comparison with controls (Table 3), especially taxa able to synthetize butyrate belonging to *Lachnospiraceae* or *Ruminococcaceae* [82–87]. However, butyrate producing bacteria can be isolated and cultivated from fecal samples of patients with AD [175] and a recent study failed to detect a significant difference in the relative abundance of the main SCFA producers between patients with AD and healthy controls (Table 3) [88]. Interestingly, a recent analysis of the gut microbiota composition and fecal metabolomics profile of 21 Chinese patients with AD and 44 controls with normal cognitive function revealed a paradoxical increase of representation of some SCFA-producing taxa, such as *Ruminococcaceae* and *Faecalibacterium prausnitzii*, in fecal samples from AD patients [89]. In the light of the existing literature, such findings suggest that the relationship between gut microbiota composition and cognitive dysfunction may be strongly influenced by

Table 3
Overview of the findings of studies that have investigated the potential of SCFA synthesis of the intestinal microbiota in patients with AD or other forms of dementia

Author, year [ref]	Country	Sample size	SCFA-producing taxa depleted in AD	Other key findings
Cattaneo et al., 2017 [82]	Italy	40 amyloid-positive with dementia 33 amyloid-negative with dementia 10 healthy controls	*Eubacterium rectale*	The abundance of *E. rectale* was inversely related to brain amyloid deposition
Vogt et al., 2017 [83]	US	25 patients with AD 25 age- and sex-matched controls	*Ruminococcaceae* *Bifidobacterium*	Correlations between the abundance of bacterial taxa and markers of neural inflammation
Zhuang et al., 2018 [84]	China	43 patients with AD 43 age- and sex-matched controls	*Lachnospiraceae*	The abundance of *Ruminococcaceae* was overall increased in AD subjects
Liu et al., 2019 [85]	China	33 patients with AD 32 patients with amnestic MCI 32 healthy controls	*Ruminococcaceae* *Ruminococcus* spp	The abundance of *Enterobacteriaceae* was the key feature distinguishing patients with AD from other categories
Haran et al., 2020 [86]	US	24 nursing home residents with AD 33 nursing home residents with other types of dementia 51 nursing home residents without dementia	*Eubacterium* *Roseburia* *Lachnoclostridium* *Butyrivibrio* *Faecalibacterium*	Depletion of taxa with the potential of synthetizing butyrate is distinctive of AD in comparison with other categories
Ling et al., 2021 [87]	China	100 patients with AD 71 age- and gender-matched controls	*Roseburia* *Faecalibacterium* *Butyricicoccus*	The abundance of SCFA producers inversely correlated with parameters of cognitive function and functional performance
Zhou et al., 2021 [88]	China	60 patients with AD (30 with neuropsychiatric symptoms) 32 healthy controls	None	The composition of microbiota of AD patients was significantly different than controls, but the abundance of the main SCFA-producing taxa was not different.
Xi et al., 2021 [89]	China	21 patients with AD 44 controls with normal cognition	None	The abundance of *Ruminococcaceae* and *Faecalibacterium* was increased, and not depleted, in AD patients.

AD, Alzheimer's disease; MCI, Mild Cognitive Impairment; US, United States.

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external factors that were not considered as possible confounders, including dietary patterns and fiber intake. Geographical location of study participants may also represent an important issue: recent data indicate that study location represents the main factor explaining inter-individual variability in microbiome studies [176, 177].

The gut mycobiome (i.e., ensemble of fungal populations symbiotically living with the host in the gut lumen) could also interact with the intestinal microbiome in patients with AD. A recent study has shown that reduced representation of SCFA producers, including *Ruminococcus, Lachnospira*, and *Roseburia*, is associated with a distinct intestinal mycobiome composition in patients with AD [178].

Overall, this evidence supports the assumption that patients with AD could have reduced SCFA synthesis in their gut microbiota. However, the descriptive, cross-sectional design of these studies, and the absence of direct measurement of SCFA levels in feces or other biological samples does not allow to make any inference on the causal relationship between SCFA depletion and dementia. Furthermore, none of these studies considered nutritional intake of fibers as covariate.

In experimental models of AD, SCFAs exhibit a neuroprotective effect at multiple levels [179]. More specifically, they contribute to modulate the microglial cell function, reducing phagocytic activity, secretion of cytotoxins and synthesis of pro-inflammatory cytokines [179, 180]. In experimental conditions, SCFAs also inhibit amyloid-β aggregations, interfering with amyloid plaque formation and enlargement [181]. Interestingly, the brain amyloid load, measured with standardized uptake value ratio versus cerebellum through florbetapir amyloid-positron emission tomography (PET), was negatively correlated with blood butyrate levels in a group of 89 older subjects with different cognitive function, ranging from normal to overt dementia [182].

The clinical translation of these findings remains, however, uncertain. To date, studies specifically investigating the effects of increasing fiber intake on cognitive function and risk of dementia are lacking. Most studies were focused on nutritional patterns and frequency of consumption of fruit and vegetables, indicating that there could be an inverse relationship between fiber intake and cognitive performance, especially in those who already suffer from dementia or subjective cognitive complaints [24, 183–185]. Two studies also showed an inverse correlation between a dietary pattern characterized by elevated fiber intake and imaging biomarkers of AD (i.e., ^{18}F-fluorodeoxyglucose uptake on brain PET in one study [186] and white matter hyperintensities module on brain magnetic resonance in another [187]). However, a large study conducted on 162 older subjects did not find any significant correlation between fiber intake and brain amyloid-β burden measured with amyloid-PET [188]. Furthermore, none of these studies considered investigation of SCFAs in their experimental design.

From a clinical perspective, the possibility of influencing AD pathophysiology through modulation of the intestinal microbiota towards an enhanced production of SCFAs is intriguing. However, very few

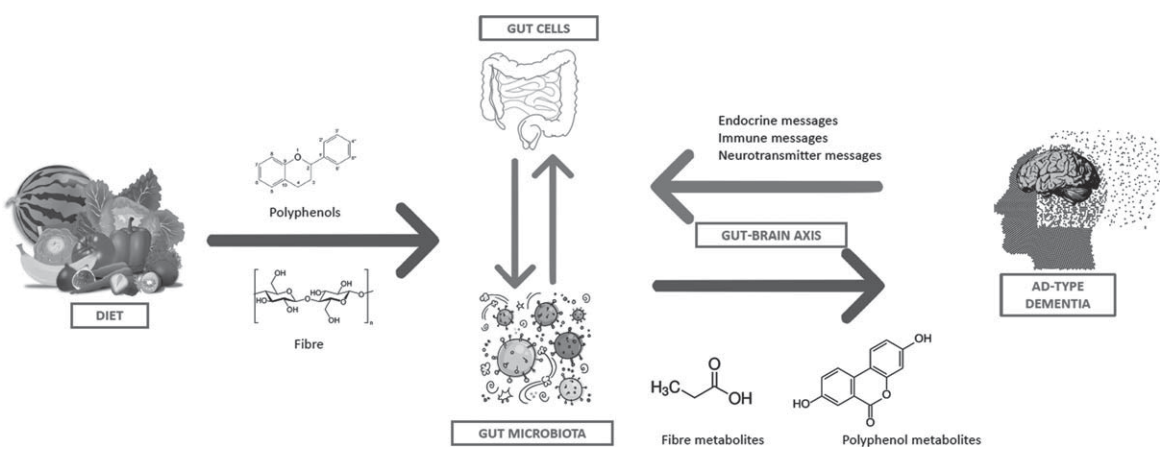


Fig. 1. Overview of the possible link between dietary fiber and polyphenol intake, gut microbiota and modulation of the pathophysiology of Alzheimer's disease.

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clinical trials with probiotics or prebiotics have considered cognition as main endpoint [189]. Three recent systematic reviews and meta-analyses have shown contradicting results, supporting the plausibility of favorable effects of probiotics or prebiotics on cognition with a low level of evidence [94, 190, 191]. None of the studied interventions were, however, specifically targeted at increasing SCFA production in the gut, and most studies were limited by small sample sizes.

Overall, the current evidence supports the concept that increasing dietary fiber intake is associated with favorable effects in the pathophysiology of AD through mediation of the gut microbiota. However, the clinical relevance of this mechanism and its therapeutic potential remains unclear.

CONCLUSIONS AND PERSPECTIVES

The current state of knowledge allows to hypothesize that the intestinal microbiome stands at the crossroads between nutrition and pathophysiology of AD, and that many of the putative beneficial effects of healthy dietary patterns on cognition could be mediated by the gut microbiota (Fig. 1). However, there is still a big gap between preclinical and clinical studies in this field. In fact, the influence of nutrition and gut microbiota on the pathophysiology and clinical course of AD has been studied mainly considering each player in a separate way, without integrating nutritional and microbiological investigations. The evidence suggesting favorable effects of gut microbiota-derived metabolites of polyphenols or fibers on markers of AD mostly comes from *in vitro* studies, and its translation in clinical practice is still difficult. As a result, there is a substantial lack of knowledge on how microbiome-centered nutritional interventions can modify cognitive function from a clinical perspective.

However, the gut microbiota could become a reasonable target for anti-dementia interventions in the foreseeable future, as suggested by preclinical studies [80, 179]. Nutritional strategies implying an increase in the intake of polyphenols and fibers have a great potential of being effective in slowing down cognitive decline in older individuals, but future studies should also consider the influence of microbiota composition in this putative association. For example, the cognitive benefits of an increase in polyphenol intake could depend on specific metabotypes of the gut microbiota and the benefits, in terms of SCFA production, of increasing fiber intake could be influenced by the pre-existing network of strains with capacity of synthetizing butyrate [192, 193].

Future studies should also consider the co-occurrence of cognitive decline and physical frailty in older individuals [194]. Interestingly, gut microbiota dysbiosis is increasingly indicated as one of the contributors to the pathophysiology of physical frailty and sarcopenia [195, 196], concurring to define the health trajectory of older individuals [197]. Effective anti-aging microbiome-centered nutritional interventions should thus consider the link between microbiota and both cognitive and physical domains, translating the evidence coming from microbiological and animal studies into clinical practice.

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Gut Microbiota as a Hidden Player in the Pathogenesis of Alzheimer's Disease

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Abstract. Alzheimer's disease (AD), the most common neurodegenerative disorder, is accompanied by cognitive impairment and shows representative pathological features, including senile plaques and neurofibrillary tangles in the brain. Recent evidence suggests that several systemic changes outside the brain are associated with AD and may contribute to its pathogenesis. Among the factors that induce systemic changes in AD, the gut microbiota is increasingly drawing attention. Modulation of gut microbiome, along with continuous attempts to remove pathogenic proteins directly from the brain, is a viable strategy to cure AD. Seeking a holistic understanding of the pathways throughout the body that can affect the pathogenesis, rather than regarding AD solely as a brain disease, may be key to successful therapy. In this review, we focus on the role of the gut microbiota in causing systemic manifestations of AD. The review integrates recently emerging concepts and provides potential mechanisms about the involvement of the gut-brain axis in AD, ranging from gut permeability and inflammation to bacterial translocation and cross-seeding.

Keywords: Alzheimer's disease, gut-brain axis, gut microbiota, systemic inflammation

INTRODUCTION

Dementia, which refers to various symptoms caused by disorders of brain function, accompanies conditions that affect memory, thinking, behavior, and emotions. Alzheimer's disease (AD) accounts for 60–80% of all dementia cases and is regarded as the most common neurodegenerative disorder. AD shows key representative pathological features, such as senile plaques composed of dystrophic neurites, reactive glial cells surrounding accumulations of amyloid-β (Aβ), and neurofibrillary tangles (NFTs) containing the hyperphosphorylated tau protein [1]. Centering on these features, the amyloid and tau hypotheses currently represent the main etiological explanation for AD. Mechanisms that constitute these hypotheses are explained below.

In neurons, Aβ generated from amyloid-β protein precursor (AβPP) can accumulate in endosomes, but is mainly secreted from the cells to form oligomers or plaques that are toxic to surrounding cells. Neurotoxic Aβ directly destroys synapses and/or lowers neuronal activity, leading to a decline in cognitive ability. Another causative agent of AD is the hyperphosphorylated and aggregated form of tau protein. In the central nervous system (CNS), tau protein is involved in microtubule assembly, structural stabilization, and molecule transport within neurons [2]. The tau protein also undergoes post-translational modifications. Some of the forms are majorly found in NFTs of the brain tissue and in the blood of AD

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patients, and thus can be utilized as blood biomarkers [3, 4]. Before the formation of NFTs, hyperphosphorylated tau can be sent from the axonal compartment of the neuron to the somatodendritic compartment, where it impairs synaptic function. Thereafter, the tau aggregates propagate in a prion-like manner from the entorhinal cortex to the neocortex of AD patients, causing serial neurodegeneration [5].

In AD, the accumulation of Aβ plaques and tau aggregates and the resulting neuronal death activate neuroglial cells (astrocytes and microglia) in the brain [6]. Astrocytes provide nutrients from the blood to support nerve cells and/or protect nerve cells by removing surrounding waste through phagocytosis. In the context of neurodegeneration, astrocytes are mainly activated around the brain lesions of patients. Sustained reactivity of astrocytes to remove damaged neurons or lesions generates increased hydrogen peroxide, which is detrimental to neurons [7]. Microglia, which are innate immune cells residing in the brain, maintain brain homeostasis through various cell-surface sensing proteins. Microglia constantly surveil the brain's microenvironment; when they detect a damage signal, they quickly move to the area and trigger immune responses, such as phagocytosis and/or secretion of pro-inflammatory cytokines. In AD, microglia usually undergo rapid activation to remove misfolded Aβ, tau, or deteriorating nerve cells at the initial stage of disease onset. However, chronic stimulation of microglia degrades their control mechanisms, resulting in an overactive inflammatory response that leads to neuronal destruction [8]. In addition, microglia that fail to remove Aβ aggregates eventually die, contributing to the formation and growth of plaques in the brain [9].

In order to enable patients to recover from the cognitive deficits and functional impairments arising from these major causes, researchers are competing to develop AD drugs. The FDA has approved four drugs to date: memantine, galantamine, rivastigmine, and donepezil [10]. Unfortunately, the approved drugs are only able to slow disease progression by facilitating intra-neuronal signal transduction through N-methyl D-aspartate receptor regulation or cholinesterase inhibition. The FDA also recently approved aducanumab, which is an Aβ-targeting antibody [11]. However, the efficacy of aducanumab is widely debated, and additional longer-term evidence is needed to determine whether it can actually prevent cognitive decline in AD patients and thereby serve as the first disease-modifying drug.

Given the centrality of the Aβ and tau hypotheses, continuous attempts have been made in the pharmaceutical industry to remove the pathogenic proteins from the brain or coax excessive immune activity. However, due to limitations such as the short-lived effectiveness of the drugs and the risk of side effects (e.g., cerebral microbleeds), no successful disease-modifying treatment has been developed thus far. Moreover, while these efforts to directly target pathogenic molecules remain valid, accruing evidence suggests that the pathogenesis of AD needs to be viewed in a broader perspective—one that does not focus solely on the brain, but also views the disease in relation to other organs. Recent experimental and clinical studies suggest that systemic changes outside the brain can be causative factors in AD [12]. In particular, metabolic dysregulation leading to obesity, diabetes, and systemic inflammation has been shown to increase the risk of AD, while physical activity and dietary modulation help lower the risk [13]. Thus, the communication between the brain and the whole body has attracted attention as a new target for AD therapeutics. From this perspective, developing a holistic understanding of the pathways throughout the body that can affect the pathogenesis, rather than viewing AD solely as a brain disease, may be key to developing a successful therapeutic agent. The gut microbiota has been recognized as a "forgotten organ" that regulates host physiology and metabolism [14], and even influences brain development and function [15]. Therefore, in this review, we focus on the role of gut microbes and discuss the potential mechanistic involvement of the gut-brain axis (GBA) in AD. Also, we describe recently emerging concepts regarding GBA involvement in AD, ranging from gut permeability and inflammation to bacterial translocation and cross-seeding.

THE GUT-BRAIN AXIS IN NEUROLOGICAL DISEASE

The gut microbiota is closely connected to the CNS through the endocrine, nervous, and immune systems, together forming the so-called GBA. The bidirectional signaling of the GBA is regulated by the gut microbiota, and many researchers have reported that the gut microbiota plays a significant role in brain development, diseases, and the associated physiological, psychological, and behavioral changes [16]. Many studies examining the effects of the gut microbiota on the CNS have been mainly conducted in germ-free (GF) mouse models. By observing the behavioral and neurological differences in GF and

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specific-pathogen-free (SPF) mice, researchers have elucidated mechanisms by which the gut microbiota affects brain development and function. For example, the turnover rates of noradrenaline, dopamine, and serotonin were found to be elevated in the striatum of GF mice compared to SPF mice, and GF mice showed altered expression of anxiety- and synaptic plasticity-related genes [17]. In addition, although adult neurogenesis of the hippocampus decreases with age, this age-dependent decrease in neurogenesis was found to be delayed in GF mice, affecting their cognitive functions related to behavior, motor control, learning, and memory [18]. Another study suggested that manipulating the gut microbiota with probiotic bacteria can restore cognitive function, memory impairment, and anxiety disorder, and recover intestinal inflammation in inflammatory bowel disease (IBD) model mice [19]. These findings supported the idea that the gut microbiota contributes to CNS functions, prompting researchers to investigate the underlying mechanisms responsible for regulating the GBA.

The gut microbiota has attracted attention as an active regulator of the GBA in various neurological diseases. For instance, the neurotransmitter, dopamine, is synthesized in the enteric nervous system at a level similar to that in the brain. A metagenomics analysis of 1,054 people found that dopamine-producing bacteria were lacking in the guts of patients with depression [20]. About 90% of serotonin (a hormone that affects appetite, sleep, mood, memory, and learning) accumulates in the intestine, and certain intestinal microorganisms can increase serotonin synthesis [21]. Serotonin can directly stimulate intestinal nerve cells, and the endocrine cells that synthesize serotonin can form a synapse to the brain via the vagus nerve to transmit signals [22]. These findings support the importance of the gut microbiota in maintaining mental health through endocrine regulation, and clearly indicate that intestinal bacteria play active roles in the neural communication between the gut and brain.

The concept that the gut microbiota is a potential regulator of resident immune cell functions in the gut, periphery, and brain suggests that the immune system should be viewed as another mechanism underlying the GBA [23]. Fecal microbiota transplantation from patients with multiple sclerosis (an autoimmune disease) into GF mice increased the number of inflammatory T cells in peripheral lymphoid tissues and accelerated the multiple sclerosis pathology [24]. In a stroke model, researchers elucidated a mechanism by which gut-derived immune cells interact with the CNS via the commensal gut microbiota [25]. Short-chain fatty acids (SCFAs, representative metabolites of gut microbes) are well known to be involved in gene expression and hormone secretion, mainly in the gut. Interestingly, however, the defects in the maturation and function of microglia in the CNS of GF mice were restored by SCFAs [26]. In another work, neuroinflammation was alleviated by binding the tryptophan metabolism by-products of gut bacteria to the aryl hydrocarbon receptors of astrocytes [27]. These studies provide convincing evidence that the metabolites of gut microbes can directly affect the activity of innate immune and glial cells residing in the brain. Alongside this work showing that the gut microbiota can control brain function via the endocrine, neuronal, and immune systems, other studies have shown that, conversely, the brain can alter the gut environment. For instance, stress hormones (e.g., norepinephrine) released into the blood under psychological and physical stress were found to negatively affect the bacterial composition and function of the intestine by regulating bacterial gene expression and inducing community-specific growth [28]. As researchers continued to unravel the detailed mechanisms by which gut microbes regulate signal transduction between the gut and brain, the field increasingly recognized that gut microbes could be associated with various pathologies, such as the neurodegenerative diseases, amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and AD.

ALS is a neurodegenerative disease characterized by the gradual loss of the motor neurons responsible for motor, speech, and cognitive abilities [29]. Transgenic model mice overexpressing mutant superoxide dismutase 1 (SOD1) or chromosome 9 open reading frame 72 (C9orf72), the most common genetic causes of ALS, exhibit pathological changes in reaction to nicotinamide-producing and immune-stimulating bacteria, respectively [30, 31]. PD is another chronic brain disease in which motor and cognitive impairments occur due to alpha-synuclein (α-syn) accumulation and cell death among dopaminergic neurons of the substantia nigra [32]. Enteric α-syn was found to propagate to the brain through the vagus nerve, leading to PD-related neuropathology [33]. The finding led to the hypothesis that the onset of PD starts in the gut, not the brain, and brought about the breakthrough concept that targeting the accumulation of α-syn in the enteric nervous system could be an important strategy for treating PD.

As with the above-described neurodegenerative diseases, alterations in the composition and diversity

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of gut microbiota in AD patients and animal models of AD have been reported, extending our knowledge on AD pathogenesis and GBA-related AD phenotypes.

THE GUT MICROBIOTA AND AD

AD patients: Evidence from human samples

The advancement of sequencing technology has improved researchers' ability to analyze bacterial taxa. This has enabled scientists to isolate and distinguish microorganisms, opening up previously unknown realms of diverse microbes. Early studies utilizing this technology identified perturbations in the gut microbiome in various diseases. As the link between the gut and the brain is increasingly being solidified through numerous studies examining the GBA, the gut microbiome's influence on the overall physiological condition, including that of the brain, is becoming evident. Evidence from human samples of various origins has revealed the implications of the gut microbiome in AD.

Stool (fecal) samples of AD patients

Analysis of human fecal samples is widely used as a proxy for examining the gut microbiota for pragmatic reasons, such as the ease and non-invasiveness of sample acquisition. Moreover, human fecal contents have been solid linked to the composition of the gut microbiome and its metabolic function. For example, in a study involving 713 fecal metabolites, 95% and 82% of the fecal metabolites were significantly associated with 99.7% of the microbial metabolic pathways and 90% of the gut microbial species, respectively [34]. Accordingly, the growing body of human evidence regarding the link between the gut microbiota and AD is mainly centered around evidence obtained from fecal samples.

Vogt et al. discovered that *Firmicutes* and *Bifidobacterium* were decreased, whereas *Bacteroidetes* was increased, in the microbiome of AD patients compared to that of control participants [35]. Zhuang and colleagues also reported that the gut microbiota is significantly altered in AD patients, but these authors observed different alterations: In their study, AD patients showed a mild decrease in *Bacteroidetes,* whereas *Firmicutes* were almost identical between the control and AD groups [36]. The caveat of these findings is that discoveries made at this phylum level may be highly variable and controversial, given that *Bacteroidetes* and *Firmicutes* make up approximately 80% of the human gut microbiota [37] and the ratio between them changes with the host's age [38, 39]. Moreover, AD is a multifactorial disease affected by environmental factors; thus, the discrepancy in the findings of the abovementioned studies may reflect regional differences in lifestyle or dietary habits [40, 41]. The discrepancy might also arise from using different sampling or analytic methodologies [42, 43] and possibly, different stages of AD progression that the patients are in. Going forward, further data collection and standardization between institutions could enable researchers to perform efficient meta-analyses and obtain more consistent and reliable results. Cattaneo et al. identified a difference in the abundance of specific bacterial taxa in amyloid negative/positive patients compared to healthy controls. Compared to both healthy and amyloid-negative patients, amyloid-positive patients exhibited a greater abundance of *Escherichia/Shigella* and a reduced abundance of *Eubacterium rectale*. Interestingly, the pro-inflammatory cytokines, interleukin 1 beta (IL-1β), NOD-like receptor pyrin domain-containing protein 3 (NLRP3), and C-X-C motif chemokine ligand 2 (CXCL2), were positively correlated with the abundance of *Escherichia/Shigella* and negatively with that of *Eubacterium rectale* [44]. These findings suggested that certain microbial species can induce systemic inflammation and impact the brain amyloid burden of the host.

Human fecal samples also provide valuable resources for *in vitro* applications in mechanistic studies. For instance, *in vitro* T84 intestinal epithelial cell functional assays performed using patients' stool samples revealed that P-glycoprotein expression was significantly lower in AD patients [45]. Intestinal P-glycoprotein secretes endocannabinoid into the intestinal lumen to control neutrophil infiltration and is thus considered to be a crucial regulator of intestinal inflammation and homeostasis [46]. The decreased expression of P-glycoprotein in AD patients implies the presence of disrupted homeostasis and increased inflammation. In addition, the authors used metagenomic sequencing to distinguish microbial composition between healthy controls, AD patients, and patients with other types of dementia, and found that AD patients were characterized by lower proportions of species of the critical butyrate-producing genus, *Butyrivibrio*, and members of the *Eubacterium* genera. Butyrate derived from microbes directly induces epithelial anti-inflammatory IL-10 receptor α-subunit, resulting in repression of the tight junction (TJ) protein, claudin-2, which is known to

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promote barrier permeability [47]. As butyrate is essential for the ability of colonic epithelial cells to maintain the gut barrier function, a decreased butyrate level could explain the increased gut permeability observed in AD patients.

Blood samples of AD patients

Blood circulates throughout the body and carries various bacterial metabolites secreted from the gut [48, 49], and is thus a valuable resource for explaining the intricate interplay between the gut microbiome and systemic metabolism [34]. Association studies between amyloid pathology and blood levels of SCFAs and lipopolysaccharide (LPS) suggest that these bacterial metabolites can mediate gut dysbiosis and AD. For example, in a study using florbetapir amyloid positron emission tomography imaging, brain amyloid was positively correlated with blood LPS, acetate, and valerate, but negatively correlated with butyrate [50]. Another study suggested that butyrate-producing microbial species are decreased in AD patients [45], further supporting the notion that gut epithelial integrity is impaired in AD. Furthermore, acetate and valerate showed significant associations with pro-inflammatory cytokines, such as CXCL2. Interestingly, several studies showed that acetate and valerate have anti-inflammatory effects on glial activation [51–53].

As LPS and SCFAs are regarded as metabolites produced almost exclusively by gut microbes in the human body [54, 55], the changes in their level strongly indicate the changes in gut microbes. While the level changes of LPS and SCFAs in blood may not be solely related to AD, their significant correlation with brain amyloid implies that AD pathology is one of the factors attributable for their increase in blood level and AD plays a role in inducing systemic inflammation.

CSF samples of AD patients

The ability of gut metabolites to reach the CNS was confirmed by the detection of these metabolites in cerebrospinal fluid (CSF) [56]. Vogt et al. reported that CSF carries trimethylamine N-oxide (TMAO), which is a microbial metabolite that has been implicated in cerebrovascular and metabolic diseases [57, 58]. Furthermore, they discovered that the TMAO level in CSF is higher in individuals with mild cognitive impairment and AD dementia than in healthy controls, and that it is associated with AD biomarkers, such as phosphorylated tau (p-tau) and neurofilament light chain protein (NfL) [59].

As TMAO is produced from trimethylamine, which is exclusively produced by anaerobic microorganisms residing in the mammalian gut [60], detection of TMAO in CSF provides obvious evidence that bacterial metabolites can directly enter CSF and cause changes to the brain. TMAO level in CSF may not be specifically related to AD. However, based on its significant correlation with canonical AD biomarkers such as p-tau and NfL, it is conceivable that AD can act as one of the contributing factors in allowing the increase of TMAO level in CSF by impairing the gut.

Therapeutic strategies in patients

As the gut microbiota has been increasingly acknowledged as contributing to regulating overall physiologic homeostasis, increasing efforts have been made to treat patients by altering their gut microbiome. In diseases where the link between gut bacterial imbalance and disease onset has been thoroughly investigated, gut microbiome modulation has become a viable treatment strategy. For example, in a gastrointestinal (GI) disorder patient with irritable bowel syndrome (IBS), the administration of gut microbiota from a healthy donor can significantly change the intestinal bacterial profile and improve fatigue and quality of life [61]. For IBD patients, fecal microbiota transplantation (FMT) has been used in clinical trials for over two decades to treat the chronic inflammatory conditions of the GI system [62].

In one brief clinical trial spanning 12 weeks, AD patients administered with probiotic milk containing mainly genus *Lactobacillus* exhibited improvements in their Mini-Mental State Exam (MMSE) scores, insulin metabolism, and triglyceride levels; however, the changes in the levels of oxidative stress and inflammation were negligible, indicating the need for further studies [63]. The same group also reported that co-supplementation of probiotics and selenium to AD patients for 12 weeks improved their cognitive function, as assessed by MMSE, and reduced the levels of serum lipid metabolites, such as triglycerides, very-low-density lipoprotein, and low-density lipoprotein [64]. One preliminary study found that supplementing AD patients with probiotics influences the gut bacteria composition and tryptophan metabolism in serum [65]. The authors speculated that the increased serum kynurenine levels seen upon probiotic supplementation were caused by macrophage activation, but it remains unclear how this might offer a therapeutic benefit in AD.

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AD model mice: Experimental models

There are fundamental differences in the microbiomes of humans and mice. For example, the relative compositions within a phylum can differ, as evidenced by the finding that genera *Mucispirillum* is exclusively present in rodents [66]. However, there is high similarity between human and murine gut microbiota: Both have *Bacteroidetes* and *Firmicutes* as major phyla, and 90% and 89% of microbes are shared at the phylum and genus levels, respectively [67]. Thus, analysis of the mouse microbiome can provide valuable insights into human physiology.

The prominent feature that distinguishes an AD model from others is the accumulation of amyloid in the brain. Thus, most studies using AD models take advantage of amyloid pathology driven by human transgenes, such as APP and presenilin (PS), harboring familial AD mutations known to accelerate the pathology. Early findings by Shen and colleagues showed that the histological and behavioral AD manifestations in APP/PS1 mice depended on the presence of a specific gut microbiome state, and that the gut microbiome exhibited an age-related decrease in diversity. They found that, compared to wild-type (WT) mice, AD mice had significantly increased *Odoribacter* and *Helicobacter* at the genus level [68]. Another study in APP/PS1 mice investigated the shift in gut microbiota composition across the lifespan [69]. The researchers found that bacterial profiles were similar between APP/PS1 and WT mice at a young age, before the pathology set in, but started to diverge at 6 months with increases in *Proteobacteria*, especially genus *Sutterella*. Interestingly, *Sutterella* had been shown to correlate with the tumor necrosis factor-alpha (TNF-α) level and aggravated social behavior in a female BTBR mouse model of autism spectrum disorder [70]. This opened the question of whether certain microbes are responsible for inducing inflammatory responses in neurological disorders.

Comparison of the fecal microbiome and SCFAs of APPswe/PS1ΔE9 transgenic model mice versus WT mice revealed differences [71]. The diversity of the fecal microbiome differed, especially at 8-12 months of age, with *Proteobacteria* found to be dramatically increased in the AD model mice. Interestingly, *B. pullicaecorum* was reduced in AD mice. This butyrate-producing microbe is known for its potential to strengthen epithelial barrier functions and has been proposed as a promising probiotic candidate for IBD patients [72, 73]. Moreover, butyrate is essential for the maintenance of a healthy gut with an intact barrier, and its level might be disrupted in an AD gut [45]. In Tg2576 mice harboring a Swedish APP mutation known to lead to early-onset AD, the abundance of *Bacteroides* was correlated with aging and plaque pathology [74]. Interestingly, in humans, neurotoxins from *Bacteroides* have been hypothesized to drive the AD process [75]. In the same model, another group found that intestinal epithelial barrier dysfunction occurs before cerebral Aβ aggregation is detectible. These changes were accompanied by elevated levels of plasma cytokines, such as IL-9, vascular endothelial growth factor, and interferon-inducible protein 10 [76].

Chen and colleagues extensively analyzed the gut microbiota composition of the well-known 5XFAD mouse model of AD [77]. The microbial communities were the same at 3 months of age and were characterized by a lower abundance of *Firmicutes* and a higher abundance of *Bacteroidetes*. At 6 months of age, however, the abundance of the two dominant phyla, *Firmicutes* and *Bacteroidetes*, showed a marked difference between the groups: Pro-inflammatory phyla, such as *Proteobacteria* and *Bacteroidetes* were drastically increased only in 5XFAD mice, while anti-inflammatory *Firmicutes* took up most of the portion in WT mice. At the genus level, 5XFAD mice showed increases in *Helicobacter*, *Prevotella*, and *Sutterella* [77, 78].

Although tau is another major pathological hallmark of AD, tau models have been used to a lesser extent than amyloid models in AD microbiome studies. This may be because pathological tau is found widely in other tauopathies and is not as exclusive as Aβ in constituting the AD phenotype. However, evidence on the alterations in the microbiome in tau models of AD has begun to accumulate, and the implications of these studies could also apply to other tauopathies. At 3, 6, and 10 months of age, the P301L tau transgenic mouse model showed increased and decreased abundances of *Bacteroidetes* and *Firmicutes*, respectively, compared with WT mice [79]. Interestingly, similar phylum-level trends were seen in humans [35]. Thus, further studies on restoring the balanced ratio between these two dominant phyla in the gut microbiota could potentially lead to the development of a therapeutic intervention for humans.

Meanwhile, in the novel ADLP (AD-like pathology) mouse, which exhibits both amyloid and tau pathology [80], analyses of the gut microbiota revealed chronic intestinal and systemic inflammation resulting from impaired integrity of the epithelial barrier [81]. In triple-transgenic model

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(3xTg-AD) harboring PS1 (M146V), APP (Swe), and tau (P301L) transgenes [82], relatively decreased abundances of Actinobacteria and TM7 compared to wild-type were observed already at 3 months of age [83]. Also, 3xTg-AD mice had lower concentrations of Anaerostipes, which plays a crucial role in gut health by producing butyric acid than wild-type [84]. Further investigations are required to reveal the synergistic effect of amyloid and tau on altering the gut microbiota.

Evidence from observational studies therefore strongly indicates that there is a significant link between gut dysbiosis and AD. In support of this link, animal models primarily designed to mimic the diseased brain also exhibit alterations in the gut microbiome. Researchers have therefore implemented various strategies to actively manipulate the microbiome in animal models, in an effort to better understand how gut perturbations can induce AD phenotypes.

Germ-free models

GF model animals are bred in isolators that keep them from contacting any microorganism. Using this model, researchers can observe how a pathology changes in the complete absence of microbes or investigate how select microbes affect the disease by generating gnotobiotic animals. When select microbiota is transplanted, the initial colonization effect can be observed without any interference from pre-existing microbes.

Harach and colleagues found that cerebral Aβ pathology is drastically reduced in APP/PS1 transgenic mice that completely lack a gut microbiota. Interestingly, when GF AD model mice were colonized with microbiota from their conventionally raised counterparts, the cerebral amyloid level was increased. The microbiota from the WT counterpart did not induce amyloid pathology as much as that from the disease model, strongly suggesting that the composition of the microbial environment is associated with the development of amyloid pathology [85]. Fujii et al. created a gnotobiotic mouse model by transplanting GF C57BL/6N mice with human gut microbiota from a healthy volunteer and an AD patient. The mice that received the healthy microbiota performed better on several behavioral tests, such as the object recognition and object location tests. Moreover, the fecal levels of neurotransmitters and CNS metabolites, such as gamma-aminobutyrate, taurine, and valine, were significantly decreased mice that received the diseased microbiota [86].

Antibiotics treatment

Antibiotics-treated mice can be a more affordable and easily accessible alternative to GF mice, as the former do not require high-cost facilities and cumbersome isolators. However, antibiotics can selectively deplete specific types of microbes via different mechanisms of action, meaning that the incomplete ablation of microbes should always be kept in mind. Moreover, the potential side effects of antibiotics on pathological phenotypes should be carefully monitored.

In AD research, antibiotics are often used to create dysbiosis within the gut to uncover how the perturbed microbiota impacts disease progression. Minter and colleagues initially reported that antibiotics treatment of APP/PS1 mice perturbed the innate gut microbiome and influenced neuro-inflammation and amyloidosis [87]. The authors found that long-term application of a broad-spectrum antibiotic cocktail significantly shifted the gut microbial composition and diversity. Surprisingly, this modulation of gut microbiota was sufficient to decrease Aβ plaque deposition in the brain, increase the level of soluble Aβ, alter the circulating cytokine signatures, and attenuate plaque-associated glial reactivity. This prompted the same research group to investigate how antibiotics-mediated perturbation of the microbial diversity of APP/PS1 mice during post-natal development impacts amyloid pathology when the animals aged [88]. Contrary to the chronic exposure performed in the former study, acute treatment at this stage was sufficient to reduce brain Aβ deposition in aged model mice. Also, the antibiotics-treated group had fewer plaque-localized microglia and astrocytes. As the early post-natal development period is critical for neuro and immune development, these results showed that the gut microbiota is involved in brain development and the onset of AD.

Although antibiotics treatment studies provide helpful insights, they do not clarify the involved bacterial strain(s) or mechanism(s). For example, while the synergistic alterations of gut microbial consortia achieved by applying an antibiotics cocktail of kanamycin, metronidazole, gentamicin, colistin, and vancomycin was shown to reduce amyloidosis in APPPS1-21 mice, the same effect was not achieved in mice treated with the individual antibiotics, complicating efforts to isolate the causative bacterial strain(s) for AD [89]. Chen and colleagues sought to utilize antibiotics to elucidate the underlying mechanism that links gut dysbiosis and AD. In the 5XFAD model, they initially found that gut dysbiosis

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was aggravated with age, leading to activation of the CCAAT/enhancer binding protein β/asparagine endopeptidase pathway. The team applied antibiotics to see if gut microbes mediate this pathway, and found that this treatment diminished the signaling and attenuated amyloidogenic processes, ultimately yielding cognitive improvements [77].

Despite the potential beneficial effects of antibiotics treatment in transgenic AD mouse models mentioned above, the debate over the efficacy of antibiotics in treating AD has never reached a consensus. While some studies report that antibiotics effectively removed amyloid plaques from the brain [87, 88], others report that cognitive impairment was worsened following gut dysbiosis [90] or antibiotics treatment had no beneficial effects on cognitive function in AD [91]. The controversy is mainly due to intrinsic limitations and biases regarding the studies. The initial composition of the gut microbiota at the time of antibiotics treatment could be different due to numerous factors such as disease state, environmental factors, and dietary habits. Also, as certain types of antibiotics selectively remove specific bacterial types [92], the effect depends on the type and combination of antibiotics. The treatment time point, period, and dosage could also be essential factors.

Probiotics treatment

Probiotics are live microorganisms that have beneficial effects on our bodies when consumed. Numerous probiotic products are commercially available and consumed by humans as dietary supplements. Since the concept is already familiar to consumers, it would be easy to implement the administration of probiotics as a therapeutic strategy for curing AD.

In a study conducted with 3xTg-AD model mice, a probiotics formulation of nine live bacterial strains (called SLAB51) significantly reduced oxidative stress in the brain by activating sirtuin 1 (SIRT1)-dependent mechanisms. There was no significant change in brain amyloid, but there were changes in the brain levels of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and consequent alterations in protein and lipid oxidation states. These results indicated that probiotics supplementation could be a promising approach to treat AD and other neurological disorders that involve oxidative stress [93]. In an animal model of AD made by intracerebroventricular injection of Aβ into mice, oral administration of *Bifidobacterium breve strain A1* (*B. breve* A1) had beneficial effects on behavior. Interestingly, while *B. breve* A1 administration had only minor effects on the overall composition of the gut microbiome, it increased the plasma acetate level and suppressed Aβ-induced gene expression changes in the brain [94]. In Aβ-injected rats, behavioral benefits were observed, and long-term potentiation was suppressed upon administration of probiotics, indicating that probiotics could even exert positive effects on synaptic plasticity [95]. Similarly, treatment with *Lactobacilli* and *bifidobacteria* was found to ameliorate memory and learning deficits and reduce oxidative stress in Aβ-injected rats [96].

In a mammalian gut microbiota composed of enormously diverse microbes, it is unlikely that a single pathogenic microbial type could be responsible for the dysbiosis of the entire microbiome. Likewise, it is not expected that a single microbial type will play a vastly protective role in AD. Rather, restoring the overall homeostatic condition of the gut should be the primary focus in efforts to treat AD. Therefore, obtaining well-balanced gut microbiota from healthy controls and settling them in the guts of patients by transplantation could be an effective therapeutic tactic. This tactic is usually implemented in the form of the FMT, as mentioned above. Indeed, FMT has already shown efficacy in mouse models of AD.

In a study conducted by Sun et al., FMT to APP/PS1 mice improved cognitive deficits, reduced the brain deposition of Aβ, and decreased the levels of phosphorylated tau, $A\beta_{40}$, $A\beta_{42}$, cyclooxygenase-2 (COX-2), and cluster of differentiation molecule 11b [97]. In an $ADLP^{APT}$ mouse model exhibiting both amyloid and tau pathology, FMT from healthy WT mice into $ADLP^{APT}$ mice significantly alleviated AD pathology. The formations of Aβ plaques and NFTs were reduced, glial reactivity was attenuated, cognition was improved, the aberrant gene expression related to intestinal macrophage activity in the colon was reversed, and the proper population of circulating monocytes in blood was restored [81]. These results from FMT studies indicate that colonizing the gut with a balanced set of microbes aimed at restoring the homeostatic condition can be more beneficial to AD symptoms than transplanting a specific subset of microbes.

Modulation of diets

Administration of dietary nutrients, which range from micronutrients (polyphenols, vitamins, minerals, etc.) to macronutrients (carbohydrates, fat, protein, etc.), can significantly alter the microbial composition of the gut [98]. Therapy based on dietary

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modulation is already being used to treat metabolic diseases, such as obesity, diabetes, and IBS [99]. Supplements comprising metabolites that help restore the homeostatic microbial community in the gut (as an alternative to those containing the organisms themselves) are being widely investigated for their potential to treat AD. For example, sodium oligomannate (GV-971) was found to suppress gut dysbiosis and the associated phenylalanine/isoleucine accumulation in 5XFAD mice, leading to cognitive improvements [100]. Moreover, oligosaccharides derived from *Morinda officinalis* were found to alter the gut microbiota and metabolome in APP/PS1 mice [101].

Calorie restriction (CR) can be another alternative. In 15-month-old female Tg2576 mice, long-term CR feeding was found to reduce the Aβ plaque load. Sustained CR also reduced the expression of γ-secretase complex components, such as PS enhancer 2 and PS 1 [102]. Similarly, Cox et al. found that CR slowed age-related microbiota changes in Tg2576 mice [74]. The authors postulated that this was related to the type strain of *Bacteroides fragilis*, which is known to increase Aβ plaques in the brain. The highly varied carbohydrate and polysaccharide utilization genes of these *Bacteroides* species are essential for their ability to maintain colonization, but the CR diet limits calories specifically from carbohydrates. Thus, the CR diet might have directly impacted *Bacteroides*.

Together, these findings indicate that there is a solid link between gut dysbiosis and AD. In order to cure AD patients, various research groups have focused on restoring the homeostasis of the gut microbiome. The mechanism that governs the connection between AD and the GBA is unclear, however, and must be elucidated in detail if we hope to refine and optimize related treatment strategies. In the next section, we discuss the possible mechanisms by which gut dysbiosis could lead to AD pathogenesis.

POSSIBLE MECHANISMS THROUGH WHICH THE GUT MICROBIOTA CONTRIBUTES TO AD

Cross-seeding effect

Since the gut microbiota can excrete a considerable amount of amyloid peptide and LPS [103], gut dysbiosis might perturb the overall composition of microbial-derived molecules. The majority of bacterial amyloid peptides contribute to adhesion, host cell invasion, and host-pathogen interaction by forming a biofilm [104]. Biofilm-forming bacteria are much more resistant to antibiotics, viruses, and other harmful substances, which greatly contributes to the protection and growth of bacterial communities [105]. Chapman et al. proposed that *curli*, the first identified functional amyloid, is mainly produced by *E. coli* and *Salmonella* species and shares structural and physical properties with the pathogenic amyloids [106]. Therefore, the biofilm-forming gut bacteria might directly affect the AD pathogenesis through the cross-seeding of neuronal amyloid aggregates by bacterial amyloids. CsgA, which is the major *curli* subunit, has a typical cross-β architecture reminiscent of human pathological amyloids, and CsgA seeds were shown to accelerate AD-associated Aβ fibrillation [107, 108]. Structural similarity between amyloids is required for the epitaxial heteronucleation that forms the underlying mechanism of the cross-seeding reaction. Importantly, full-length CsgA has a fibril structure sufficiently similar to that of $A\beta_{1-40}$ and $A\beta_{1-42}$, enabling it to act as a template for fibril elongation [109]. Gram-negative bacteria produce amyloid peptides for biofilm formation, such as FapC from *Pseudomonas fluorescens* [110]. Structurally, FapC possesses several Aβ-binding sequences and a region that enables it to be incorporated into Aβ nanofibrils via seeding. FapC was absorbed onto the Aβ fibril surface and altered the morphology and toxicity of Aβ in a zebrafish AD model [111]. Many amyloid peptides are produced by the gut microbiome, and the amyloid-producing enteric bacteria might cause nucleation and fibrillation of brain amyloids in AD [112]. Similar to bacterial amyloid, LPS, which is a major component of the outer membrane of Gram-negative bacteria, was also found to promote Aβ fibrillization *in vitro* [113]. *In vivo*, bacterial LPS injection increased Aβ deposition and tau aggregation in the hippocampus of model mice [114, 115]. Given this, it is likely that LPS detected in the brain of AD patients can promote the formation of Aβ plaques [116]. Cross-seeding of bacterial amyloid misfolds and LPS are known to either access the brain directly via the autonomic nervous system in a manner similar to prion disease [117], or cross the defective blood-brain barrier (BBB) in AD.

High gut permeability and gut inflammation

Under the healthy condition, the gut epithelium plays a pivotal role in biological functions, such as the absorption of nutrients, ions, and water, and performs

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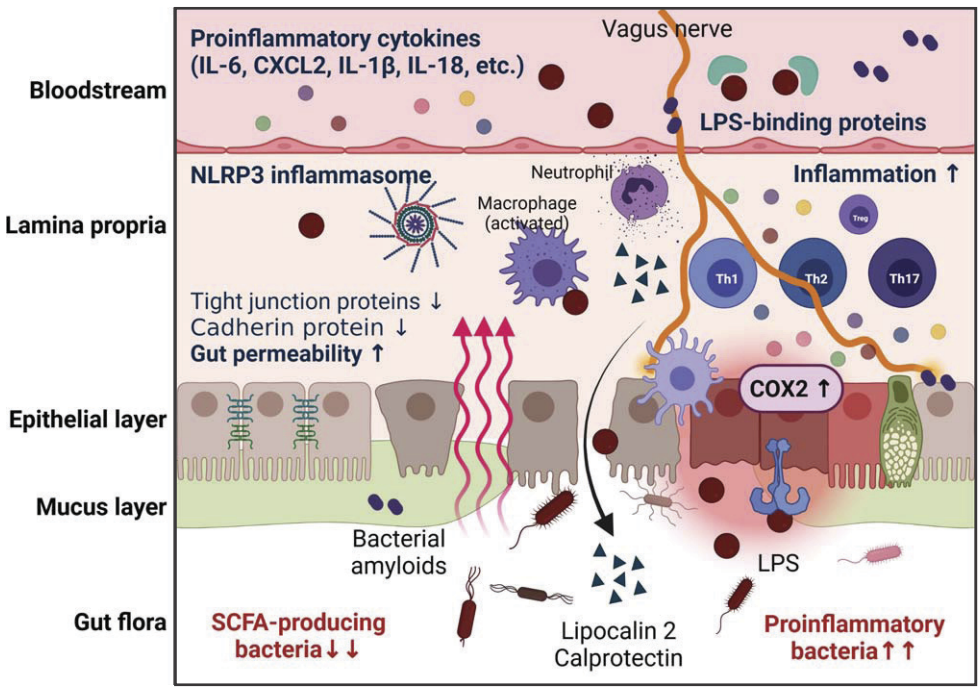


Fig. 1. Potential mechanisms through which gut dysbiosis contributes to gut pathology in AD. The imbalanced gut microbial composition induces impairment of the gut barrier; this is accompanied by mucus layer degradation, epithelium damage, and gut inflammation. The gut dysbiosis-mediated increase in gut permeability facilitates the translocation of bacteria into the circulation to cause systemic inflammation. The increased pro-inflammatory bacteria also induce aberrant immune activation, which reinforces and perpetuates the inflammation. High gut permeability and chronic gut inflammation induce translocation of bacteria-derived molecules and inflammatory mediators into the bloodstream and brain.

physical barrier functions to protect the host against invasion of gut bacteria and toxins [118]. The gut barrier is the first line of defense against pathogenic molecules and harmful microbes, and an imbalance of the gut microbiota leads to barrier dysfunction [119, 120]. Gut barrier breakdown and increased permeability have been described in GI disorders, including IBD (Crohn's disease and ulcerative colitis) [121]. Patients with IBD show a higher prevalence of anxiety and depressive symptoms and exhibit issues with concentration, memory, and executive function [122, 123]. The psychomotor and cognitive impairments seen in GI disease support the hypothesis that high gut permeability may negatively impact brain functions.

The gut mucus layer

The gut barrier is a multiple-layer system composed of the mucus layer, the epithelial layer, and the underlying lamina propria [124]. The mucus layer consists of an outer layer that is colonized by commensal bacteria and an inner layer that protects the gut epithelial cells from the bacteria and potential pathogens in the lumen. Damage to the inner layer can increase the direct contact between the bacteria and the gut epithelium, resulting in gut barrier dysfunction and allowing bacteria to penetrate through the epithelial tissue into the circulation [125]. The mucus layer comprises large glycoproteins, called mucins, that are predominantly produced and secreted by goblet cells [126]. Therefore, the regulation of the secretory goblet cells is key to maintaining the mucus barrier. Commensal bacteria and their metabolic byproducts (e.g., SCFAs such as acetate, propionate, and butyrate) have been implicated in the differentiation of goblet cells to synthesize and secrete mucin [127–129]. Some bacterial residents in the mucus layer can degrade and utilize the mucus component as their carbon and energy sources [130]. Taken together, the available evidence indicates that the microbial composition determines the properties and integrity of the mucus layer, influencing its permeability (Fig. 1).

AD patients are characterized by decreased populations of butyrate-producing species, such as members of genera *Butyrivibrio* (*B. hungatei and B. proteoclasticus*) and *Eubacterium* (*E. eligens,*

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E. hallii, and E. rectale), as well as *Clostridium* sp. strain SY8519, *Roseburia hominis*, and *Faecalibacterium prausnitzii* [45]. Another study found that butyrate producers in the gut, such as members of the *Lachnospiraceae* and *Ruminococcaceae* families, were decreased in AD patients compared to healthy controls [131]. Furthermore, the potential butyrate producer, *Lachnospiraceae NK4A136 group*, was found to be reduced in patients with dementia [132]. Consistent with the results from AD patients, Zhang et al. showed that the microbiota composition and diversity were perturbed and the SCFA level was reduced in APP/PS1 mice compared to WT mice [71]. Jiminez et al. provided evidence that supplementing mice with a high concentration of butyrate could reduce the severity of a pathogenic bacterial insult through epithelial barrier repair and mucus secretion [133]. Sodium butyrate treatment was also shown to have profound effects on brain Aβ levels, associative learning, and cognitive function in 5XFAD mice [134]. These findings suggest that increasing butyrate-producing bacteria may be a promising therapeutic strategy for AD.

Mucolytic bacteria, such as *Akkermansia muciniphila* and *Bacteroides thetaiotaomicron*, have protective effects on cognitive deficits and amyloid pathology in mouse models of AD [135, 136]. Although it was previously thought that mucin degradation was detrimental to gut health, it is now accepted as a normal physiological process that promotes mucus layer turnover [137]. Research findings that abundant mucin-degrading bacteria can improve gut barrier function [138–140] suggest that such bacteria may be beneficial microbes in AD.

The gut epithelial layer

The gut epithelium is a single-cell layer that separates the luminal gut microbiota from the host. Its integrity and permeability are maintained by adhesive complexes, such as desmosomes, adherens junctions (AJs), and tight junctions (TJs) [141]. Epithelial TJs, which represent the uppermost apical component of intracellular junctions, consist of transmembrane proteins (claudin, occludin, etc.) and cytosolic scaffold proteins (zonula occludens (ZO-1)). TJs regulate the paracellular movement of ions, solutes, and water across the gut epithelium, and also constitute biological barrier against pathogens and their toxins. AJs and desmosomes, which are located beneath TJs, provide essential adhesive and mechanical properties that also contribute to barrier function [142]. The gut epithelium is not a static system relative to the external environment; rather, its structure is greatly affected by enteric pathogens and their secreted toxins. The interaction of gut bacteria with epithelial cells has been proposed as a major contributor to TJ remodeling and gut permeability [143, 144], and specific bacteria-induced mechanisms of TJ disruption have been elucidated. For example, *Clostridium difficile* and *Escherichia coli*-induced TJ disruption was suggested to reflect changes in the cytoskeleton and the redistribution of proteins, including occludin and ZO-1, from TJs. Other enteric pathogens, such as *Bacteroides fragilis* and *Vibrio cholera*, were found to secrete metalloprotease and disrupt TJs by proteolytic degradation of TJ proteins [143]. As researchers recognized that the intestinal epithelial response to enteric pathogens can lead to perturbation in barrier functions, studies began to focus on the relationship of gut dysbiosis to the leaky gut. Increased gut permeability has frequently been reported in AD patients and model mice with imbalanced gut microbial composition and gut inflammation, and the involvement of increased gut permeability in disease progression is a research topic of growing interest (Fig. 1).

In APP/PS1 mice, findings of dilated TJs and downregulation of TJ proteins, including occludin, claudin 1, claudin 5, and ZO-1, indicated that intestinal barrier integrity is impaired. Observed increases in serum diamine oxidase and d-lactate, which are indicators of gut permeability, further supported the idea that there is severe gut barrier dysfunction in these mice [145]. To determine the effect of AD conditions on the integrity of colonic epithelial TJs and AJs, distal colon cryosections from young 5xFAD and their age-matched non-Tg littermates were stained for TJ (occludin and ZO-1) and AJ (E-cadherin and β-catenin) proteins. The results revealed that the junctional distributions of occludin and ZO-1 were reduced and the distal colon expression levels of E-cadherin and β-catenin were decreased in 5xFAD mice [146]. Fluorescein isothiocyanate-dextran assay of plasma, which can easily measure the pericellular permeability of the gut epithelium, revealed that AD model mice, including 5XFAD, 3xTg-AD, $ADLP^{APT}$, and APP knock-in mice, showed higher gut permeability than control WT mice [77, 81]. Due to the high gut permeability seen in AD patients, gut bacteria-derived pathogens, such as LPS and endotoxins, may be translocated into the systemic circulation and/or brain [147]. In AD brain lysates, the presence of *Escherichia coli K99* and bacterial LPS was identified, and LPS was found to localize with $A\beta_{1-40/42}$ in amyloid plaques and around

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vessels in the brain [116, 148]. A 16s rRNA sequencing analysis showed that the bacterial load is higher in AD brain compared to control brain, and the top-hit bacterial species were proposed to be microbial contributors to AD-associated neuroinflammation [149]. Taken together, these data indicate that the gut microbial environment in AD favors gut barrier disruption, which in turn induces the infectious agents to translocate into the blood and brain (Fig. 1).

The gut lamina propria

In healthy conditions, the gut immune system prevents the invasion of pathogenic bacteria. However, when dysbiosis occurs, harmful bacteria-derived enterotoxins perturb the immune system, causing chronic inflammation to increase gut permeability [150]. Located underneath the epithelial layer, the lamina propria is a thin layer of connective tissue that consists of innate and adaptive immune cells, such as neutrophils, macrophages, T cells, and B cells. The lamina propria plays a pivotal role in the communication between the microbiome and immune cells to maintain gut barrier homeostasis [151]. Pattern recognition receptors (PRRs) on the gut epithelial cell surface detect bacterial antigens and transmit signals from bacteria to adjacent immune cells [152]. The PRRs sense microbial antigens through pathogen associated molecular patterns (PAMPs), such as bacterial glycans, nucleic acids, and peptides. Upon the recognition of a PAMP, PRRs trigger intracellular signal transduction that leads to the upregulation of inflammatory genes, the production of pro-inflammatory cytokines, and the recruitment of innate and adaptive immune cells [153]. Macrophages can phagocytose the infectious agents and play a critical role in initiating and resolving inflammation by shifting between the pro-inflammatory M1 and anti-inflammatory M2 phenotypes [154]. Activated macrophages and dendritic cells present the antigens to naive T cells, leading to the differentiation and activation of various effector T cell subsets (Th1, Th2, Th17, and regulatory T cells (Tregs)) or the production of IgA from B cells [155, 156]. The gut immune cells are involved in host defense against pathogenic bacteria and govern the sophisticated modulation of unnecessary immune activity to maintain tissue homeostasis (Fig. 2).

The gut microbiota composition is crucial for regulating the balance and homeostasis of various helper T (Th) cell populations in the lamina propria. During the colonization of the ubiquitous gut microorganism, *Bacteroides fragilis,* in animals, a bacterial polysaccharide presented by intestinal dendritic cells mediates the establishment of a proper Th1/T-h2 cell balance, which supports healthy immunologic functions [157]. In adaptive immunity, the balance achieved by reciprocal regulation between Tregs and Th17 cells is vital to preventing a chronic, overactive, or ineffective immune response. In particular, colonic Tregs, which are important for maintaining gut immune homeostasis via protective immunosuppressive molecules, can be activated or expanded by SCFAs, gut microbiota-derived bacterial fermentation products, and specific spore-forming bacteria (*Clostridium clusters IV* and *XIVa*) [158, 159]. The differentiation of Th17 cells was found to correlate with the presence of Gram-negative members of the *cytophaga-flavobacter-bacteroidetes (CFB)* phylum in the intestine [160]. These findings indicate that commensal bacteria are pivotal in developing the immune system and maintaining immune homeostasis, whereas an imbalanced gut microbiota activates an uncontrolled immune response that leads to prolonged production of inflammatory cytokines. This is accompanied by deterioration of the barrier function of the gut epithelium via the disassembly of TJs, and eventual cell death [161]. Consequently, inflammatory mediators are permitted to enter the circulatory system and cause systemic inflammation, which is the major hallmark of chronic inflammatory disease.

In the elderly, an aging-induced shift in the normal gut microbiota composition is associated with the manifestation of immunosenescence and the development of a pro-inflammatory environment (inflamm-aging) [162]. The contributions of pro-inflammatory intestinal bacterial taxa and the peripheral inflammatory state have been reported in the elderly suffering from brain amyloidosis and cognitive impairment. A positive correlation was found between pro-inflammatory cytokines (IL-6, CXCL2, NLRP3, and IL-1β) in the blood and the number of pro-inflammatory intestinal bacteria belonging to the *Escherichia/Shigella* taxon in stool samples of patients with dementia. As expected, a negative correlation was found between pro-inflammatory cytokines and the number of intestinal bacteria of the *E. rectale* taxon, which are known for their anti-inflammatory effects [44]. In AD patients, the numbers of pro-inflammatory bacteria, such as *Gammaproteobacteria*, *Enterobacteriales*, and *Enterobacteriaceae* of phylum *Proteobacteria*, were reported to increase with clinical severity [131].

Calprotectin is a cytosolic protein that is released by neutrophils in response to inflammation. It is

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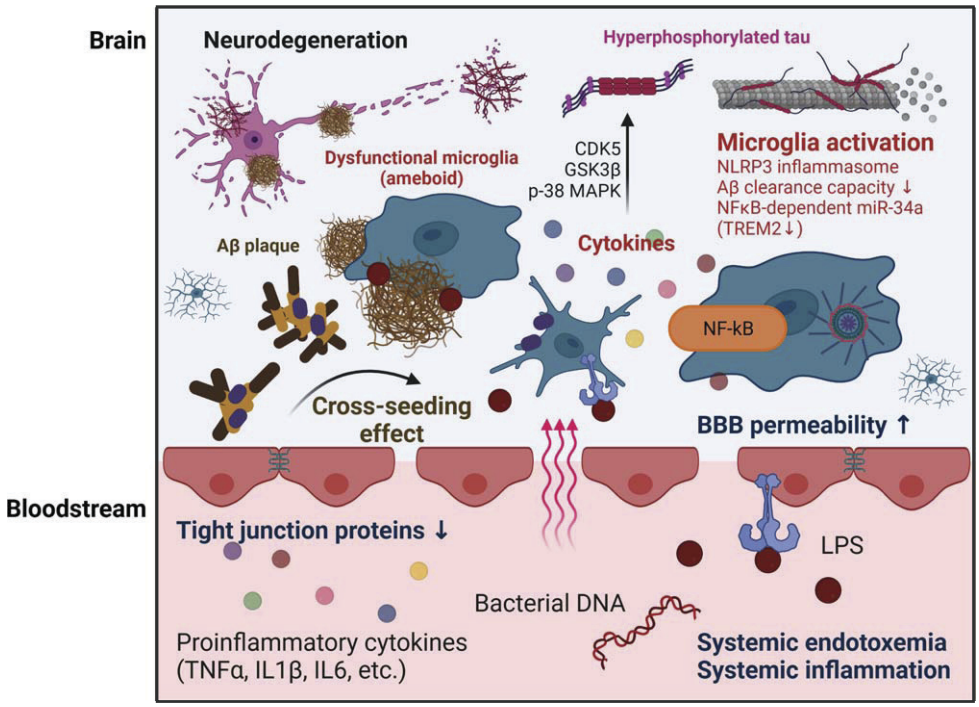


Fig. 2. Potential mechanisms through which gut dysbiosis contributes to brain pathology in AD. The crossing of bacteria-derived amyloids and LPS through the permeable BBB can promote amyloid aggregation and provoke microglia activation in the brain. The cross-seeding ability of bacterial amyloid involves a synergic effect on Aβ aggregation in the brain. The loss of BBB integrity due to bacterial LPS and/or circulatory inflammatory cytokines initiates the activation of microglia, which are resident immune cells in the brain. The persistent microglial activation driven by chronic systemic inflammation due to gut dysbiosis and gut permeability results in uncontrolled inflammatory responses and loss of microglia function. This neuroinflammation increases Aβ accumulation and facilitates tau phosphorylation, synaptic loss, and neuronal damage in AD.

noticeably elevated in both plasma and stool samples of patients with inflammatory conditions, including IBD [163]. The gut inflammatory state can be indirectly assessed by evaluating the level of calprotectin. This level was found to be significantly increased in the CSF and brains of AD patients, and calprotectin was found to promote the co-aggregation of Aβ [164]. Another study found that the fecal calprotectin level was elevated in nearly 70% of AD patients, prompting the researchers to hypothesize that the calprotectin found in feces may have been translocated from the leaky gut into the blood, ultimately contributing to neuroinflammation [165]. Another representative biomarker for gut inflammation, lipocalin 2 (LCN2), is secreted primarily from intestinal epithelial cells and myeloid cells in response to tissue injury, bacterial infection, or inflammation [166]. A higher concentration of fecal LCN2, indicative of intestinal inflammation, was found in ADLP^{APT} mice compared to WT mice [81]. Moreover, the immunoreactivity of the general intestinal regulator and pro-inflammatory protein, COX-2, was increased in the intestinal tissues of AβPP/PS1 mice compared to those of WT mice [167].

NLRP3, a member of the NLR family of innate immune receptors, induces the assembly of inflammasome complexes in the presence of microbial ligands, triggering the activation of caspase-1 and the secretions of IL-1β and IL-18 [168]. Many previous studies have reported that the NLRP3 inflammasome contributes to intestinal inflammation and the pathogenesis of IBD [169]. Shukla et al. observed increased NLRP3 inflammasome expression and IL-1β production in the gut of 5XFAD mice with compromised gut barrier function, as evident from the loss of TJ and AJ proteins [146]. In another study providing direct evidence, fecal transplantation from AD patients to APP/PS1 transgenic mice was reported to induce intestinal NLRP3 inflammasome activation and pro-inflammatory cytokine production to exacerbate neuroinflammation and cognitive deficits [170]. Thus, NLRP3 inflammasome activation caused by gut dysbiosis plays a critical role

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in neuroinflammation and AD-related neuropathology. In sum, the information gathered above suggests that the imbalanced composition of gut microbiota in AD induces neuropathology through gut inflammation and the concomitant release of inflammatory mediators (Fig. 1).

Bacterial translocation and systemic inflammation

The loss of gut barrier integrity is detrimental to the host because it allows bacteria-derived molecules to be translocated into the circulatory system and consequently induces systemic endotoxemia [171]. For example, in the peripheral blood of patients with IBD, several studies detected live intestinal bacteria by assessing the bacterial DNA concentration in patients' sera [172–174]. Thus, leaky gut syndrome is considered to be an initial event that takes place at the onset of IBD. Leaky gut syndrome is a well-characterized phenomenon in other metabolic diseases similar to IBD, which also have a high probability of accompanying endotoxemia. A number of studies showed that in a mouse model with high-fat-diet-induced diabetes and in patients with type 2 diabetes, live gut bacteria and bacteria-derived LPS were translocated into the blood, where they induced metabolic endotoxemia and inflammation [175–177]. In support of this notion, it is known that patients with IBS who share the same risk factor, the bacterial translocation, are also highly susceptible to metabolic disease such as obesity-induced type 2 diabetes [144, 178–180]. These results support the idea that bacterial translocation and systemic inflammation can influence the pathologies of AD patients with loss of gut barrier function.

Blood-brain barrier disruption

In the absence of infection, levels of bacterial endotoxin in the bloodstream may be mostly attributed to the permeable gut barrier. Thus, it is hypothesized that the dysbiotic gut microbiota in AD may increase gut permeability and impair BBB integrity, allowing infectious agents and/or inflammatory factors to access the brain. The BBB, which is a component of the neurovascular unit, comprises neurons, glial cells (astrocytes, microglia, oligodendrocytes), and vascular cells (pericytes, vascular smooth muscle cells, endothelial cells) [181]. It has increasingly been recognized that BBB damage can provoke neuroinflammation and is sufficient to modify neuronal activity in many CNS diseases, including AD [182–184]. Recent neuroimaging studies in patients with early AD revealed that BBB breakdown is seen in several brain regions prior to brain atrophy or dementia [185–187]. Biomarker studies have also detected BBB disruption in AD before cognitive decline or other brain pathologies [188–190]. These studies suggested that the BBB leakage in AD might be part of a cascade leading to cognitive impairment and dementia. Thus, it is notable that the translocation of gut microbes and their derivatives to blood can cause BBB impairment and neuroinflammation, contributing to the pathogenesis of AD (Fig. 2).

The endothelial cells of the BBB separate the brain from the circulation to protect the CNS from neurotoxins in the periphery, while simultaneously playing a crucial role in transporting serum factors into the brain to maintain brain homeostasis [191]. Lipoteichoic acid and LPS, which are the major cell wall components of Gram-positive and Gram-negative bacteria, respectively, are known to induce inflammation and impair the structure and function of the BBB. Lipoteichoic acid and LPS interact directly with the endothelial cells of the BBB via toll-like receptors (TLRs; TLR2, TLR4) to trigger the release of pro-inflammatory cytokines associated with increased BBB permeability [192]. Indirectly, lipoteichoic acid and LPS activate the body's response to blood endotoxin, resulting in elevated plasma concentrations of cytokines (TNF-α, IL-1β, and IL-6) and triggering the degradation of TJ proteins of endothelial cells to decrease the paracellular integrity of BBB [193, 194]. The disassembled BBB may allow endotoxin to cross the BBB and accelerate the aggregation of Aβ and tau within the AD brain [195]. Overall, BBB breakdown promotes the entry of blood-derived molecules and microbial agents into the CNS and is associated with neuroinflammation and neurodegeneration [196] (Fig. 2).

Braniste et al. reported that GF mice displayed increased BBB permeability compared to SPF mice with normal gut microbiota. The increased BBB permeability seen in GF mice was associated with reduced expression of the TJ proteins, occludin and claudin-5, in endothelial cells. Moreover, fecal transfer of normal gut microbiota or transplantation of SCFA-producing bacteria into GF mice reduced BBB permeability and restored the expression of TJ proteins [197]. Hoyles et al. found that propionate, an SCFA produced from dietary substrates by colonic bacteria, has beneficial effects on the BBB, alleviating the effects of deleterious inflammatory and oxidative stimuli. Propionate inhibits

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pathways related to non-specific microbial infections via a cluster of differentiation 14 (CD14)-dependent mechanism. It also suppresses the expression of low-density lipoprotein receptor-related protein 1 and protects the BBB from oxidative stress via nuclear factor erythroid-2-related factor 2 signaling [198]. These findings suggest that certain bacterial types can aid BBB maintenance, helping to delay AD pathology.

Neuroinflammation

Microglia are the primary resident macrophages in the CNS and are considered to be immune sentinels that are responsible for initiating an innate immune response upon receipt of diverse damage signals in the brain [199]. Under normal conditions, homeostatic microglia (resting microglia) continuously survey their local environment via highly motile processes. In response to brain injuries or immunological stimuli, microglia are capable of rapidly transitioning to an activated state characterized by alterations in their proliferation rate, morphology, and phagocytic activity, and thereafter scavenge the CNS for damaged neurons, infectious agents, and/or foreign substances [200]. Microglia are equipped with a wide range of membrane receptors that enable them to sense alterations in the local environment, and activated microglia produce the inflammatory factors, including cytokines and chemokines, which enable them to fulfill their immune functions [201]. However, prolonged microglial activation leads to the excessive release of pro-inflammatory cytokines and reactive oxygen species, resulting in detrimental consequences for neurons and other glial cells in the brain. Indeed, accumulating evidence indicates that a chronic microglial activation status is implicated in the pathology of neurodegenerative diseases [202], and uncontrolled inflammatory responses and microglial dysfunction due to persistent microglial activation were revealed as major contributors to AD pathology [203].

Due to microbial dysbiosis and the resulting increased gut permeability, certain pro-inflammatory cytokines that are elevated in plasma can cross the BBB and likely contribute to the neuroinflammation initiated by microglia. The peripheral inflammatory cytokines that are used as diagnostic biomarkers for AD [204] could be reflecting gut dysbiosis. The loss of BBB integrity following increased gut permeability in AD may allow bacterial components and metabolites to enter the CNS and trigger aberrant microglial activation [205]. Based on recent studies showing that microglia activation is profoundly affected by gut inflammation and bacteria-derived materials, the imbalanced gut microbiota composition and high gut permeability underlying these symptoms can be suggested as major risk factors for AD (Fig. 2).

Gut inflammation. In mice that received intragastric administration of LPS and ethanol, increased levels of BBB-permeable pro-inflammatory cytokines (TNF-α, MCP-1, IL-1β) in the liver and serum were found to induce microglia activation, leading to morphological changes with reduced neurogenesis [206]. The gut inflammation induced by intracolonic administration of 2, 4, 6-trinitrobenzene sulfonic acid was found to increase hippocampal susceptibility to TNF-α-mediated neuronal excitability arising from microglial activation [207]. 3xTg-AD mice (an AD mouse model) were reported to be susceptible to gut infection, which can trigger inflammation, resulting in exaggerated microglial activation [208]. A systemic immune challenge was shown to predispose WT mice to develop age-associated AD-like phenotypes and cognitive decline [209]. Several studies have thoroughly documented the neuroinflammation, amyloidogenesis, tau phosphorylation, and memory impairment that arises following LPS-induced systemic inflammation in AD model mice [115, 210]. Tejera et al. investigated microglial changes in APP/PS1 mice following peripheral immune challenge with intraperitoneal LPS injection. The microglia exposed to peripheral LPS challenge were particularly poor in their Aβ clearance capacity. The impaired phenotype of microglia was restored by NLRP3 knockout, indicating that the NLRP3 inflammasome is deeply involved in the microglial activation that occurs during systemic inflammation [211]. These results suggest that peripheral inflammation contributes to the progression of AD. This hypothesis is supported by clinical studies showing that both acute and chronic systemic inflammation are followed by increased cognitive decline in AD [212]. Thus, systemic inflammation due to microbial dysbiosis can affect microglia in AD, further exacerbating neuroinflammation and accelerating the progression of AD.

Bacterial LPS and gut microbiota-secreted amyloids. Bacterial LPS and gut microbiota-secreted amyloids are also known to directly contribute to the microglial inflammatory response. LPS binds to microglial receptors (TLR2, TLR4, and CD14) and activates the signaling of the myeloid differentiation primary response gene 88-dependent nuclear factor

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kappa B (NF-κB). The NF-κB signaling pathway can evoke inflammatory responses through a cascade of cytokines and chemokines [213]. These inflammatory mediators eventually act on components of AD pathogenesis, including Aβ production/deposition and tau phosphorylation. For example, the inflammatory cytokines, IL-1β, IL-6, IL-18, TNF-α, and interferon-gamma, increase the synthesis and processing of APP in astrocytes and/or neurons. Most inflammatory cytokines are linked to the activation of several tau kinases in the brain, including cyclin dependent kinase 5, glycogen synthase kinase-3β, and p-38 mitogen-activated protein kinase, to provoke abnormal tau phosphorylation at AD-associated residues [214]. Hence, the neuroinflammatory processes triggered by bacterial LPS can contribute to the formation of both Aβ plaques and NFTs and can perpetuate as a vicious cycle, thereby playing active roles in AD progression.

In addition to LPS, amyloids produced by some bacterial species can easily pass through a compromised gastrointestinal tract or BBB and accumulate in the AD brain [215]. Bacterial amyloids are also recognized as PAMPs by TLR1/2 and CD14 to induce the innate immune response through NF-κB signaling, thereby eliciting the production of pro-inflammatory chemokines and cytokines [216]. Therefore, it has been suggested that microglial activation by bacterial amyloids in the brain may alter the misfolded protein load in neurodegenerative diseases. Chen et al. verified this hypothesis by comparing rats exposed to *curli*-producing bacteria or *curli*-deficient bacteria. Compared to rats exposed to mutant bacteria unable to synthesize *curli*, rats exposed to *curli*-producing bacteria displayed increased neuronal α-syn deposition in both gut and brain, along with enhanced inflammation indicated by microgliosis and astrogliosis [217]. In the AD brain, both LPS and microbial amyloids can alter microglial function through NF-kB-mediated pathways. NF-kB-dependent microRNA-34a (miRNA-34a) can downregulate a triggering receptor expressed on myeloid cells 2 that is directly involved in Aβ peptide sensing, phagocytosis, and clearance in microglia [218]. LPS-induced NF-kB stimulates miRNA-146a and miRNA-155; these microRNAs potently modulate complement factor H (CFH), which regulates the alternative pathway of complement activation in innate immunity [219]. Given the findings that systemic CFH deficiency contributes to excessive activation of the complement pathways associated with autoimmune, host tissue damage, and persistent/chronic inflammatory responses [220, 221], it is reasonable to assume that NF-kB stimulating microRNAs may act on microglia-mediated inflammation.

Microbe-derived metabolites. Microbe-derived metabolites also contribute to microglial activation. SCFAs are the major end products of bacterial fermentation in the colon [222]. It has been well described that supplementation of GF WT mice with SCFAs reversed the phenotype and functions of stunted microglia through other peripheral myeloid cells that express free fatty acid receptor 2 [26]. However, since there is evidence that SCFAs can cross the BBB [223], the possible direct effect of SCFAs on microglia cannot be excluded. A recent study found that SCFA supplementation of GF APP/PS1 mice altered the microglial transcriptomic profile and caused apolipoprotein E upregulation in microglia, but not astrocytes, to promote Aβ deposition [224]. The results underscore that microbiota-derived SCFAs can be critical mediators along the GBA and potentially increase Aβ plaque load via altering microglia. Alteration of the gut microbiota was found to elevate the levels of phenylalanine and isoleucine in the periphery in 5XFAD mice to provoke pro-inflammatory Th1 cell infiltration to the brain with microglia activation. GV-971, a sodium oligomannate that inhibits phenylalanine and isoleucine accumulation, was found to attenuate neuroinflammation and reverse cognitive impairment, and was accordingly proposed as a treatment for AD pathology caused by gut dysbiosis [100]. Another study provided evidence that the butyrate-producing probiotic bacterium, *Clostridium butyricum* (CB), can attenuate microglia-mediated neuroinflammation via its metabolites [225]. Together, these studies show that microbial-related products can mediate microglia activity and may serve as potential therapeutic targets acting through the GBA in AD.

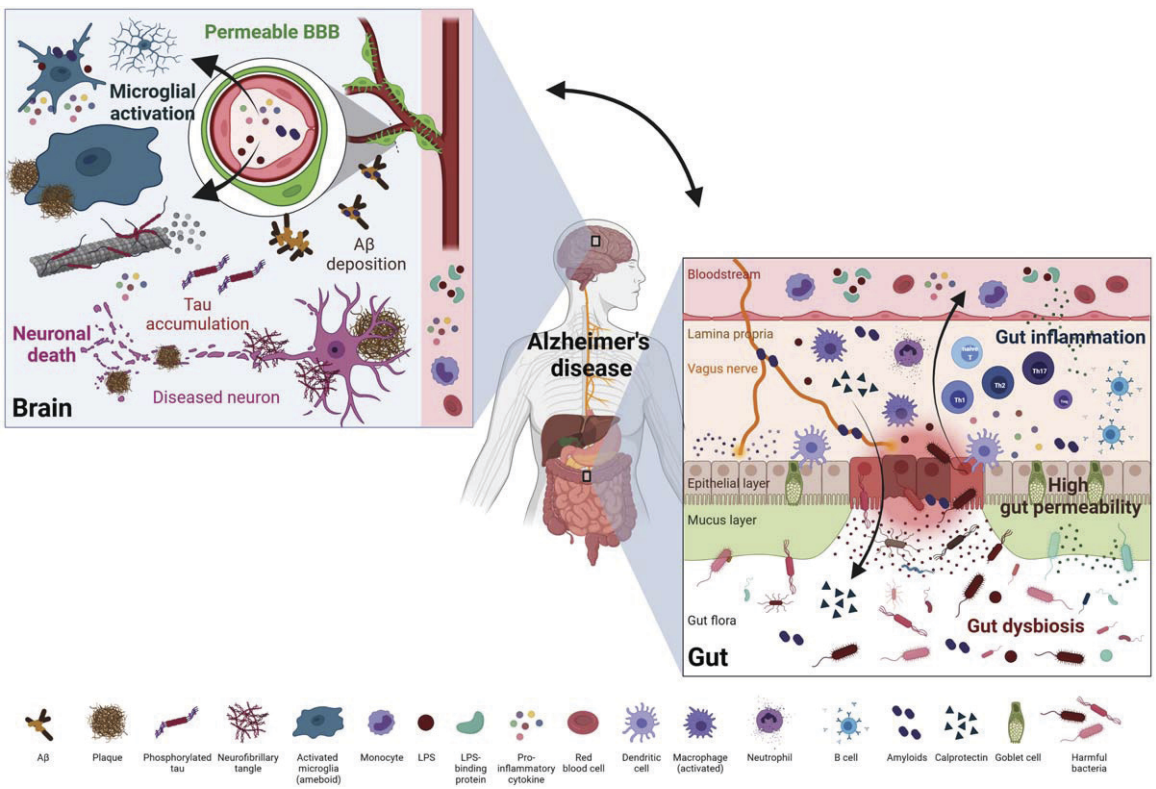
CONCLUSION

In summary, an imbalanced gut microbial composition in AD negatively impacts the properties and integrity of the gut barrier, as well as gut permeability. Gut dysbiosis and high gut permeability perturb gut immunity, thereby causing persistent inflammatory responses. A highly permeable gut also allows bacteria-derived molecules and inflammatory mediators to translocate into the bloodstream and the brain. Bacteria-derived substances such as amyloids and LPS cross the permeable BBB to promote amyloid aggregation and microglia activation in the brain.

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The persistent microglial activation driven by peripheral inflammation results in a blunted phagocytic activity and chronic inflammatory response. This neuroinflammation increases Aβ accumulation and facilitates tau phosphorylation. Thus, the imbalance in the gut microbiota can be proposed as a hidden player in triggering the neurodegeneration of AD (Fig. 3).

The extensive evidence presented in this review for possible mechanisms by which gut dysbiosis accelerates the onset of AD raises several points that need to be addressed. First of all, since the stages in which



Possible mechanism	Cause	Effect
Cross-seeding	Bacterial amyloids and LPS ↑	AD-associated Aβ fibrillation
Gut permeability (Gut mucus layer)	Butyrate-producing bacteria ↓	Gut barrier disruption Endotoxin translocation into brain Aβ plaque formation Neuroinflammation
	SCFAs levels ↓	
	Mucolytic bacteria ↓	
Gut permeability (Gut epithelial layer)	Epithelial TJs and AJs proteins ↓	
Gut inflammation (Gut lamina propria)	Pro-inflammatory intestinal bacteria	Pro-inflammatory cytokines in blood BBB disruption Microglial activation (Neuroinflammation)
	Inflammatory mediators (Calprotectin, LCN2, COX, NLRP3)	
Bacterial translocation into brain	Lipoteichoic acid and LPS	BBB disruption Microglial activation (Neuroinflammation)
	Bacterial amyloids	
	Microbe-derived metabolites	

Fig. 3. Graphical summary and table of the mechanisms of the gut-brain axis in AD. LPS, lipopolysaccharide; AD, Alzheimer's disease; Aβ, amyloid-beta; SCFAs, short chain fatty acids; TJs, tight junctions; AJs, adherens junctions; LCN2, lipocalin 2; COX, cyclooxygenase; NLRP3, NOD-like receptor pyrin domain-containing protein 3; BBB, blood-brain barrier.

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AD patients are in at the time of sampling are not specified in most studies, the relationship between gut microbes and different stages of AD is still unclear. One study shows that pro-inflammatory bacteria such as Enterobacteriales of the phylum Proteobacteria are progressively enriched from healthy control to mild cognitive impairment to AD, and the changes are significantly correlated with the clinical severity [131]. Another study involving 3xTG-AD mice suggests that gut microbiota alterations are already present in a pre-clinical stage of 3 and 5 months, at which no robust pathology is observed [83]. These studies collectively point out the importance of taking different AD stages into account. Chronologically ordering the alterations in gut microbiota and the concomitant AD lesions will further clarify the cause-and-effect relationship between AD pathogenesis and dysbiosis of gut microbiota. Furthermore, a longitudinal study spanning the course of disease progression or a cross-sectional study using strictly characterized cohorts of various disease stages will help broaden our understanding of it.

It is worth envisaging the possibility that gut microbiota alterations are the consequences of AD brain pathology rather than vice versa. Transgenic mouse models are driven by powerful transgenes that overexpress representative pathological proteins. The very fact that these mouse models of AD, which are designed to have pathological proteins overexpressed only in the brains, show alterations in gut microbiota indicates that the brain pathology can be a preceding event. Thus, a study on the link between AD and gut microbiota should always consider the interconnectivity and bidirectionality of GBA and retain a holistic viewpoint.

Lastly, the limitations of animal models of AD must always be kept in mind. AD animal models retain poor genetic diversity, cannot fully recapitulate physiologically-relevant disease phenotypes, and carry gene mutations for familial AD cases, representing only a minor portion of the overall patient population [226–228]. As failed clinical trials have told from the past, a successful amelioration of pathology in animal models does not necessarily mean that the treatments will work the same way in human physiology. Bacterial species that are beneficial in animals could potentially have no apparent effect or even be detrimental in humans.

In conclusion, the alteration of gut microbiota composition (gut dysbiosis) plays a critical role in the progression of AD. Multiple animal experiments focused on manipulating the gut microbiota in ways that have benefits in AD are paving the way for developing new therapeutic targets. There are still limitations to the translation of animal research results to patients, such as those arising from complications related to age, sex, genetic factors, and environmental factors. However, in the years to come, accumulating experimental evidence obtained through epidemiologic studies will provide a solid ground on which human clinical trials can stand. Given the growing body of evidence linking gut dysbiosis and AD pathogenesis, researchers should pay more attention to curing AD by modulating the gut microbiome. Improving a healthy gut environment via restoring balance to the gut microbiota composition could eventually be a breakthrough strategy for the treatment of AD.

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Investigating Casual Associations Among Gut Microbiota, Metabolites, and Neurodegenerative Diseases: A Mendelian Randomization Study

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Abstract.

Background: Recent studies had explored that gut microbiota was associated with neurodegenerative diseases (including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS)) through the gut-brain axis, among which metabolic pathways played an important role. However, the underlying causality remained unclear.

Objective: Our study aimed to evaluate potential causal relationships between gut microbiota, metabolites, and neurodegenerative diseases through Mendelian randomization (MR) approach.

Methods: We selected genetic variants associated with gut microbiota traits ($N = 18{,}340$) and gut microbiota-derived metabolites ($N = 7{,}824$) from genome-wide association studies. Summary statistics of neurodegenerative diseases were obtained from IGAP (AD, 17,008 cases; 37,154 controls), IPDGC (PD, 37,688 cases; 141,779 controls), and IALSC (ALS, 20,806 cases; 59,804 controls) respectively.

Results: Greater abundance of *Ruminococcus* (OR, 1.245; 95%CI, 1.103–1.405; $p = 0.0004$) was found significantly related to higher risk of ALS. Besides, our study found suggestive associations of Actinobacteria, Lactobacillaceae, *Faecalibacterium, Ruminiclostridium,* and *Lachnoclostridium* with AD, of Lentisphaerae, Lentisphaeria, Oxalobacteraceae, Victivallales, Bacillales, *Eubacteriumhalliigroup, Anaerostipes,* and *Clostridiumsensustricto1* with PD, and of *Lachnospira, Fusicatenibacter, Catenibacterium,* and *Ruminococcusgnavusgroup* with ALS. Our study also revealed suggestive associations between 12 gut microbiome-dependent metabolites and neurodegenerative diseases. Glutamine was related to lower risk of AD. For the serotonin pathway, serotonin was found as a protective factor of PD, while kynurenine as a risk factor for ALS.

Conclusion: Our study firstly applied a two-sample MR approach to detect causal relationships among gut microbiota, gut metabolites, and neurodegenerative diseases. Our findings may provide new targets for treatments and may offer valuable insights for further studies on the underlying mechanisms.

Keywords: Alzheimer's disease, amyotrophic lateral sclerosis, gastrointestinal microbiome, mendelian randomization analysis, Parkinson's disease

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INTRODUCTION

Neurodegenerative diseases are characterized by progressive loss of structure or function of neurons in the central or peripheral nervous system, which involves irreversible long-term motor or cognitive impairments [1]. The prevalence of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), are rising worldwide with the increasing life expectancy. In recent years, emerging evidence has indicated that gut microbiota derived metabolites including short-chain fatty acids (SCFAs) [2, 3] and neurotransmitters such as glutamate [4], serotonin [5, 6], and γ-aminobutyric acid (GABA) [7] may play a central role in the gut-brain axis alterations and risk of neurodegenerative diseases [8]. However, few consistent links connecting gut microbiota and diseases or their associated metabolic pathways were found.

Increasing number of cross-sectional studies have implicated the association between gut microbiota and neurodegenerative diseases, including AD, PD, and ALS [9]; however, such associations differed across studies. For example, an observational study ($N = 25$) found a significantly decreased abundance of Ruminococcaceae and Actinobacteria and significant increase in abundance of Bacteroidetes in patients with AD compared with control individuals [10]; while another cross-sectional study ($N = 43$) showed an opposite outcome of those microbiota [11]. Similarly, the association between gut microbiota and PD or ALS also differed in different studies [12–15]. The results of those small observational studies should be considered with caution due to participant selection bias, confounding bias, and reverse causation. However, it is crucial to identify whether those relationships were robust causal associations or spurious correlations.

Mendelian randomization (MR) approach, which uses genetic variants as instrumental variables (IVs), has been widely accepted to determine the causal effect of exposures on diseases [16]. As single nucleotide polymorphisms (SNPs) are of random allocation and is independent of confounders, MR is similar to randomized controlled trial and circumvent the limitations of previous observational studies.

Therefore, our study firstly applied a two-sample MR approach to detect causal relationships among gut microbiota, metabolites, and neurodegenerative disorders including AD, PD, and ALS, using summary statistics from the largest genome-wide association studies (GWAS) so far.

MATERIALS AND METHODS

Data sources and instruments

Summary statistics applied for investigating traits had the largest sample sizes with similar populations, and exposure and outcome statistics were obtained from different consortia, sample overlap tended to be little [17]. Details of the contributing GWAS consortiums are listed in Supplementary Table 1.

Gut microbiota

We leveraged summary statistics from most comprehensive exploration of genetic influences on human gut microbiota so far. The MiBioGen consortium recruited 18,340 participants of multiple ancestries (including European, American Hispanic/Latin, East Asian, etc.) from 24 cohorts [18]. After extracting DNA from fecal samples, 16S rRNA gene sequencing was utilized to characterize the gut microbiome using SILVA as a reference database [19], with truncation of the taxonomic resolution to genus level.

Gut metabolites

Considering the important roles of gut metabolites in microbiota-host crosstalk, we also leveraged summary-level data from a GWAS of the human metabolome conducted among European-descent subjects (TwinsUK and KORA, $N = 7824$). The GW AS tested all 486 metabolite concentrations present in both datasets at each SNP. Then we applied HMDB [20] to obtain a list of 81 gut microbiota derived metabolite traits from all the quantified metabolites in the GWAS.

Neurodegenerative diseases

We utilized the GWAS summary statistics from the largest and most recent datasets for AD, PD, and ALS so far. We obtained the corresponding genetic variants from the International Genomics of Alzheimer's Project (IGAP) including 17,008 cases and 37,154 controls [21], the International Parkinson's Disease Genomics Consortium (IPDGC) including 37,688 cases and 141,779 million controls [22], and the International Amyotrophic Lateral Sclerosis Genomics

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Consortium (IALSC) including 20,806 cases with ALS and 59,804 controls [23]. Cases of those neurodegenerative diseases were all clinically confirmed using published criteria.

Ethical approval for each study had been obtained in all original articles [18, 21–24], and no ethical approval for the current analyses was needed as they were based on publicly available summary statistics.

Selection of instrumental variables

To ensure the validity of the conclusion, our study applied several quality control steps to select instrument variables. First, SNPs significantly related to gut microbiome were selected as instrumental variables. Two thresholds were used to select the instrumental variable. In order to obtain more comprehensive results and increase sensitivity to IVs, SNPs smaller than the locus-wide significance level (1×10^{-5}) was selected as instrumental variables in primary analysis. We also selected exposure-related SNPs at genome-wide significance ($p < 5 \times 10^{-8}$) as instrumental variables in secondary analysis to maximize specificity. Second, we manually checked all the identified SNPs by PhenoScanner GWAS database (http://www.phenoscanner.medschl.cam.ac.uk/) and excluded variants for the linkage disequilibrium (LDlink: https://ldlink.nci.nih.gov/; LD, R2 < 0.001). All GWAS were assumed to be coded on the forward strand. Third, we also computed the F-statistic of each exposure, and SNPs that had F-statistics less than 10 were excluded to avoid weak instrument bias. Finally, for gut microbiota instruments, a total of 8,269 host SNPs were identified, which were associated with 200 gut microbiota traits (9 phyla + 16 classes + 20 orders + 36 families + 119 genera), and for gut metabolite instruments, 3,134 SNPs associated with 81 traits were included in our study. Summary statistics of these significant SNPs are assessed in Supplementary Tables 2 and 3.

Statistical analyses

We applied two sample MR as our main statistical methods to estimate causal associations between each instrument-exposure (gut microbiota and metabolite) and instrument-outcome (AD, PD, and ALS). The MR approach was based on three key assumptions: 1) the genetic variant must be truly associated with the exposure; 2) the genetic variant should not be associated with confounders of the exposure-outcome relationship; and 3) the genetic variant should only be related to the outcome of interest through the exposure under study [25].

Primary analyses were performed using Inverse-variance weighted (IVW) method, which essentially assumed the intercept was zero, and our results were corrected for multiple hypothesis testing using the Benjamini and Hochberg false discovery rate (FDR), as significance threshold was set at FDR-corrected p-values < 0.05 [26], while associations with $p < 0.05$, but not reaching the FDR-controlled threshold were reported as suggestive of association. Power calculations were conducted based on the website http://cnsgenomics.com/shiny/mRnd/ [27] (see Supplementary Table 6).

To validate assumption 3 and improve the robustness of the findings, we also undertook a series of sensitivity analyses including MR-Egger regression, weighted mode, weighted median, simple median methods, and robust adjusted profile score (MR.RAPS) method, which provided different assumptions about horizontal pleiotropy [28, 29]. However, the MR-Egger method had the lowest power among the 6 methods and was based on the instrument strength independent of the direct effects (INSIDE) assumption, with no measurement error in the SNP exposure effects (NOME) assumption [30]. Therefore, MR Egger was performed when I2GX was > 0.9 [31].

Cochran Q statistic and leave-one-out sensitivity analysis were also adopted to the SNPs that may influence the outcome through an unaccounted causal pathway, and Steiger analysis was performed to explore direction of causal effects [32]. Furthermore, MR-Egger intercept and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) global test were used to detect the presence of pleiotropy [33].

At last, we conducted multivariable MR (MVMR) analyses using IVW method to estimate the direct and indirect effect of each exposure on an outcome, as we found a high degree of IV overlap across gut microbiota (Lentisphaerae at phylum level, Lentisphaeria at class level, and Victivallales at order level) in univariable MR analyses on PD [34]. Furthermore, we also conducted multivariable MR-Egger analyses to evaluate the horizontal pleiotropy for direct and indirect effects. The IVs used for MVMR analysis were listed in Supplementary Table 8.

A flowchart of our study was provided in Fig. 1. The MR analyses were performed in the R version 4.0.2 computing environment using the latest Two SampleMR (https://github.com/MRCIEU/TwoSam

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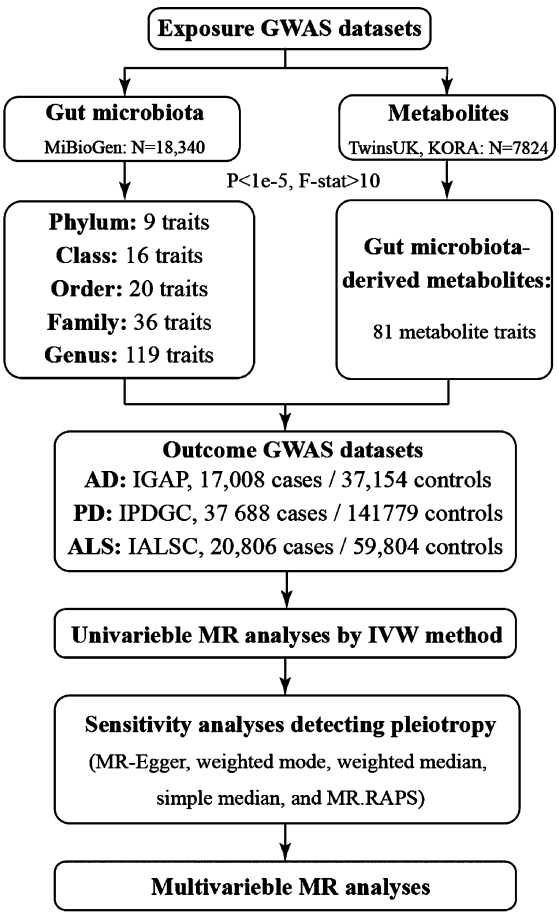


Fig. 1. Flowchart of current study. AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis.

pleMR), MVMR (https://github.com/WSpiller/MVMR), and MRPRESSO (https://github.com/rondolab/MR-PRESSO) packages.

RESULTS

Associations between gut microbiota and neurodegenerative diseases

By the means of IVW method, results reaching a threshold of $p < 0.05$ are presented in Fig. 2. Causal effects were estimated by odds ratio (OR), which represented increased risk of binary outcomes (AD, PD, ALS) per SD increase in abundance of gut microbiota feature. By the means of IVW method, we found suggestive associations of host-genetic-driven increases in Actinobacteria at class level (OR, 1.027; 95%CI, 1.006–1.048; $p = 0.013$); Lactobacillaceae at family level (OR, 1.027; 95%CI, 1.006–1.048; $p = 0.014$); *Lachnoclostridium* at genus level (OR, 1.03; 95%CI, 1.005–1.056; $p = 0.019$) and higher risks of AD, while genetically increased in *Faecalibacterium* at genus level (OR, 0.975; 95%CI, 0.954–0.997; $p = 0.028$) were associated with protective effects on the risk of AD. We also found suggestive causal effect of *Ruminiclostridium6* at genus level (OR, 1.025; 95%CI, 1.006–1.045; $p = 0.009$) on higher risk of AD, while *Ruminiclostridium9* (OR, 0.969; 95%CI, 0.943–0.996; $p = 0.009$) on lower risk of AD. However, after calculating FDR, we found that all q-values were over 0.05, suggesting no significant associations. In addition, associations between the gut microbiota traits and risk of AD were consistent in sensitivity analyses (see Table 1). MR-Egger intercept (we calculated I2GX, which were all over 0.9) and Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) were applied to test the directional pleiotropy, and all p values were over 0.05, suggesting no significant pleiotropy, while Cochran Q statistic of both the IVW test and the MR-Egger regression was used to test the heterogeneity, and no notable heterogeneity across instrument SNP effects was indicated (see Supplementary Table 7). However, we had limited power (less than 80%) to test causal effects of those gut microbiota features on AD.

Causal relationship between gut microbiota and other neurodegenerative diseases were also analyzed by the same process. Our study revealed that genetically increased abundance of Lentisphaerae at phylum level (OR, 0.836; 95%CI, 0.724–0.965; $p = 0.015$); Lentisphaeria at class level (OR, 0.847; 95%CI, 0.728–0.986; $p = 0.032$), and Victivallales at order level (OR, 0.847; 95%CI, 0.728–0.986; $p = 0.032$) were potentially associated with a protective effect of PD. In contrast, no notable effects of the three gut microbiota features on the risk of PD could be observed after mutual adjustment using multivariable MR method (see Supplementary Table 7). In addition, genetically increased abundance of Oxalobacteraceae at family level (OR, 1.13; 95%CI, 1.003–1.273; $p = 0.044$); Bacillales at order level (OR, 1.144; 95%CI, 1.013–1.292; $p = 0.03$); *Eubacteriumhalliigroup* (OR, 1.253; 95%CI, 1.055–1.487; $p = 0.01$), and *Clostridiumsensustricto1* (OR, 1.354; 95%CI, 1.068–1.716; $p = 0.012$) were related to higher risk of AD; while *Anaerostipes* (OR, 0.744; 95%CI, 0.587–0.944; $p = 0.015$) was related to protective effect of PD (Fig. 2).

Besides, genetically increased *Lachnospira* (OR, 1.315; 95%CI, 1.063–1.628; $p = 0.012$); decreased

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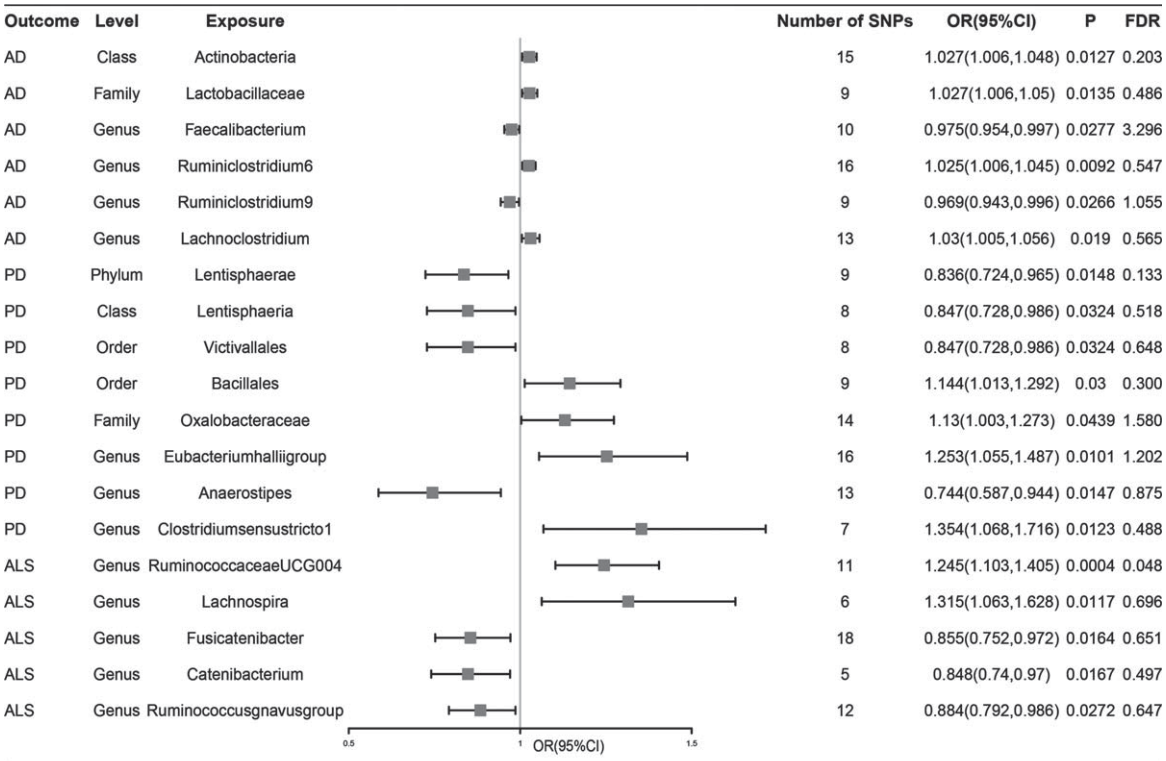


Fig. 2. Associations of genetically predicted gut microbiota with risk of neurodegenerative diseases using IVW method. OR, odds ratio; CI, confidence interval; FDR, False discovery rate.

Fusicatenibacter (OR, 0.855; 95%CI, 0.752–0.972; $p = 0.016$) and *Catenibacterium* (OR, 0.848; 95%CI, 0.74–0.97; $p = 0.017$) were potentially related to a higher risk of ALS. Our study also revealed that increased *RuminococcaceaeUCG004* (OR, 1.245; 95%CI, 1.103–1.405; $p = 0.0004$) and decreased *Ruminococcusgnavusgroup* (OR, 0.884; 95%CI, 0.792–0.986; $p = 0.027$) were related to a higher risk of ALS. Among all those results, we found a significant causal effect of increased *RuminococcaceaeUCG004* on risk of ALS (FDR-corrected p-value < 0.05) (Fig. 2).

Those estimate effects mentioned above were considered robust (Table 1) with no directional pleiotropy or heterogeneity was significant (see Supplementary Table 7), and MR power calculation results were showed in Supplementary Table 6.

Associations between gut metabolites and neurodegenerative diseases

Among 81 gut microbiota-derived metabolites incorporated in our MR analyses, we found 11 suggestive estimate effects of gut metabolite on neurodegenerative diseases. Those metabolites were classified into two types: host-derived or dietary molecules [35].

With regard to host metabolites transformation, our study suggested that increased abundance of taurodeoxycholate, which was a product of primary bile acids (OR, 1.16 for risk ratio of ALS per SD unit of taurodeoxycholate; 95%CI, 1–1.345; $p = 0.050$) was associated with higher risk of ALS. However, no steroid hormone was proved relevance to neurodegenerative diseases.

For the dietary molecules, amino acids, complex plant polysaccharides and polyphenols were considered to exert impact on brain function. In tryptophan metabolism, our study revealed that serotonin (OR, 0.535; 95%CI, 0.292–0.979; $p = 0.043$) was a protection factor of PD, while kynurenine (OR, 1.756; 95%CI, 1.113–2.769; $p = 0.015$) was a risk factor of ALS. In arginine metabolism, dimethylarginine (OR, 1.826; 95%CI, 1.003–3.321; $p = 0.049$) was suggested to be related to higher risk of ALS. Phenylacetate, a modulator of central adrenergic functions (OR, 1.064; 95%CI, 1.008–1.124; $p = 0.024$). Other ammino acids such as glutamine (OR, 0.803; 95%CI,

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Table 1
Sensitivity analyses of MR analyses of neurodegenerative diseases on gut microbiota by MR Egger, simple mode, weighted median, and weighted mode

Outcome	Exposure		Weighted mode		Weighted median		Simple mode		MR Egger		RAPS	
	Level	Microbiota	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*
AD	Class	Actinobacteria	1.048 (1.009,1.088)	0.03	1.039 (1.011,1.068)	0.01	1.022 (0.979,1.067)	0.33	1.08 (1.023,1.141)	0.02	1.031 (1.01,1.052)	0.00
AD	Family	Lactobacillaceae	1.015 (0.981,1.051)	0.41	1.022 (0.996,1.049)	0.10	1.01 (0.978,1.043)	0.56	0.986 (0.936,1.038)	0.60	1.024 (1.002,1.046)	0.03
AD	Genus	Faecalibacterium	0.98 (0.946,1.015)	0.29	0.977 (0.949,1.007)	0.13	0.979 (0.942,1.018)	0.31	0.979 (0.938,1.021)	0.35	0.976 (0.952,1)	0.05
AD	Genus	Ruminiclostridium6	1.01 (0.974,1.048)	0.59	1.019 (0.992,1.048)	0.17	1.015 (0.97,1.062)	0.53	1.005 (0.958,1.053)	0.85	1.025 (1.004,1.045)	0.02
AD	Genus	Ruminiclostridium9	0.984 (0.926,1.045)	0.61	0.984 (0.947,1.022)	0.40	0.983 (0.921,1.05)	0.63	0.96 (0.852,1.082)	0.53	0.967 (0.938,0.996)	0.03
AD	Genus	Lachnoclostridium	1.004 (0.939,1.074)	0.91	1.023 (0.99,1.058)	0.17	1.006 (0.944,1.073)	0.85	1.031 (0.939,1.133)	0.53	1.034 (1.007,1.062)	0.01
PD	Phylum	Lentisphaerae	0.745 (0.555,0.999)	0.08	0.762 (0.629,0.921)	0.01	0.743 (0.538,1.026)	0.11	0.715 (0.431,1.186)	0.23	0.832 (0.71,0.975)	0.02
PD	Class	Lentisphaeria	0.751 (0.559,1.009)	0.10	0.783 (0.641,0.957)	0.02	0.747 (0.539,1.037)	0.13	0.743 (0.45,1.225)	0.29	0.844 (0.714,0.996)	0.04
PD	Family	Oxalobacteraceae	1.202 (0.934,1.547)	0.18	1.177 (1.007,1.376)	0.04	1.194 (0.901,1.583)	0.24	1.422 (0.856,2.362)	0.20	1.133 (0.994,1.291)	0.06
PD	Order	Victivallales	0.751 (0.555,1.015)	0.11	0.783 (0.64,0.959)	0.02	0.747 (0.536,1.042)	0.13	0.743 (0.45,1.225)	0.29	0.844 (0.714,0.996)	0.04
PD	Order	Bacillales	1.221 (0.931,1.601)	0.19	1.179 (0.996,1.397)	0.06	1.215 (0.934,1.581)	0.18	1.133 (0.614,2.092)	0.70	1.164 (1.017,1.331)	0.03
PD	Genus	Eubacterium hallii group	1.462 (0.97,2.202)	0.09	1.361 (1.075,1.723)	0.01	1.526 (0.967,2.407)	0.09	1.329 (0.916,1.928)	0.16	1.274 (1.056,1.538)	0.01
PD	Genus	Anaerostipes	0.588 (0.336,1.029)	0.09	0.747 (0.54,1.034)	0.08	0.6 (0.342,1.052)	0.10	0.579 (0.26,1.288)	0.21	0.739 (0.569,0.961)	0.02
PD	Genus	Clostridium sensustricto 1	1.416 (0.942,2.128)	0.15	1.413 (1.043,1.915)	0.03	1.404 (0.915,2.154)	0.17	1.728 (1.009,2.959)	0.10	1.358 (1.041,1.77)	0.02
ALS	Genus	Ruminoco ccaceae UCG 004	1.259 (0.92,1.723)	0.18	1.251 (1.06,1.476)	0.05	1.263 (0.934,1.707)	0.17	0.76 (0.398,1.45)	0.18	1.271 (1.114,1.45)	0.00
ALS	Genus	Lachnospira	1.15 (0.745,1.776)	0.56	1.298 (0.987,1.708)	0.01	1.431 (0.933,2.196)	0.16	2.518 (0.707,8.97)	0.43	1.326 (1.046,1.682)	0.02
ALS	Genus	Fusicatenibacter	0.882 (0.635,1.225)	0.46	0.838 (0.703,0.999)	0.06	0.873 (0.617,1.234)	0.16	0.702 (0.423,1.166)	0.23	0.848 (0.735,0.979)	0.02
ALS	Genus	Catenibacterium	0.924 (0.757,1.128)	0.48	0.884 (0.756,1.034)	0.05	0.927 (0.731,1.176)	0.45	0.617 (0.159,2.39)	0.19	0.841 (0.723,0.978)	0.02
ALS	Genus	Ruminococ cusgnavus group	0.811 (0.628,1.047)	0.14	0.887 (0.775,1.014)	0.12	0.776 (0.599,1.004)	0.57	0.921 (0.542,1.563)	0.53	0.875 (0.772,0.991)	0.04

OR, Odds ratios for associations of genetically predicted gut microbiota traits with neurodegenerative diseases; CI, confidence interval; MR, Mendelian randomization; RAPS, robust adjusted profile score; AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis.

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Table 2
Sensitivity analyses of MR analyses of neurodegenerative diseases on gut microbiota dependent metabolites features by MR Egger, simple mode, weighted median, and weighted mode

Outcome	Exposure	MR Egger		Simple mode		Weighted median		Weighted mode		RAPS	
		OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*
AD	Mannitol	0.962 (0.911,1.016)	0.19	1 (0.946,1.058)	0.99	0.976 (0.944,1.009)	0.15	0.981 (0.935,1.029)	0.45	0.969 (0.944,0.994)	0.02
AD	Glutamine	0.617 (0.441,0.864)	0.03	0.972 (0.654,1.445)	0.89	0.727 (0.573,0.923)	0.01	0.714 (0.547,0.932)	0.04	0.801 (0.655,0.98)	0.03
AD	Phenylacetate	1.046 (0.965,1.134)	0.31	1.058 (0.946,1.184)	0.36	1.063 (0.99,1.14)	0.09	1.063 (0.989,1.142)	0.14	1.065 (1,1.133)	0.05
PD	Isoleucine	0.847 (0.677,1.06)	0.19	0.758 (0.517,1.11)	0.19	0.818 (0.667,1.004)	0.06	0.819 (0.679,0.987)	0.07	0.786 (0.661,0.935)	0.01
PD	Hydrocinnamate	2.296 (0.914,5.772)	0.10	1.942 (0.75,5.029)	0.19	2.026 (1.092,3.761)	0.03	1.942 (0.937,4.024)	0.10	1.754 (1.09,2.821)	0.02
PD	Serotonin	0.672 (0.178,2.545)	0.57	0.418 (0.11,1.595)	0.22	0.498 (0.218,1.14)	0.10	0.459 (0.15,1.404)	0.19	0.535 (0.285,1.006)	0.05
PD	Arabinose	1.939 (0.379,9.935)	0.51	2.345 (0.752,7.319)	0.24	2.156 (0.862,5.39)	0.10	2.184 (0.836,5.708)	0.21	2.153 (0.931,4.983)	0.07
ALS	Hippurate	1.2 (0.611,2.357)	0.61	1.909 (0.992,3.673)	0.07	1.405 (0.963,2.051)	0.08	1.277 (0.784,2.079)	0.34	1.587 (1.152,2.187)	0.00
ALS	Kynurenine	1.956 (0.772,4.956)	0.17	1.791 (0.567,5.654)	0.33	1.682 (0.885,3.197)	0.11	1.791 (0.859,3.733)	0.13	2.056 (1.318,3.206)	0.00
ALS	Dimethylarginine	1.525 (0.262,8.872)	0.64	3.646 (0.655,20.297)	0.15	2.202 (0.913,5.315)	0.08	3.073 (0.831,11.369)	0.10	1.853 (0.968,3.545)	0.06
ALS	Taurodeoxycholate	1.132 (0.683,1.876)	0.64	1.125 (0.783,1.616)	0.54	1.138 (0.922,1.404)	0.23	1.158 (0.833,1.608)	0.40	1.162 (0.99,1.364)	0.07

OR, Odds ratios for associations of genetically predicted gut microbiota-derived metabolite traits with neurodegenerative diseases; CI, confidence interval; MR, Mendelian randomization; RAPS, robust adjusted profile score; AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis.

0.667–0.968; $p = 0.022$) and isoleucine (OR, 0.791; 95%CI, 0.678–0.923; $p = 0.003$) were revealed as protective factors of AD and PD respectively. Besides, phenylalanine (PAA), one of phenylalanine derivatives (OR, 1.064; 95%CI, 1.008–1.124; $p = 0.024$) was indicated to increase the risk of AD. We also found that hippurate, a product of polyphenols (OR, 1.531; 95%CI, 1.142–2.051; $p = 0.004$), was associated with high risk of ALS. Gut microbiota also generated a protective factor of AD, mannitol (OR, 0.993; 95%CI, 0.988–0.998; $p = 0.009$), and a risk factor of PD (OR, 2.143; 95%CI, 1.026–4.476; $p = 0.043$).

Furthermore, those results were judged to be reliable without pleiotropy through sensitivity analyses (Table 2, Supplementary Table 7). However, no significant association was revealed (FDR-corrected p-values > 0.05), and MR power calculation results were showed in Supplementary Table 6.

The results of MR analysis which applied a SNP selecting threshold of genome-wide significant level of $p < 5 \times 10^{-8}$ were shown in Supplementary Tables 10 and 11. However, the results should be considered with caution as the number of the IVs applied in analysis was not much enough to perform sensitivity analysis.

DISCUSSION

In the present MR study, we found significant association of increased abundance of genera *RuminococcaceaeUCG004* and higher risk of ALS. Besides, we found suggestive evidence of causal associations of Actinobacteria, Lactobacillaceae, *Faecalibacterium*, and *Ruminiclostridium, Lachnoclostridium* with AD, of Lentisphaerae, Lentisphaeria, Oxalobacteraceae, Victivallales, Bacillales, *Eubacteriumhalliigroup, Anaerostipes,* and *Clostridiumsensustricto1* with PD, and of *Lachnospira, Fusicatenibacter, Catenibacterium,* and *Ruminococcusgnavusgroup* with ALS. Additionally, metabolites including amino acids, bile acids, amino acids, polyphenols produced by gut microbiota were also potentially related to the risks of neurodegenerative disorders, indicating their important roles in gut microbiota-brain axis.

A previous MR study has suggested that increase in *Blautia* and elevated γ-aminobutyric acid (GABA) were related to lower risk of AD [36]. However, our study failed to repeat these findings, nor *Blautia* or GABA including putrescine, glutamate, arginine, or ornithin which produces GABA were found related

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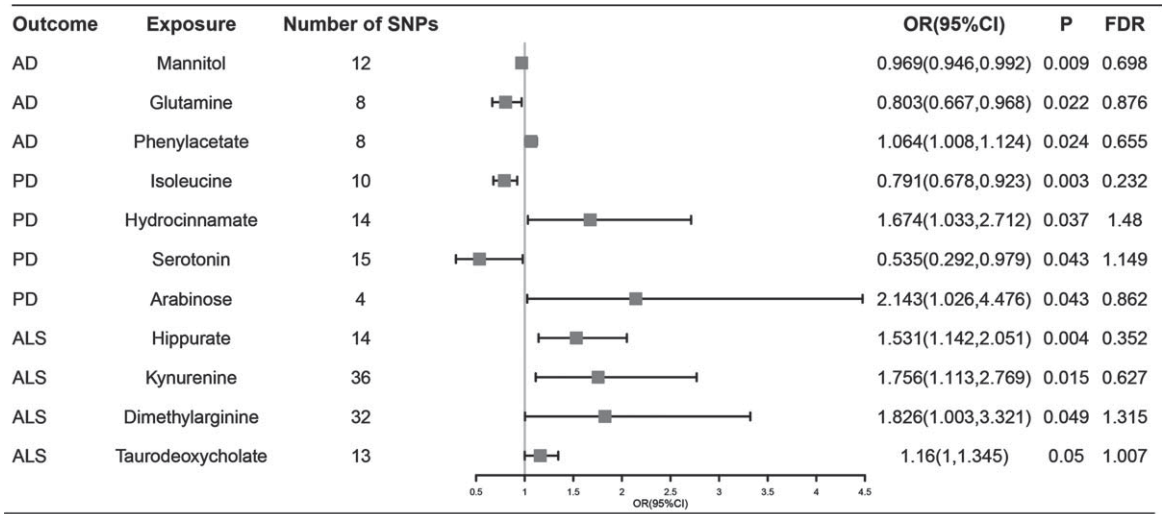


Fig. 3. Associations of genetically predicted gut microbiota-dependent metabolites with risk of neurodegenerative diseases using IVW method. OR, odds ratio; CI, confidence interval; FDR, False discovery rate.

to risk of AD, which is potentially due to lack of significance of results and scale of GWAS. Another MR study proved no causal association of trimethylamine N-oxide (TMAO) or its precursor with AD, which was consistent with our results [37]. Additionally, our finding of Actinobacteria at family level as a risk factor of AD was opposite to previous studies [10], while the findings of relationships between Lactobacillaceae and *Faecalibacterium* with AD were in accordance with the result of previous cross-sectional studies [11]. Interestingly, genera *Ruminiclostridium6 and Ruminiclostridium9* represent different effects on risk of AD in our analysis results, which remind us that inconsistencies in results of previous clinical studies were potentially due to insufficiently digging deeper into classification of genera level of gut microbiota. Besides, our study suggested that phenylacetate, which was a potential tracer of glibal metabolism was related to increased risk of AD [38]. In addition, mannitol, a microbial metabolite was found as protective factor of AD, which may provide new ideas for disease interventions.

Our study revealed suggestive causal effect of increased abundance of phylum Lentisphaerae, class Lentisphaeria, and order Victivallales on protective effects of PD; however, no direct effect revealed after multivariable MR analysis, while no relevant result was reported in previous studies either, therefore, such results should be treated with caution. Other associations of Family Oxalobacteraceae, Order Bacillales, *Eubacteriumhalliigroup, Anaerostipes*, and *Clostridiumsensustrictol* with risk of PD were in accordance with the result of previous cross-sectional studies [12, 13, 39]. In a previous clinical study, which compared the fecal microbiota of 25 ALS patients with 32 controls, significant higher abundance of uncultured Ruminococcaceae at genus level was observed in ALS patients [14]. However, our study found significant association between *RuminococcaceaeUCG004* and higher risk of ALS, and suggestive association between *Ruminococcusgnavusgroup* and lower risk of ALS. Inconsistent results between these studies may likely be attributed to small study sample sizes of previous observational studies, sample heterogeneity, and different sequencing technologies. Therefore, a standardized classification system for gut microbiota at genus level or even more specific level is crucial to direct mechanism research and provide more accurate clinical guidance.

Tryptophan is broken down by the microbiota into indole derivatives and also tryptamine and kynurenine metabolites, and those metabolites were considered important in gut-brain axis [40, 41]. Previous studies have revealed that glutamate signals are destroyed by serotonergic overdrive, and serotonergic dysfunction is associated with the development of motor and non-motor symptoms and complications in PD [42]. Moreover, kynurenine pathway (KP) of tryptophan degradation is involved with several neuropathological features present in ALS including neuroinflammation, excitotoxicity, oxidative stress, immune system activation, and dysregulation of energy metabolism [43]. Previous clinical studies

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have revealed that serum kynurenine in controls were lower than that in ALS [44]. Our study proved that serotonin was protective factor of PD, while kynurenine was risk factor of ALS, and those molecules may become potential biomarkers to assess the progression of relative diseases. In addition, other amino acid such as glutamine and isoleucine were found causally associated with lower risk of AD and PD. Actually, up to 50% of all α-amino groups of glutamate and glutamine are derived from leucine. Leucine is a regulator of the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), which is critical on protein synthesis and degradation, autophagy as well as maintenance of glutamate homeostasis, and may have effects on the neuronal solute transport and the excitatory neurotransmitter function [45]. Moreover, in the glutamate-glutamine cycle, synaptically-released glutamate is rapidly transported into astrocytes, and glutamine is then released by astrocytes through SN-type glutamine transporters into the extracellular fluid. Aβ has been shown to reduce the surface expression of GLT-1and to impair astrocyte glutamate uptake [46, 47]. A recent study demonstrated that altered astrocyte glutamine synthesis directly impaired neuronal GABA synthesis in brain slices of the 5xFAD mouse model of AD [48], and our results provided clinical evidence to confirm that reduction of glutamine in peripheral blood was causally associated with occurrence of AD.

Bacterial metabolites produced from polyphenol precursors were also found at levels sufficient to exert biological effects enter circulation [49]. *In vitro* cultures have shown that polyphenol metabolites such as ferulic acid are able to exert protective effects on neuronal cultures and neurodegenerative models, mostly through a decrease in inflammatory responses [50, 51]; however, *in vivo* evidence remains lacking. Our study suggested hippurate belongs to the group of uremic toxins as a risk factor of ALS, which may indicate potential treatment of disease. Since those neurodegenerative diseases develop through a long prodromal phase, it is plausible that our findings may inform early interventions by targeting the microbiota via gut microbiota transplantation, psychobiotics, or antibiotics in the future. Plus, gut microbiome is explicitly bidirectionally associated with diet [52]. Recent studies have found that Mediterranean diet adherence is found to reshape gut microbiota composition with increased abundance of *Bacteroides, Prevotella, Ruminococcus,* and F*aecalibacterium* [53]. Those gut microbiota may play a role through generating healthy SCFAs and producing anti-inflammation effects in preventing AD, PD, and ALS [54–56]. Therefore, more in-depth investigations are needed to find out mechanisms underlying the relationships among diet, gut microbiota, and occurrence of different neurodegenerative diseases.

Among the strengths of the study are the most comprehensive MR study on association of gut microbiota and metabolite traits with neurodegenerative diseases, and the largest sample size so far. However, our study still suffers from several limitations. First, most of the results did not survive a strict FDR correction. However, MR was a hypothesis-driven approach, and it could be used to detect some causal relationships regardless of FDR adjusting when some biological evidence exists. Second, 16S rRNA gene sequencing describes gut microbiota from genus to phylum level only, and metagenomic and multi-omic approaches may offer opportunities to target gut microbiota composition at a more specific level, avoiding bias if species of more specific level associated with neurodegenerative diseases. Third, our findings might have been affected by weak instrument bias as we included a loose cutoff of exposure-related SNPs at a threshold of $p < 1 \times 10^{-5}$, although we had excluded SNPs that had F-statistics less than 10 to avoid weak instrument bias, and the result based on exposure-related SNPs at genome-wide significance ($p < 5 \times 10^{-8}$) should be considered with caution due to low sensitivity of IVs. Finally, gut microbiota is affected by several environmental factors including diet, lifestyle, and medication, whereas the original studies lacked detailed information on disease severity or medication status, further subgroup analysis was hard to be performed so far.

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All data used in the current study were based on summary-level data that have been made publicly available. Summary data from genome-wide association studies for the gut microbiota (Kurilshikov et al.) is available at https://www.mibiogen.org/. Metabolomic GWAS summary statistics (Shin et al.) are available for download from the metabolomics GWAS server (http://metabolomics.helmholtz-muenchen.de/gwas/index.php?task=download). Summary level data for AD (Jansen et al.) can be obtained from https://ctg.cncr.nl/, for PD (Nalls et al.) from https://pdgenetics.org/resources (participants from 23andMe Inc. were excluded), and for ALS (Nicolas et al.) from http://als.umassmed.edu. All data gen-

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erated or analyzed during this study are included in this published article and its Supplementary Material.

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SUPPLEMENTARY MATERIAL

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Relationship Between the Gut Microbiota and Alzheimer's Disease: A Systematic Review

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Abstract.
Background: In recent years, scientific research on the gut microbiota and their relationship with some diseases, including neurological ones, has notably increased. As a result of these investigations, the so-called gut-brain axis arises. Despite its influence on the evolution and development of cognitive impairment, the gut-brain axis is little defined and demonstrated.
Objective: To provide the best scientific evidence available on the relationship between the gut microbiota and Alzheimer's disease.
Method: Systematic and narrative review of the information generated in the last 5 years in national and international databases, in English and Spanish.
Results: Eight observational studies were selected, carried out in humans and, therefore, suitable for inclusion in this review.
Conclusion: The results of these studies support the hypothesis that there is a relationship between the gut microbiota and cognitive disorders through the gut-brain axis. However, today, there is a substantial lack of human studies, especially clinical trials, which makes it difficult to formulate clinical recommendations on this topic.

Keywords: Alzheimer's disease, gut-brain axis, gut microbiome, microbiota, Parkinson's disease

INTRODUCTION

Neurological diseases

The increase in life expectancy and the constant aging of the population carries the risk of developing some conditions, in particular, neurodegenerative diseases such as dementia. In this regard, the United Nations in 2019 reported that there were 703 mil-

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lion people over 65 years of age and projected that by 2050 there will be around 1.5 billion. In high-income countries, there will be an increase from 226 to more than 285 million people (MP) between 2019 and 2030, respectively [1]. This fact shows that the elderly represent an increasingly significant percentage, compared to the general population.

Aging constitutes a greater risk of contracting diseases related to some type of dementia, which affects more than 55 MP; however, it is expected to increase, by 2030, to more than 78 MP [2, 3]. The global cost of dementia is estimated to be "$817.9 billion USD" [4]. Likewise, Alzheimer's disease (AD) is reported as the sixth cause of death and the fifth in people over 65 in the US [5].

In this sense, AD is the most common type of dementia, representing between 60% and 80% of cases [6]. Derived from neuronal degeneration, and learning and memory impairment, people living with the disease are affected in their activities of daily living, causing dependency, disability, and even mortality [7].

It has been reported that AD is characterized by an accumulation of amyloid-β (Aβ) peptides, followed by the formation of the hyperphosphorylated tau protein, accumulations of which cause neuroinflammation, which in turn triggers failure in synaptic activity and neuronal death [8, 9]. In the same vein, it has been noted that the intestinal and blood-brain barrier, having greater permeability as a consequence of aging or disease, are related to the pathogenesis of AD and other neurodegenerative disorders, because dysbiosis allows the passage of cytokines induced by lipopolysaccharides/amyloids or other small pro-inflammatory molecules [8, 9].

Microbiota

The intestinal microbiota, located in the human digestive tract, is made up of an ecosystem of around 100 billion microorganisms such as bacteria, archaea, fungi, and viruses [10]. Its composition depends on various factors, such as age, sex, diet, and race. Likewise, it is conditioned by exogenous factors such as the geographical environment, physical exercise, the consumption of medications, mainly antibiotics, smoking, alcohol consumption, and a lifestyle that causes disorders such as stress, among others [10, 11].

These last three factors are increasingly present, from an early age, in the daily routine of many people, as well as in the consumption of conventional medicines, which are abused on numerous occasions [12, 13]. As the body ages, the regenerative capacity of tissues decreases, affecting the structure of the microbiota. The transition between the adult and elderly microbiota is also characterized by a reduction in bacterial diversity, mainly of *Bifidobacteria, Bacteroidetes*, and *Lactobacillus* [14].

All these factors lead to dysbiosis, a phenomenon that consists of "qualitative or quantitative alterations of the microbiota of the digestive tract, which produce overgrowth or reduction of the microbiota" [15]. However, it is not yet clear to what extent each of these factors influences the quality of the microbiota, since it constantly changes, varying its composition throughout the day.

The fact that the intestinal microbiota is dynamic and dependent on each individual [13] makes its study particularly complex. Various studies have confirmed the importance of the microbiota in metabolic, endocrine, and protective functions against infectious and inflammatory processes.

Microbiota-gut-brain axis and neurological diseases

In addition, it collaborates in neurological function, which establishes the so-called microbiota-gut-brain axis [9, 16]. In contrast, a deficient microbiota is associated with the development of inflammatory bowel diseases, type 2 diabetes mellitus, obesity, and neurodegenerative diseases [8, 10, 13]. In turn, although it has not yet been systematically verified, it has been suggested that a gut with poor microbiota could lead to an "unhealthy brain" [17, 18].

The microbiota-gut-brain axis is a two-way communication system that allows intestinal microorganisms to communicate with the brain, and vice versa. However, the factors of intestinal dysbiosis lead to a lower proportion of bacteria that synthesize butyrate, which generates inflammatory activity. This, in turn, triggers a compromise of the blood-brain barrier, which directly influences the accumulation of Aβ or other pro-inflammatory molecules that promote neuroinflammation and the development of AD [8, 9].

Factors affecting microbiota-gut-brain axis

Diet is recognized as the superlative modulator of the gut microbiota, so much so that cognitive health related to AD can benefit from modulating a diet focused on unsaturated fats, fruits, vegetables, and whole grains [19].

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In this sense, it is known that a high fat diet can alter the composition of the intestinal microbiota by reducing several bacterial groups, such *as Bifidobacterium spp.*, *Eubacterium rectal,* the group of *Clostridium coccoides*, and bacteria related to *Bacteroides*. This, in addition to promoting "metabolic endotoxemia", a consequence of the increase in lipopolysaccharide, which causes chronic inflammation, insulin resistance, and diabetes mellitus. The intestinal microbiota participate in the conversion of plant polysaccharides to short chain fatty acids (SCFA), predominantly acetate, propionate, and butyrate [18].

It is possible that alteration of the intestinal microbiota is related to a decrease in the levels of SCFA such as butyrate, since it facilitates the microglial activation induced by the increase in the levels of lipopolysaccharide or bacterial amyloids in the CNS, which may be involved in the development of AD, suggesting another mechanism for the development of AD [19].

Currently, there have been several studies on the use of supplements that directly target gut bacteria in AD. These have shown in clinical trials that a 12-week consumption of a probiotic combination of *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum,* and *Lactobacillus fermentum*, has significant positive effects on cognitive functioning, although no effects on oxidative stress or inflammation were observed [20].

This work aims to provide the best scientific evidence available on the relationship between gut microbiota and cognitive disorders, through the microbiota-gut-brain axis, in addition to identifying whether this relationship could be used for the prevention and treatment of dementia, specifically AD, which affects the cognitive process.

METHODS

Design

This systematic review focuses on a comprehensive search for available scientific information about the relationship between the gut microbiota and AD. For this, a systematic method was used to guide the identification, data collection, evaluation, and identification of available scientific evidence.

Search strategy

To search for information, only observational scientific articles were included, whether transversal or longitudinal, published from January 2016 to May 2020, available in open access, published in English, Spanish, and Portuguese. In addition, articles exclusively carried out in humans were searched, due to the lack of practical information in a first screening.

For information search, the DeCS and MeSH thesauri were used, for an adequate exploration of the databases. The following Boolean operators were used: AND, to establish the logical operations between concepts, OR, to retrieve documents where at least one of the specified arguments appears, and NOT to indicate that the keyword before the operator should not appear in our search.

The databases consulted were Pubmed, Scielo, Scopus, Web of Science, and BVS, making different combinations with the following keywords: "gastrointestinal microbiome", "microbiota", "Alzheimer's disease", "Parkinson's disease", and "gut microbiota".

Study selection

The results of each search were individually checked for relevance within the scope of this systematic review; the title and full abstract were read. Documents were excluded by screening title and abstract, and those articles that did not adhere to the subject of the composition of the microbiota and the relationship with the gut-brain axis or because they were carried out in animals, as well as because they did not comply with any of the established goals, were excluded.

Evaluation of methodological quality

To assess quality, the Newcastle-Ottawa scale tool [21] was used, which consists of eight items collected in three groups that were evaluated: selection of study groups, comparability of groups and determination of exposure or outcome of interest, which assesses the quality of cohort, cross-sectional or case-control studies. The Newcastle-Ottawa scale [22] is rated by several stars according to the items evaluated; an article of high quality was considered one that has between 7 and 9 stars, moderate between 4 and 6 stars and poor between 1 and 3 stars.

RESULTS

In a first search, 599 articles were obtained, of which, after eliminating duplicates and filtering by

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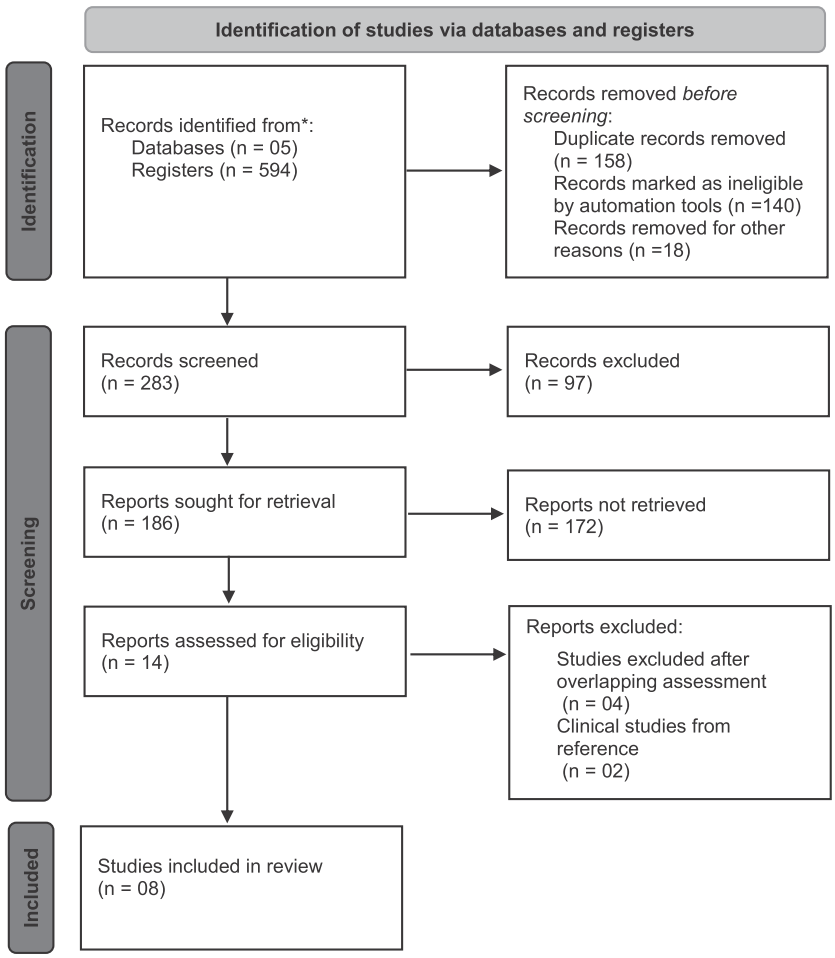


Fig. 1. PRISMA 2020 flow diagram for new systematic reviews which included searches of databases and registers only. *Pubmed, Scielo, Scopus, Web of Science and BVS.

inclusion criteria and relevance of the study, 283 articles remained. Later, after reading the abstract, 172 articles were eliminated, leaving 14 documents to evaluate the methodological quality. In the end, eight documents were included after removing the low-quality documents (Fig. 1).

In the selected studies, the US [23–27] was found as the country with the highest production in this context (5), followed by China (1) [28], Taiwan (1) [29], and Japan (1) [30].

Regarding the design of the studies carried out, both cross-sectional [23, 26, 28, 30] and longitudinal studies have been used alike, with 50% of the selected studies being of a cross-sectional type and the remaining 50% of a longitudinal type [24, 25, 27, 29].

Concerning the sampling and sample size, three of them used a stratified sampling [25, 28–30], two used an intentional sampling [24, 26], one with simple random sampling [23], and another that did not specify the type of sampling [27]. The type of sample was also varied, the most common being the selection of participants with dementia, participants with mild cognitive impairment, and healthy participants. In terms of size, these samples range from 95 to 161,490 participants.

The age ranges of the frequent variations were fundamentally from 40 years onwards, although the greatest specific weight was made up of people over 60 years of age, because they are the patients with the highest incidence of mental illnesses and cognitive disorders.

Regarding the gender of the participants, in the studies there was a higher prevalence of women compared to men, although in some studies, the proportion was quite equal (Table 1).

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Table 1
Characteristics of included studies

Author (y) country	Aim	Design	Sample size	Description and an analysis of data	Statistics analysis	Main results
Vogt et al. (2017) USA [23]	To perform DNA sequencing of RNA isolated from fecal samples to characterize IM samples in individuals with and without AD. Examine relationship between IM and AD measured by CSF biomarkers.	Case-control	Cases: $n = 25$ Control: $n = 94$	Cases recruited by the Wisconsin Alzheimer's Disease Research Center and controls from the ADRC study and the Wisconsin Registry for the Prevention of Alzheimer's. For the microbiome richness estimates, the abundance-based coverage estimator and Chao1 have been used.	Analysis of fecal samples and CSF analysis to see the relationship of microbial load and greater pathology of AD. Non-parametric models.	The result showed that the IM of the participants with AD has decreased the microbial richness and diversity with a different composition than the control participants. Differentially abundant levels of genera were found to correlate with CSF biomarkers of AD pathology. The analyzes revealed that the gut microbiome of participants with AD has decreased microbial diversity and is different in composition from control individuals.
Haran et al. (2019) USA [24]	To demonstrate that stool samples from elderly people with AD can induce levels of P-gp expression *in vitro*, those samples from elderly people without dementia or with other types of dementia.	Longitudinal	108	Participants recruited from one of the nursing homes located in central Massachusetts. Beta regression model with zero inflammation to predict gender proportion based on clinical covariates.	Collection of stool samples and analysis performing metagenomics and *in vitro* sequencing. Generalized linear mixed models using the *Package glmmTMB R*.	We observed that the microbiome of the elderly with AD shows a lower proportion and prevalence of bacteria. There is a potential link between IM and AD being the modulation of intestinal homeostasis through increases in inflammatory and microbial inflammatory metabolism.
Liu et al. (2019) China [28]	To identify differences in the microbiome between AD patients and normal cognitive healthy controls. To assess the possible association between II and the clinical characteristics of AD.	Prospective and Cross sectional	97	Participants recruited from the Memory Clinic, Department of Neurology at the first affiliated hospital of the Zhejiang University School of Medicine. Multivariate logistic regression models based on the relative abundance of the fecal microbiota in a gradual manner. Analysis of fecal samples.	Analysis of fecal samples. Student's t and Mann-Whitney U.	It is shown that the fecal microbiome composition of patients with AD was altered, characterized by depleted SCFAs-producing Firmicutes, and enriched the inflammation of Proteobacteria that correlate with the severity of AD.

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Table 1
(Continued)

Author (y) country	Aim	Design	Sample size	Description and an analysis of data	Statistics analysis	Main results
Vogt et al. (2018) USA [25]	To identify the influence of TMANO derived from IM in AD.	Case-control.	414	Participants recruited from the Wisconsin Alzheimer's Research Center and the Wisconsin Registry for the Prevention of Alzheimer's study who had undergone lumbar puncture with CSF collection, as well as TMANO and biomarker quantification. Multiple linear regression model in R to determine the differences of TMANO in CSF in groups.	Examinations of levels about TMANO in CSF. Non-normal distribution.	It is demonstrated that the intestinal metabolite derived from the microbiota TMANO is elevated in the CSF of individuals with dementia due to MCI and AD, in additional, the levels of TMANO in CSF are associated with CSF biomarkers of AD pathology and degeneration. neuronal. In addition, they report the role of IM in AD.
Mahmoudian Dehkordi et al. (2019) USA [26]	To check that altered BA is associated with cognitive impairment in AD.	longitudinal cohort	1,.464	Participants enrolled in the Alzheimer's Disease Neuroimaging Initiative. Separate binary logistic regression models to examine the cross-sectional association of each metabolite with the baseline diagnosis.	Associations of BA profiles that include selected proportions with diagnosis, cognition, and AD-related genetic variants adjusted by co-founders and multiple tests are evaluated. Student's t and Pearson's Chi-square.	An association was demonstrated between the altered BA profile, the genetic variants involved in AD and cognitive changes in the disease.
Chen et al. (2016) Taiwan [29]	To assess the risk of dementia after the diagnosis of IBS.	Longitudinal	161,.490	Participants recruited from the Taiwan National Health Insurance Research Database with and without IBS. Univariate and multivariate Cox ratio risk regression models to examine the effect of IBS on dementia risk. Follow-up of cases from the index date to the onset of dementia or December 31, 2011. Student's t and Pearson's Chi-square.	Follow-up of cases from the start date to the onset of dementia or December 31, 2011. Student's t and Pearson's Chi-square.	It is observed that IBS is associated with an increased risk of dementia, and this effect is obvious only in patients older than or equal to 50 years. Pearson.
Araos et al. (2018) USA [27]	To evaluate the diversity and composition of the IM colonized with Clostridium difficile, in people with advanced dementia.	Longitudinal Retrospective analysis and prospective observational study.	362	Participants are older people from 35 Long Term Care Centers in Boston. Linear Discriminant Analysis effect size method.	Analysis of rectal samples and extraction of fecal DNA. Non-parametric models.	Prevention of colonization by *Clostridium difficile* would improve AD patients.
Saji et al. (2019) Japan [30]	To investigate the relationship between the composition of IM and dementia in Japanese patients, using a comprehensive assessment of cognitive function.	Case-control.	128	Patients who visited the memory clinic at the National Center for Geriatrics and Gerontology. Multivariate logistic regression models to identify variables independently associated with dementia.	Fecal samples were collected, the gut microbiome was analyzed and stratified into three entertypes. Student's t, Wilcoxon's rank sum and Pearson's $\chi 2$.	Components of the gut microbiome, particularly Bacteroides and other bacteria, have been shown to be independently associated with dementia and these associations are stronger than those of traditional dementia biomarkers.

AD, Alzheimer's disease; BA, bile acids; CSF, cerebrospinal fluid; IBS, irritable bowel syndrome; IM, intestinal microbiota; MCI, mild cognitive impairment; P-gp, P-glycoprotein; SCFAs, short chain fatty acids; TMANO, Trimethylamine N-oxide.

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The samples of the eight studies selected had been obtained from various fields such as research centers (25%), clinics (25%), and databases (25%), but it should be noted that the significantly larger samples were collected from the databases.

According to the results of the different studies carried out, it can be deduced that there is a relationship between the variable "deficient and not very diverse microbiota in bacteria" and the "person with Alzheimer's disease". On the contrary, there is a relationship between the "microbiota rich and very diverse in bacteria" and the "mentally healthy person". This relationship is statistically significant, with $p < 0.05$, and clinically relevant in light of the results obtained [23–26, 28–30].

Regarding the evaluation of methodological quality, the eight documents have a good methodological implementation according to the Newcastle-Ottawa scale [21, 31], the highest percentage of quality (62.5%) is within an optimal range; therefore, they can infer a good methodology in studies.

On the other hand, some of the selected studies may have some type of bias, due to factors such as lifestyle and the diet, and their small sample size.

DISCUSSION

All the studies show that dementia and other cognitive disorders are associated with a deficient intestinal microbiota, without prejudice to the fact that, as expressly indicated in some of the studies, it would be convenient to carry out other complementary investigations that confirm and enrich the conclusions reached in these studies.

Thus, Vogt (US), in his study "Alterations of the intestinal microbiome in Alzheimer's disease" [23], shows that the intestinal microbiome of participants with AD presents a decrease in microbial diversity, in addition to being different in its composition from that of the individuals in the control group. Likewise, he added that intestinal bacterial communities may be a therapeutic target for AD patients. The latter is very relevant, since he talks about the possibility of treating and curing diseases through the intestinal microbiota.

Chen [29] mentioned that irritable bowel syndrome is associated with an increased risk of dementia. Furthermore, he noted that this effect is obvious only in patients ≥ 50 years of age. This indicates that having a good microbiota is especially important in the elderly.

On this point, he agrees with Saji [30], who shows that components of the gut microbiome, particularly Bacteroides and "other" bacteria, are independently associated with dementia. These associations are stronger than those of traditional dementia biomarkers. However, more studies are needed to reinforce and complement this line of work.

In this sense, knowledge about the bi-directional biochemical communication between brain and intestine and the influence of a microbiota which is bacteriologically poor and not very varied on AD is still scarce in the scientific literature, especially regarding the complete process of AD.

This lack of knowledge about mental disorders is especially serious if we consider that, worldwide, more than forty-seven million people suffered from some type of dementia in 2015, and it is estimated that this number will have tripled by the year 2050.

The concept of the microbiota-gut-brain axis originated from studies carried out in mice. This axis is a bidirectional communication system of which, due to the small number of studies carried out in humans so far, there is still little and limited knowledge of its operation and implications in brain functions. However, research supports the hypothesis that the microbiota may be the cause of some of the most serious neurodegenerative and psychiatric disorders, including AD.

The latest human research has demonstrated a novel association between the gut microbiota and dementia, suggesting that the gut microbiota can modulate host brain function through the microbiota-gut-brain axis. Thus, this review shows how the gut microbiota of people suffering from AD differs from that of subjects without dementia.

In this way, it has been possible to verify that alterations in the intestinal microbiota are associated with a wide spectrum of degenerative and psychiatric neurological pathologies that range from autism, multiple sclerosis, and Parkinson's disease, to the most widespread dementias, such as AD [24, 25, 27, 29].

Although it is still not entirely clear how the gut influences the development of neuropathologies, substantial research evidence supports the existence of a gut-brain axis that allows two-way communication between the gut and the brain through various pathways, including immune, endocrine, and neuronal mechanisms [32].

However, studies have shown that alterations in the intestinal microbial communities in AD patients can produce pathophysiological changes in the brain [23, 26, 28].

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As mentioned by Vogt et al. [25, 33], Mahmoudian-Dehkordi et al. [26], and Chen et al. [29], these findings provide more information on gut microbial involvement in AD and help to understand the gut-brain axis. However, more research, including experimental work, is needed to determine the precision of the causal relationship between gut microbiota and pathogenesis of AD.

On the one hand, an interesting fact that the studies reveal is that the incidence of dementia increases with age, is higher among men than among women, and usually coincides with a higher incidence of comorbidity. In this sense, people over fifty years of age showed a greater risk of suffering from dementia, a risk that progressively increased with age [24, 25, 27, 29].

On the other hand, it should be noted that some of the studies analyzed in this work could have some type of bias, mainly due to the size of the sample and because diet, lifestyle, exposure, and other factors may contribute to the differences in the microbiota of the groups.

However, in late 2021, from a double-blind multicenter randomized clinical trial, Kim et al. [34] found that the relative abundance of inflammatory-causing gut bacteria was significantly reduced at week 12 in a probiotic group ($p < 0.05$). Furthermore, the probiotic group showed greater improvement in the mental flexibility test and stress score than the placebo group ($p < 0.05$). Unlike placebo, probiotics significantly increased the serum level of brain-derived neurotrophic factor (BDNF) ($p < 0.05$). Notably, gut microbes displaced by probiotics *(Eubacterium and Clostridiales)* showed a significant negative correlation with serum BDNF level only in the probiotic group (RS = –0.37, RS = –0.39, $p < 0.05$).

With this, they showed the benefits of supplementation with probiotics, since they promote mental flexibility and relieve stress in healthy older adults, in addition to causing changes in the intestinal microbiota.

Furthermore, these results can be supported by the findings of Tran et al. [35], in which the influence on the structure and function of the intestinal microbiome in humans and mice was explored, where there was evidence of a link between the genotypes of apolipoprotein E (*APOE*) and the composition of the intestinal microbiome. In this study, they point out that the loss of butyrate- and SCFA-producing bacteria in *APOE4* carriers could drive the impact of the *APOE4* allele on neuropathology. This suggests a possible role of the buryate-producing bacteria of the intestinal microbiota as a point of intervention to mitigate the impact of the *APOE* genotype in the development of AD.

Nevertheless, additional studies with larger samples are needed to corroborate and expand what has been discovered so far. But above all, more longitudinal studies are required that cover the presymptomatic stages of the disease. Also, studies are needed to establish the influence of changes, composition, and activity of the intestinal microbiota in patients with AD, so that it can be studied in different periods, in addition to carrying out follow-up investigations in the patient to define the cause-effect relationship begun by Vogt et al. [25, 33], MahmoudianDehkordi et al. [26], and Chen et al. [29].

Study limitations

The fact that the intestinal microbiota is unique, dynamic, changing, and easily adjustable by external factors, such as diet and lifestyle, makes studying it difficult. However, evidence is being generated that highlights the effect of inflammation on intestinal homeostasis. A route of action for research is outlined in the search for strategies that preserve the proportion and prevalence of gut microbiota by modulating inflammation.

Furthermore, despite these findings, the information available comes primarily from animal studies. Therefore, many clinical trials in humans are necessary, providing us with better and more precise knowledge of the intestinal microbiota before making the appropriate recommendations for clinical practice.

Conclusion

There is a high association between the decrease in the richness of the microbiota and the incidence of AD. According to the evidence found, the existence of a gut-brain axis and its relationship with the gut microbiota in dementia is highly probable. In addition, the composition of gut microbiota, such as *Bacteroides* or bacteria such as *Clostridium difficile*, is a factor that is specifically associated with dementia and AD.

The ability to modulate the intestinal microbiota reveals a relevant field to conduct research and develop new strategies to prevent cognitive and other disorders.

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DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/21-5224r4).

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Section 3

Role of Gut Microbiome and Microbiota in Cognitive Deterioration and Alzheimer's Disease

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Vascular Cognitive Impairment and the Gut Microbiota

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Abstract. Vascular cognitive impairment (VCI), the second most common cause of dementia in elderly people, is a term that refers to all forms of cognitive disorders that can be attributed to cerebrovascular disease such as manifestations of discrete infarctions, brain hemorrhages, and white matter lesions. The gut microbiota (GM) has emerged recently as an essential player in the development of VCI. The GM may affect the brain's physiological, behavioral, and cognitive functions through the brain-gut axis via neural, immune, endocrine, and metabolic pathways. Therefore, microbiota dysbiosis may mediate or affect atherosclerosis, cerebrovascular disease, and endothelial dysfunction, which are the predominant risk factors for VCI. Moreover, the composition of the GM includes the bacterial component lipopolysaccharides and their metabolic products including trimethylamine-*N*-oxide and short-chain fatty acids. These products may increase the permeability of the intestinal epithelium, leading to systemic immune responses, low-grade inflammation, and altered signaling pathways that are associated with the pathogenesis of VCI. In this review, we discuss the proposed mechanisms of the GM in the maintenance of VCI and how it is implicated in acquired metabolic diseases, particularly in VCI regulation.

Keywords: Atherosclerosis, cerebrovascular disease, endothelial dysfunction, gut microbiota, lipopolysaccharides, short-chain fatty acids, trimethylamine-*N*-oxide, vascular cognitive impairment

INTRODUCTION

Vascular cognitive impairment (VCI) is a term that refers to all forms of cognitive disorders that can be attributed to cerebrovascular disease, encompassing dementia and milder forms of impairment [1, 2], and it is the second most common cause of dementia in elderly people [3]. The prevalence of VCI in those aged 65 or older is approximately 5% and doubles every 5.3 years [3, 4]. There is substantial evidence suggesting that conventional risk factors such as atherosclerosis [5], cerebrovascular disease (CVD) [2], endothelial dysfunction [6], toll-like receptors (TLRs) [7], and insulin resistance (IR) [8] play important roles in the development of VCI. In addition, inflammation is thought to be a factor involved in

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the pathogenesis of VCI [8, 9]. Furthermore, growing evidence suggests that the human gut microbiota (GM) plays a potential role in VCI.

The GM can be defined as all the species within the ecosystem and is considered the largest microbial host in the human body, containing approximately 10^{14} microbes. Over 99% of the microbiota in the gastrointestinal (GI) tract is anaerobic bacteria dominating the environment along with species including viruses, protozoa, archaea, and fungi [10]. The GM plays many pivotal roles in maintaining dynamic metabolic ecological balance, conferring many health benefits on the host organism including pathogen displacement, barrier fortification, immune system development, vitamin production, and nutrient absorption, and it is often referred to as the forgotten organ [11].

Recent studies report that the brain and the GI tract are engaged in constant bidirectional communication through the brain-gut axis via neural, immune, endocrine, and metabolic pathways [12, 13]. First, the GM can affect actions in the peripheral and central nervous system by various means of communication including vagal nerve and adrenergic nerve activation, as well as the production of many neurotransmitters and neuromodulators or by evoking the synthesis and release of neuropeptides from enteroendocrine cells. Second, the GM can regulate the hypothalamic-pituitary-adrenal (HPA) axis activation state [13]. The release of cortisol as a result of HPA axis activation can govern the activation state of brain microglia and effect cytokine release, as well as attract monocytes from the periphery to the brain [14]. Finally, the GM can also influence the immune system by changing the levels of pro-inflammatory cytokines and anti-inflammatory cytokines in the blood to affect the central nervous system and alter brain function [10]. Consequently, dysfunction and alterations of the GM composition contribute to neural disorders such as Alzheimer's disease [15], multiple sclerosis [16], and Parkinson's disease [17]. There are also many studies suggesting that the GM is closely related to VCI. In this review, we summarize the evidence of GM involvement in VCI and the developmental mechanisms of VCI.

ATHEROSCLEROSIS

As atherosclerosis is the predominant risk factor for VCI, atherosclerotic changes may play a potential role in the onset of VCI [5, 18]. Atherosclerosis is a chronic and progressive process, with the bidirectional interaction between inflammation and lipids as its pathogenic hallmark [19]. Clinically, atherosclerosis has a long asymptomatic phase, and a majority of patients will develop symptoms of chronic ischemia in affected organs over years or acute ischemic events such as myocardial infarction or ischemic stroke during plaque destabilization and thrombus formation [20]. Recent studies have found that the GM is closely associated with the development of atherosclerosis [21–23]. This result is supported by the finding of bacterial DNA, such as *Staphylococcus*, *Veillonella*, and *streptococcus* in atherosclerotic plaques [24, 25]. This observation suggests the possibility that the microbial communities in plaques may be a source of bacteria that impact plaque stability and the development of atherosclerosis. Karlsson et al. found that the genus *Collinsella* was enriched in 12 patients with symptomatic atherosclerosis, which was defined as stenotic atherosclerotic plaques in the carotid artery leading to cerebrovascular events, while *Roseburia* and *Eubacterium* were enriched in 13 healthy controls, as measured by the shotgun sequencing of the gutmetagenome [26]. In addition, the number of *Lactobacillales* and the ratio of *Firmicutes* to *Bacteroidetes* increased in patients, while the number of *Bacteroidetes* increased in the control group [27]. Consequently, alterations in the composition of the GM might modulate the atherosclerosis process. There are three main pathways by which microbiota might affect atherogenesis [23]. First, gut dysbiosis might cause a harmful inflammatory response, especially involving lipopolysaccharides (LPS), that aggravates plaque development or triggers plaque rupture [23, 28]. LPS, also called endotoxins, are an important component of the outer membrane of gram-negative bacteria [29]. LPS and their receptor cluster of differentiation 14 (CD14) form a complex that is identified by the immune cell surface toll-like receptor 4 (TLR4) and activates several signal transduction responses that lead to a series of nonspecific inflammatory responses [30, 31]. Second, the metabolism of cholesterol and lipids by the GM may affect the development of atherosclerotic plaques through bile acids and the bile acid receptor [23, 32]. It has been suggested that bacterial bile salt hydrolase (BSH) regulates host lipid and cholesterol metabolisms [33, 34]. The expression of cloned BSH enzymes in the GI tract of gnotobiotic or conventionally raised mice significantly altered plasma bile acid signatures and regulated the transcription of key

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genes involved in cholesterol metabolism (Abcg5/8), lipid metabolism (Pparγ, Angptl4), gastrointestinal homeostasis (RegIIIγ), and circadian rhythm (Dbp, Per1/2) in the liver or small intestine [33]. Third, the metabolic potential of the GM has been identified as a contributing factor in atherosclerosis development [23]. In particular, trimethylamine-*N*-oxide (TMAO), which is carried by trimethylamine (TMA) via the portal circulation to the liver where it is converted by a family of enzymes called host hepatic flavin monooxygenases (FMOs) [35], mainly enhances platelet hyperreactivity and thrombosis risk to regulate atherosclerosis [36]. Short-chain fatty acids (SCFAs), which are additional important metabolites generated by the GM, stimulate host G-protein–coupled receptor (GPR) pathways that impact renin secretion and blood pressure regulation to accelerate atherosclerosis [37]. Therefore, the GM may contribute to atherosclerosis.

CEREBROVASCULAR DISEASE

CVD causes vascular brain injuries that can lead to VCI [2, 38]. The GM plays an emerging role in the course of CVD. The gut microbiome of CVD patients is clearly different. Stroke and transient ischemic attack patients show more opportunistic pathogens, such as *Enterobacter*, *Megasphaera*, *Oscillibacter*, and *Desulfovibrio*, and fewer commensal or beneficial genera including *Bacteroides*, *Prevotella*, and *Faecalibacterium* [39]. Increased bacterial counts of the *Atopobium* cluster and *Lactobacillus ruminis* and decreased numbers of the *Lactobacillus sakei* subgroup were reported in patients with ischemic stroke [40]. Furthermore, strong evidence proves that the microbiome drives cerebral cavernous malformations [41], common vascular malformations that can cause hemorrhagic strokes. Moreover, the outcomes of CVD are affected by the GM. C. butyricum significantly attenuated cognitive dysfunctions and histopathological changes in vascular dementia mice [42] and attenuated cerebral ischemia/reperfusion injuries via modulation of the GM [43]. Antibiotic-induced alterations in the GM reduce ischemic brain injuries [44], and the depletion of cultivatable GM by broad-spectrum antibiotic pretreatment significantly decreased survival after murine stroke [45]. The lack of segmented filamentous bacteria led to significantly larger brain infarcts [46]. The therapeutic transplantation of fecal microbiota normalizes brain lesion-induced dysbiosis and improves stroke outcomes [47]. Inflammation and immunity have been increasingly recognized as important contributors of the GM to CVD. There is a bidirectional communication between the gut and the brain via the HPA axis to activate microglia and affect cytokine release [13]. This communication may work through activation of NF-κB and increase intestinal permeability to promote the displacement of the GM to extraintestinal organs, the blood stream, or lymphatic fluid, thus engaging local and systemic immunity [48]. GM dysbiosis induces the polarization of pro-inflammatory T-cells in the intestinal immune compartment and in the ischemic brain and induces the migration of intestinal lymphocytes to the ischemic brain [46, 47], where they localize in the leptomeninges and enhance ischemic neuroinflammation by secreting IL-17. This secretion leads to increased chemokine production in the brain parenchyma and the subsequent infiltration of cytotoxic immune cells including neutrophils [43]. Altered GM following amoxicillin/clavulanate treatment primes intestinal dendritic cells, leading to local regulatory T-cell expansion in the small intestine and effector IL-17+$\gamma\delta$ T-cell suppression [43]. Gut dysbiosis increases the concentration of valeric acid, which is correlated with the level of high sensitivity C-reactive protein and with white blood cell counts in ischemic stroke patients [40]. Activation of TLR4 by gram-negative bacteria or lipopolysaccharides accelerates cerebral cavernous malformation formation [41]. In addition to inflammation, the GM also affects neuronal apoptosis in cerebral ischemia/reperfusion injuries. TMAO levels accelerate atherosclerosis [49] and are associated with an increased risk of major adverse cardiovascular events [50]; however, stroke and transient ischemic attack patients show significantly decreased blood TMAO levels [39]. Therefore, the role of TMAO in CVD should be clarified with more studies. In conclusion, it is possible that the GM facilitates the development of CVD.

ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is a pivotal early event in the pathogenesis of atherosclerosis, contributing to the initiation and progression of plaques, which are an important risk factor for VCI in aging patients [6, 51]. It has been reported that the metabolites of the GM are closely associated with endothelial dysfunction [52, 53]. Ma et al. found that human umbilical vein endothelial cells showed obvious impairments in cellular proliferation after TMAO treatment com-

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pared with controls. This result showed that TMAO may impair the endothelial cell self-repair capacity by activating protein kinase C/NF-κB and leading to the elevated expression of vascular cell adhesion molecule-1 and monocyte adhesion [54]. Li et al. reported that circulating levels of TMAO contribute to aging-associated endothelial dysfunction, which may impair endothelial nitric-oxide synthase (eNOS)-derived nitric oxide (NO) bioavailability by increasing vascular inflammation and oxidative stress [52]. NO, produced in endothelial cells from L-arginine by the enzyme eNOS, is one of the most important vasodilating substances [55]. TMAO may inhibit eNOS expression and activity and reduce NO production, leading to endothelial dysfunction and atherosclerosis [56]. Saisudha et al. found that TMAO may induce nucleotide-binding oligomerization domain–like receptor family pyrin-domain containing 3 (NLRP3) inflammasome formation and activation and thus contribute to endothelial injury [57]. In addition to TMAO, LPS also orchestrate inflammatory responses contributing to the development of endothelial dysfunction associated with atherosclerosis in obesity, metabolic syndrome, and diabetes. Studies have shown that LPS treatment induced endothelial alterations via activation of NF-κB signaling [53]. In summary, these studies highlight the metabolites of the GM in the development of endothelial dysfunctions.

TOLL-LIKE RECEPTORS

Evidence is accumulating that TLRs, and particularly TLR4, are important players in the development of atherosclerotic disease[58] and CVD [59]; in other words, TLRs may also play potential roles in the onset of VCI [7]. TLR2-mediated cerebrovascular dysfunction leads to decreased cerebral blood flow and cognitive impairment [60]. TLRs are pattern recognition receptors, which can recognize a variety of broadly conserved microbial components and allow the innate immune system to sense a wide variety of bacteria, viruses, fungi, and parasites to produce cytokine responses [61]. The GM is a source of TLR ligands, and their compositional changes may play a key role in the communication between the GM and the host [13, 62]. High-fat diets increase both plasma and fecal endotoxin levels and lead to dysregulation of the GM by increasing the *Firmicutes* to *Bacteriodetes* ratio; these changes lead to the induction of TLR4 and increased expression of proinflammatory cytokines [63]. Probiotics have been shown to protect against the underlying mechanisms of VCI [42] by reducing the inflammatory response and improving the mucosal barrier function that may be related to reduced levels of TLR2 and TLR4 [64, 65]. It has been found that TLR5 can relocalize to a predominantly basolateral localization in response to a *Helicobacter pylori* infection in gastric epithelial cells [66]. *Helicobacter pylori* infection is a critical risk factor for VCI [67, 68]. Moreover, the effect of TLR stimulation is additive to the effects of butyrate, and butyrate also increases the expression of TLRs including TLR4, and the NF-κB response to TLR stimulation [69]. There are two major signaling pathways that have been shown to lead to inflammation. The first pathway, which is the principal one, is activated by most TLRs and recruits myeloid differentiation factor 88 (MyD88), which activates the transcription factor NF-κB and the mitogen-activated protein (MAP) kinases p38 and c-Jun N-terminal kinase (JNK) to increase the expression of many proinflammatory genes such as IL-1β and IL-6. The other pathway is activated only by TLR3 and TLR4 and leads to the activation of both NF-κB and interferon regulatory factor 3 (IRF3), which is a transcription factor that induces an additional set of genes including antiviral genes such as interferon-beta [70, 71]. Hence, TLRs may be considered an interface between the intestinal epithelial barrier, the GM, and the immune system.

INSULIN RESISTANCE

IR contributes to pathological processes in VCI [72, 73]. IR is a complicated condition where primary metabolic tissues such as skeletal muscle, liver, and adipose tissue become less sensitive to insulin and its downstream metabolic actions under normal serum glucose concentrations, which is considered to be an early alteration of diabetes mellitus and obesity [74]. Accumulating animal model and human subject research indicates that the GM contributes to the development of IR and that intestinal flora and metabolites may pass directly through a compromised GI tract and/or pass indirectly through these protective physiological barriers to trigger cytokines or other small proinflammatory molecules [75, 76]. Diet-induced obesity promotes insulin resistance by mechanisms independent and dependent on the GM [77]. Studies show that a high-fat diet profoundly reduces the GM dominant phyla *Bacteroidetes* and increases the *Firmicutes* to *Bacteroidetes* ratio that

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has been associated with obesity [78]. Similarly, obese individuals have a differential proportion of dominant phyla in their GM, that is, fewer *Bacteroidetes* and more *Firmicutes*, compared with lean controls [79]. Turnbaugh et al. showed that the obesity trait could be transmitted from obese mice to non-obese mice by fecal transplants, and non-obese mice showed the same signs of obesity two weeks after transplants [80]. This study suggests that alterations of the GM may induce obesity. Moreover, it has been reported that the fecal concentration of SCFAs was higher in obese individuals compared with normal individuals [81]. SCFAs, a major production from the GM, accounting for up to 15% of caloric intake, thereby contributing to the development of obesity [82]. SCFAs can bind and activate free fatty acid receptor (FFAR) 2 and FFAR3 to lead to downstream signaling [81]. In addition, Bjursell et al. found that FFAR2-deficient mice did not develop obesity [83]. It has also been observed that a high-fat diet chronically increases plasma LPS levels 2-to 3-fold, which could lead to low-grade chronic inflammation, IR, and diabetes mellitus [75, 84]. The circulation of LPS initiates the activation of TLR4 and TLR2 and LPS receptor CD14, leading to increased activation of inflammatory pathways and impaired insulin signaling [85]. Therefore, the GM and its metabolites contribute to the development of IR (Table 1).

LIPOPOLYSACCHARIDES

Studies have proven that LPS impair cognition [72, 86–88]. LPS, also called endotoxins, consisting of lipid A, a core oligosaccharide and an O-antigen/O-polysaccharide, are an important component of the outer membrane of gram-negative bacteria [29]. When LPS derived from the GM enter systemic circulation [89, 90], they may lead to a deleterious pro-inflammatory systemic response [91]. Increased gut permeability and changes in the composition and diversity of the gut microbiome may be possible mechanisms that lead to endotoxemia [92]. Cani et al. found that a 4-week high-fat diet chronically increased plasma LPS concentrations two to three-fold in mice, whereas in mice who were given continuous subcutaneous infusions of LPS, adipose tissue weight gain and insulin resistance were increased to a similar extent as the high-fat diet mice [93]. LPS levels vary from two-fold increases

Table 1
GM and risk factors

Risk Factor	Animal models	Results
Atherosclerotic	Has not been reported	Has not been reported
CVD	Mice model of VaD induced by a permanent rUCCAO	Attenuated cognitive dysfunctions and histopathological changes [42]
	Diabetic mice subjected to 30 min of BCCAO	Attenuated cerebral ischemia/reperfusion injuries [43]
	Wild-type C57BL/6 mice for MCAO	Antibiotic-induced alterations in the GM reduce ischemic brain injuries [44]
	C57BL/6J mice for MCAO or sham operation	Decreased survival after murine stroke [45]
	C57BL/6 mice for MCAO	The lack of segmented filamentous bacteria led to significantly larger brain infarcts [46]
	Wild-type C57BL/6J mice for acute MCAO	Normalizes brain lesion-induced dysbiosis and improve stroke outcome [47]
Endothelial dysfunction	C57BL/6 mice	HUVECs showed obvious impairments in cellular proliferation after TMAO treatment compared with controls [54]
	Fisher-344 rats treated with 3,3-Dimethyl-1-butanol	Circulating levels of TMAO contribute to aging-associated endothelial dysfunction [52]
	C57BL/6mice with partially ligated left carotid artery	Induce NLRP3 inflammasome formation and activation [57]
Toll-like receptors	TLR2-knockout mouse induced diabetes by STZ	Decreased cerebral blood flow and cognitive impairment [60]
	C57BL/6J and TLR4-deficient C57BL/10ScNJ mice were maintained on a LFD or a HFD	Increased expression of proinflammatory cytokines [63]
Insulin resistance	Wistar and Sprague-Dawley rats for standard and HF diet	Reduces the GM dominant phyla *Bacteroidetes* and increases the *Firmicutes* to *Bacteroidetes* ratio [78]
	C57BL/6J mice	Alterations of the GM may induce obesity [80]

rUCCAO, right unilateral common carotid arteries occlusion; BCCAO, bilateral common carotid arteries occlusion; MCAO, middle cerebral artery occlusion; HUVECs, human umbilical vein endothelial cells.

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in the neocortex to three-fold increases in the hippocampus of Alzheimer's disease patients compared to age-matched controls, even to the extent of a 26-fold increase over age-matched controls [94] in the perinuclear region [95]. LPS damage the brain in many ways. LPS increase the blood–brain barrier permeability by binding to TLR4 in the endothelial cells of blood vessels [96], activate astrocytes [97], detach pericytes from the basal lamina, and alter the function of tight junctions [98, 99]. Increasing evidence indicates that dysfunction in the blood-brain barrier function may play a significant role in the pathogenesis of VCI [100, 101]. Numerous studies demonstrate that LPS stimulate the synthesis of pro-inflammatory cytokines in the brain. Interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF)-α and CC chemokine ligand 2 (CCL2) increase in various brain regions after intravenous or intraperitoneal injections of LPS [102]. These pro-inflammatory cytokines are associated with VCI [103] and are secreted mainly by microglia [102]. Microglia are activated in mouse models of VCI [104, 105]. Microglial activation is induced by LPS [86, 106]. The underlying mechanisms of the effect of LPS on inflammation is that LPS and their receptor CD14 form a complex, which is identified by the immune cell surface TLR4, that activates several signal transduction responses that lead to a series of nonspecific inflammatory responses [30, 31, 91]. This complex mainly activates the MyD88-dependent and the TRIF (TIRdomain-containing adaptor inducing interferon-β)-dependent signaling pathways to promote the expression of pro-inflammatory cytokines such as TNFα and IL-1β, which are critical for the homeostasis of intestinal and systemic immunities [91]. In conclusion, we have reason to speculate that LPS may contribute to the development of VCI.

TRIMETHYLAMINE-N-OXIDE

Recent studies highlight the participation of the GM and its metabolites in the pathogenesis of atherosclerotic diseases [107–109]. TMAO is a bacterial metabolite that may promote the process of atherosclerosis formation through a metaorganismal pathway involving nutrient precursors abundant in phosphatidylcholine and L-carnitine, such as eggs, milk, liver, red meat, poultry, shell fish and fish, and the sequential actions of the GM which initially forms TMA, followed by the host hepatic flavin monooxygenase-dependent conversion into TMAO [110, 111]. In addition, it has been found that some bacteria are closely related to the level of TMAO in the host [111, 112]. An increase in the proportion of *Firmicutes* may be related to an increase of TMAO in the host, while *Methanobacterium* may reduce TMAO levels. A critical role for dietary choline and the GM in TMAO production, augmented macrophage cholesterol accumulation and foam cell formation has been confirmed through germ-free mice [113]. Elko et al. reported that a decrease of carotid intima-media thickness may be related to a decrease in TMAO levels [114]. A longitudinal follow-up (3-year) study reported that increased TMAO levels are associated with an increased risk of major adverse cardiovascular events, such as myocardial infarction and stroke [50]. In addition, a 5-year follow up study demonstrated that circulating TMAO was a strong prognostic marker for adverse cardiac events [115]. Moreover, elevated TMAO concentrations are positively correlated with long-term mortality risk in patients with heart failure [116, 117], diabetes mellitus, and chronic kidney disease [118, 119]. In contrast, Meyer et al. reported that TMAO may not significantly contribute to advancing early atherosclerotic disease risk among healthy early-middle-aged adults (33 to 55 years) [120]. Although these findings are not consistent, the identification of TMAO as an independent risk factor for promoting atherosclerosis is recognized, although this may be influenced by the age and severity of illness. As atherosclerosis, diabetes mellitus, stroke and others are risk factors for VCI, we could thus assume that TMAO may contribute to the development of VCI.

The mechanisms through which the TMAO pathway may affect atherosclerotic diseases are quite extensive. First, TMAO may enhance platelet hyper-reactivity and thrombosis risk to accelerate atherosclerosis [36, 121]. Physiologically, platelets maintain low levels of intracellular cytosolic calcium (Ca^{2+}) as they circulate through healthy vessels. Zhu et al. reported that the direct exposure of platelets to increasing levels of TMAO was associated with the release of Ca^{2+} from intracellular stores, which enhanced stimulus-dependent platelet activation by multiple agonists such as adenosine diphosphate (ADP), thrombin, collagen and arachidonic acid. Moreover, TMAO was also found to augment inositol-1,4,5-trisphosphate (IP3) signaling pathways during platelet activation [36]. IP3, a second messenger signaling molecule produced by various phospholipase C isoforms, triggers the

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release of intracellular Ca2 + to enhance platelet hyper-reactivity [122]. Second, TMAO can promote macrophage cholesterol accumulation in a microbiota-dependent manner by increasing the cell surface expression of two proatherogenic scavenger receptors, CD36 and scavenger receptor A (SRA) [49, 123]. Moreover, it has reported that FMO3 is a powerful modifier of cholesterol metabolism and reverse cholesterol transport (RCT). A knockdown of FMO3 in cholesterol-fed mice alters biliary lipid secretion, attenuates intestinal cholesterol absorption, limits the production of hepatic oxysterols and cholesteryl esters and stimulates macrophage RCT [124]. Third, TMAO may promote vascular inflammation by activating the NLRP3 inflammasome [125–127]. The NLRP3 inflammasome is an IL-1β family cytokine-activating protein complex that responds to exogenous and endogenous danger signals involved in the development of atherosclerosis [128]. The NLRP3 inflammasome activates reactive oxygen species (ROS), particularly mitochondrial ROS, which leads to the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 and ultimately triggers vascular inflammation [126, 127]. Fourth, circulating levels of TMAO increase with age, which may impair eNOS-derived NO bioavailability by increasing vascular inflammation and oxidative stress, resulting in age-associated endothelial dysfunction and promoting atherosclerosis [52]. In conclusion, it is possible that TMAO generated from the GM promotes accelerated atherosclerosis through a variety of mechanisms that may contribute to the development of VCI.

SHORT-CHAIN FATTY ACIDS

SCFAs, a subset of key gut microbial metabolites, play an important role in host metabolism [129, 130] and inflammatory responses [131, 132], which are suggested to be associated with VCI [42, 133]. SCFAs, especially acetate, propionate, and butyrate, are produced by the GM from indigestible polysaccharides such as dietary fibers [82]. The *Bacteroidetes* phylum mainly produces acetate and propionate, whereas the *Firmicutes* phylum has butyrate as its primary metabolic end product [134]. Hiroshi et al. found that butyrate is derived from *Clostridia* [135]. Moreover, the generation of SCFAs by GM fermentation involves the metabolic actions of methanogenic *Archaea*. Samuel et al. reported that *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron* greatly increased the efficiency of bacterial fermentation and the production of SCFAs in GF mice [136]. Fecal dysbiosis reduced SCFA concentration [137]. Recent studies have shown that SCFAs play a vital role in the pathogenesis of hypertension [138, 139]. Holmes et al. found a significant correlation between SCFA levels and blood pressure (BP) in Asian study participants compared with European study participants [140]. The signaling actions of SCFAs are mediated by the G protein-coupled receptors FFAR2 (GPR43) and FFAR3 (GPR41), and olfactory receptor 78 (Olfr78), which are expressed in the GI tract, adipose tissues, immune cells, peripheral nerves and olfactory sensory neurons [139]. Pluznick et al. reported that Olfr78-deficient mice presented with lower renin concentrations and decreased BP, whereas GPR41-deficient mice did not display a hypotensive response [141]. Therefore, SCFAs may regulate BP via Olfr78 and GPR41. Alternatively, GPR43 may be involved in appetite regulation by increasing the secretion of peptide YY (PYY) and glucagon-like peptide (GLP)-1, which regulate digestive enzymes and satiety [142]. Bjursell et al. reported that FFAR2-deficient (Gpr43-/-) mice did not develop obesity and had greater insulin sensitivity and lower hepatic triglycerides and plasma cholesterol levels despite being fed a high-fat diet [83]. Similarly, Samuel et al. also reported that mice deficient in FFAR3 (Gpr41-/-) did not develop increased adiposity [143]. These results suggest that SCFAs may modulate host metabolism. Furthermore, SCFAs also serve as important modulators of immunity via activation of GPR41 and GPR43, which could recruit leukocytes and produce chemokines [144]. In addition, some researchers have demonstrated that butyrate can downregulate NF-κB signaling triggered by LPS or cytokines in different cells [145, 146]. And it can induce colonic regulatory T (Treg) cells and play a central role in the suppression of inflammatory and allergic response [135]. Although a detailed analysis of the microbiota in patients with VCI is lacking, given the alterations in the GM related to SCFAs in hypertension, obesity, and diabetes mellitus, we have reason to speculate that SCFAs may provide another mechanism for the development of VCI (Fig. 1).

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we show that the GM, the GM composition, metabolites of the GM, and the translocation

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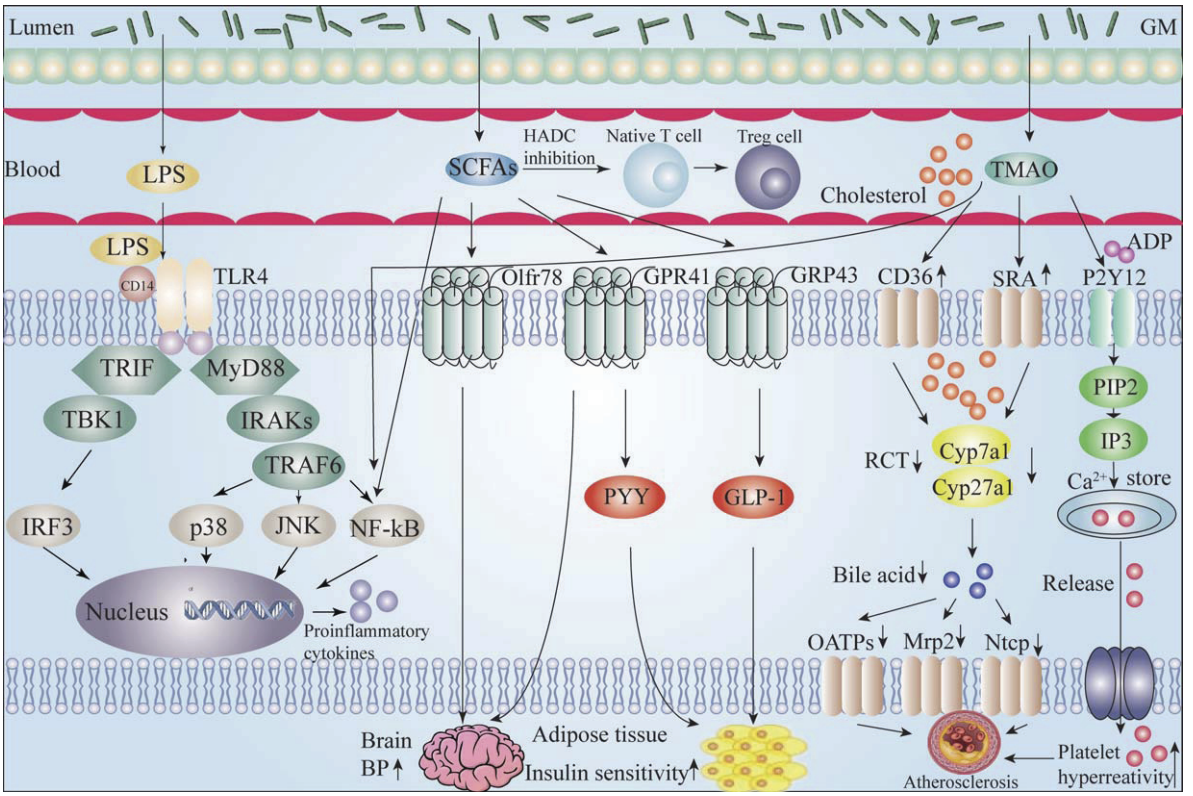


Fig. 1. The composition of the gut microbiota (GM) includes lipopolysaccharides (LPS) and their metabolic products including trimethylamine-*N*-oxide (TMAO) and short-chain fatty acids (SCFAs) may contribute to the development of vascular cognitive impairment. The broken of tight junction function leads to LPS translocation. LPS binds and activates toll-like receptor 4 (TLR4), which recruits downstream adaptor molecules such as myeloid differentiation factor 88 (MyD88) dependent and the TRIF-dependent signaling pathways to promote the expression of pro-inflammatory cytokines. TMAO may enhance platelet hyperreactivity and thrombosis risk by triggering the release of intracellular Ca^{2+} and inositol-1,4,5-trisphosphate (IP3) signaling pathway. TMAO can promote macrophage cholesterol accumulation in a microbiota-dependent manner by increasing the cell surface expression of two proatherogenic scavenger receptors CD36 and scavenger receptor A (SRA) and reduce reverse cholesterol transport (RCT). Moreover, TMAO may promote vascular inflammation by activating the NLRP3 inflammasome. SCFAs may regulate blood pressure via Olfr78 and GPR41. SCFAs can bind and activate GPR41 and GPR43 to lead to increase insulin sensitivity. And SCFAs have anti-inflammatory effects reducing the secretion of proinflammatory cytokines and chemokines and inducing the extrathymic generation of anti-inflammatory Treg cells.

of these gut-expressed signaling molecules to the central nervous system via systemic circulation or other pathways facilitates inflammatory processes that may contribute to the development of VCI. Currently, fecal microbiota transplantation has shown favorable outcomes in intestinal diseases and extra-intestinal diseases associated with microbiota dysbiosis [147, 148]. Fecal microbiota transplantation is an extreme treatment that replaces the infusion of a fecal suspension from a healthy individual into the gastrointestinal tract of the patients, which is now arguably the most effective form of *Clostridium difficile* eradication [149]. Bruce-Keller et al. found that fecal transplantation from high-fat fed mice whose microbiota was depleted with antibiotics induced significant and selective disruptions in exploratory, cognitive, and stereotypical behavior compared with conventionally housed, control-diet mice [150]. In addition, Bonfili et al. found that 3xTg-AD in the early stage of Alzheimer's disease treated with SLAB51 probiotic formulation to affect the composition of gut microbiota and its metabolites so as to counteract cognitive decline and brain damage [151]. It may through reintroducing normal flora via donor feces so that the imbalance can be corrected, the cycle interrupted, and normal bowel function re-established [152]. In addition, it has suggested that GM can activate the vagus nerve and that vagal signals from the gut can induce an antiinflammatory reflex [153]. Moreover, comparative analysis of the GM may enable further novel insights into the complex biology of VCI, which is very important to allow for take preventive mea-

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sures such as early diagnosis, identification of new therapeutic targets and development of novel drugs. Hence, the modulation of the GM or the direct targeting of GM enzymes may be a growing area for pharmaceutical and functional food industries, with the goal of decreasing the widespread growth of VCI.

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The Association of Post-Stroke Cognitive Impairment and Gut Microbiota and its Corresponding Metabolites

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Abstract.
Background: Post-stroke cognitive impairment (PSCI) is an important factor causing disabilities after acute ischemic stroke (AIS). Emerging evidence suggested that gut microbiota play an important role in cognitive impairment.
Objective: This study aimed to explore the association between PSCI and gut microbiota.
Method: 65 patients with newly diagnostic AIS finished the fecal collection on admission and cognitive assessment 3 months later in the clinic. Fecal samples were subjected to 16SrRNA gene sequencing and gas chromatography-mass spectrometry analysis. Additionally, we enrolled new 18 AIS patients, whose treatment was supplemented by probiotics, to assess the potential of microbial treatment in PSCI.
Results: PSCI patients were characterized by the significantly decreased alpha-diversity, disturbed microbial composition, and corresponding metabolites compared with non-PSCI patients. Increased *Fusobacterium* and deficiency of microbial metabolized short-chain fatty acids (SCFAs) were significantly associated with PSCI. A model based on gut microbiota and SCFAs could predict 3 months or longer PSCI early and accurately after stroke onset. While traditional probiotic administration had little effect on PSCI, it could ameliorate patients' mood, including depression and anxiety in the 3 months after stroke.
Conclusion: Our study revealed the association between PSCI and gut microbiota and its corresponding metabolites for the first time, suggesting the potential for applying microbiota and its corresponding metabolites to early clinical diagnosis and treatment of PSCI.

Keywords: Cognitive dysfunction, microbiota, predictors, probiotics, stroke

INTRODUCTION

Stroke carries the second leading cause of death and third leading cause of disability adjustment life years lost all over the world, about 80% of which are ischemic stroke [1, 2]. Post-stroke cognitive impairment (PSCI) is fairly common in acute ischemic stroke (AIS) patients and probably develops into

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dementia [3]. Within a year of stroke, the incidence of PSCI ranges from 7.4% to 41.3% [4]. However, it often takes 3 months or longer post-stroke to diagnose PSCI by the present standard criteria, such as the Montreal Cognitive Assessment scale (MoCA), the Diagnostic and Statistical Manual of Mental Disorders IV, and the Mini-Mental State Examination (MMSE). Besides, only a few drugs used in Alzheimer's disease are effective for PSCI patients, such as cholinesterase inhibitors and memantine. How to distinguish PSCI early and find newly valid treatment for PSCI is gaining a substantial amount of attention [3, 5]. With the development of metagenomics and the establishment of germ-free models, researchers have discovered that many neurological diseases are closely associated with gut microbiota, which possess many more genes than the human genome [6, 7]. The "microbiota-gut-brain" axis plays an important role in the occurrence and development of stroke and its corresponding post-stroke cognitive impairment [8–10]. Rodent studies have confirmed that disturbed gut microbiota could aggravate cognitive impairment, which still needs detailed mechanism studies and human studies to validate [11–15]. Moreover, the effect of microbial intervention on cognitive function, such as probiotics, prebiotics, and synbiotics is mixed [16–20], suggesting there is still lack of persuasive cognitive studies to expound the role of gut microbiota in cognitive impairment.

Thereby, we focused on post-stroke cognitive impairment, excluding cognitive impairment caused by other diseases in this study. We tried to find the association between PSCI and gut microbiota, its corresponding metabolites, and reveal the effect of probiotics on PSCI. Moreover, we applied gut microbiota and corresponding metabolites to the early diagnosis of PSCI.

METHODS

Enrollment of participants in this study

We performed a longitudinal research study including 83 patients between June 2018 and June 2019 in Shanghai Tenth People's Hospital, China. First, we enrolled 65 patients with newly diagnosed AIS, as confirmed by magnetic resonance imaging, who finished the fecal collection on admission and cognitive assessment 3 months later in the clinic. After we found a significant association between PSCI and gut microbiota, we enrolled 18 new AIS patients, whose treatment was supplemented by probiotics to assess the effect of probiotic administration on PSCI. All 83 enrolled patients did not have severe hearing, visual, or reading deficits and cognitive impairment before stroke, that were recorded in medical documents or reflected by patients and families. Patients were excluded due to 1) illiterate, 2) cancer, 3) exposure to antibiotics, probiotics, prebiotics, or synbiotics within 1 month before admission, 4) severe depression or anxiety, 5) cerebral hemorrhage confirmed by imaging, 6) severe intestinal diseases, such as inflammatory bowel disease. The probiotics, took by 4*10^6CFU/d * 90d, were composed of *Bifidobacterium longum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Lactobacillus fermentan*. Demographic and clinical data were gathered from the electronic medical records system. All the patients were provided with informed consents. This study has been approved by the ethical review committee of the Shanghai Tenth People's Hospital and registered in clinical trials (NCT03812445).

Fecal sample and clinical data collection

To avoid the effect of drugs and hospitalization on gut microbiota, fecal samples were collected within 36 hours of admission. Fresh fecal samples were immediately given to doctors or nurses and then gathered into the sterile tubes. These samples were stored at –80°C prior to microbial analysis. Demographic and clinical data were collected during hospitalization, including age, sex, body mass index (BMI), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglyceride, and total cholesterol. Additionally, we collected possible cognition-related indicators reported in emerging studies, including fasting glucose, homocysteine, and thyroid function: free triiodothyronine (FT3), free tetraiodothyronine (FT4), total triiodothyronine (TT3), total tetraiodothyronine (TT4), and thyroid-stimulating hormone (TSH). In addition, we used the National Institute of Health stroke scale (NIHSS) to assess the severity of stroke.

After 3 months of AIS onset, all enrolled patients were evaluated with the Beijing version of the MoCA, MMSE, Hamilton Depression Scale (HAMD), and Hamilton Anxiety Scale (HAMA) in a stable condition by senior neurologists in the outpatient department. The Beijing version of the MoCA has been widely used in China to assess cognition of stroke patients. MoCA scores range from 0 to 30. If the score was below 26, the patient was considered

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to have PSCI. Considering the influence of education, we added one point to the score of patients who received less than 12 years of education, following the instruction of MoCA [21].

16S High-throughput sequencing and targeted metabolomics

DNA was extracted from fecal samples using the QlAamp DNA Stool Mini kit (Qiagen). The DNA concentrations and quality were examined by a Nanodrop 2000 uv-vis spectrophotometer (Thermo Scientific, Wilmington, MA, USA) and 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with forward primer 314F (5′-CCTACGGGRSGCAGCAG -3′) and reverse primer 806R (5′-GGACTACVVGGGTATCTAATC -3′) on a thermocycler PCR system. PCR products were extracted from a 2% agarose gel electrophoresis and recycled by AxyPrep DNA Gel Recovery Kit (Axygen Biosciences, Union City, CA, USA), and then examined by a Nanodrop 2000 uv-vis spectrophotometer (Thermo Scientific, Wilmington, MA, USA). Purified amplicons were pooled in equimolar solutions and underwent paired-end sequencing using an IlluminaMiSeq PE250 (Illumina, San Diego, CA, USA). Targeted SCFAs metabolome was tested by 7890A-5975C gas chromatography-mass spectrometer (GC-MS) (Agilent Technologies, Palo Alto, CA, USA) [22].

Bioinformatic and statistical analysis

After quality control of the original data, we filed the raw data, identified the optimized sequences, and then used Usearch (version7.1, http://drive5.com/uparse/) to assign the raw sequences to operational taxonomic units (OTUs) based on OTUs having $\geq$97% similarity. Each OTU is considered to represent each taxonomic level, i.e., kingdom, phylum, class, order, family, and genus. The OTUs cluster analysis was performed following chimeric filtration, including alpha diversity, beta diversity, analysis of biological difference and relevance as well as prediction of functional composition. Alpha diversity was used to describe the abundance and diversity of gut microbiota, including Observed_species index, Chao1 index, Shannon index, and Simpson index analysis. Beta diversity was utilized for further analysis of species diversity of gut microbiota from fecal samples, including analysis of similarity (Anosim) and Principal Coordinates Analysis (PCoA). Anosim was applied to estimate whether the difference between groups was greater than that within groups, and if it made sense to divide AIS patients into the both groups. Analysis of biological difference and relevance were consisted of linear discriminant analysis coupled with effect size (LEfSe), Spearman analysis of gut microbiota, and MoCA. Subsequently, we used Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) to predict the microbiota-associated functional pathway based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

All quantitative data were analyzed using GraphPad prism 8.0 and SPSS 22.0 software. Clinical data were expressed as mean $\pm$ standard deviation. We used the Student's t, Pearson's Chi-square, Fisher's exact and Mann-Whitney U tests to analyze the continuous and categorical variables, respectively.

RESULTS

General descriptions of enrolled patients

The demographic and clinical data of 65 AIS patients mentioned above are listed in Table 1. As shown, there were no difference found in sex, age, and BMI. Moreover, we found no difference in some clinical indicators that may influence cognition of AIS patients, including fasting glucose, homocysteine, thyroid function, and severity of AIS (assessed by NIHSS), suggesting none of confounding clinical parameters influenced group discrimination. For further study, we compared the sub item of MoCA between the both groups (shown in Table 2), demonstrating that PSCI patients had significantly lower scores, especially in visuospatial/executive function, language, abstraction, and delayed recall compared with non-PSCI patients.

Lower diversity and disturbed composition of gut microbiota in PSCI subjects

From all fecal samples of enrolled patients, a total number of 3,196,723 clean reads were obtained, with an average count of 49,180 clean reads per sample, which were clustered into 696 OTUs based on $\geq$97% similarity. The Venn diagram suggested that 533 OTUs were shared by both groups while 113 and 48 OTUs were unique to non-PSCI and PSCI

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Table 1
Demographic and clinical characteristics of PSCI subjects versus non-PSCI subjects

	PSCI ($n=30$)	Non-PSCI ($n=35$)	p
Female/male	5/25	11/24	0.25
Age (y)	64.9 ± 8.13	64.06 ± 8.67	0.69
BMI (kg/m^2)	23.41 ± 2.91	24.01 ± 2.48	0.37
Fasting glucose (mmol/L)	7.20 ± 3.30	6.79 ± 2.36	0.58
Homocysteine (μmol/L)	15.73 ± 9.08	13.42 ± 4.70	0.19
TG (mmol/L)	1.55 ± 0.72	1.91 ± 1.05	0.12
TC (mmol/L)	4.24 ± 0.95	4.56 ± 1.10	0.22
LDL-C (mmol/L)	2.71 ± 0.88	2.92 ± 0.96	0.35
HDL-C (mmol/L)	1.09 ± 0.22	1.08 ± 0.27	0.86
Thyroid function			
FT3 (pmol/L)	4.68 ± 0.47	4.58 ± 0.56	0.46
FT4 (pmol/L)	16.16 ± 1.95	16.35 ± 2.38	0.73
TT3 (nmol/L)	1.62 ± 0.29	1.54 ± 0.23	0.18
TT4 (nmol/L)	102.35 ± 19.08	98.11 ± 20.57	0.40
TSH (mIU/L)	1.31 ± 0.67	1.84 ± 1.76	0.11
NIHSS	2.87 ± 1.68	3.66 ± 2.89	0.18

Data were expressed as mean ± standard deviation. BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FT3, free triiodothyronine; FT4, free tetraiodothyronine; TT3, total triiodothyronine; TT4, total tetraiodothyronine; TSH, thyroid-stimulating hormone; NIHSS, National Institutes of Health stroke scale.

Table 2
Sub item of MoCA scores of patients with or without PSCI

	Patients without PSCI ($n=30$)	Patients with PSCI ($n=35$)	p
MoCA	26.9 ± 1.24	17.46 ± 4.16	**
Visuospatial/executive function	4.07 ± 0.83	2.43 ± 1.36	**
Naming	2.87 ± 0.35	2.34 ± 0.84	*
Attention	5.67 ± 0.55	4.09 ± 1.31	**
Language	2.6 ± 0.62	1.17 ± 0.79	**
Abstraction	1.6 ± 0.56	0.8 ± 0.76	**
Delayed recall	3.87 ± 0.86	1 ± 1.26	**
Orientation	5.77 ± 0.5	5.03 ± 1.04	*

*$p<0.01$, **$p<0.001$.

subjects, respectively (Supplementary Figure 1). Alpha diversity was utilized to assess difference in the richness (Chao1 and observed species indices) and diversity (Shannon and Simpson indices) of gut microbiota. Compared with non-PSCI subjects, PSCI subjects had significantly lower Shannon index ($p=0.027$), observed species ($p<0.01$), and Chao1 index ($p<0.01$), whereas no significant difference was observed in Simpson index ($p=0.17$) between the both groups (Fig. 1A).

Moreover, the three-dimensional principal coordinate analysis based on unweighted UniFrac distance demonstrated that significantly different composition of gut microbiota was found between the both groups (Fig. 1B). For further verification, unweighted UniFrac Anosim was performed ($R=0.228$, $p=0.001$) (Supplementary Figure 2), confirming the distinct bacterial structure between the two groups.

Significantly different abundance of microbiota in PSCI versus non-PSCI subjects

Linear discriminant analysis effect size analysis and Wilcoxon rank-sum test were utilized to identify the significantly different bacterial taxa between the both groups. At the level of phylum, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the main phyla in the both groups (53.37% versus 48.75%; 34.67% versus 36.31%; 7.24% versus 9.29%, and 1.24% versus 4.27%, respectively). There were 4 taxa found to be distinct between the both groups, including Fusobacteria, Synergistetes, Verrucomicrobia, and Euryarchaeota (all $p<0.05$). The relative abundance of Fusobacteria was significantly increased in PSCI group, whereas that of the remaining 3 taxa were decreased in PSCI group (Fig. 1D). At the genus level, 20 taxa were identified for discriminating PSCI subjects from non-PSCI subjects (LDA score>2, $p<0.05$), including enriched abundance of *Fusobacterium*, *Bacteroides*, *Clostridium XlVa*, *Gemella,* and *Flavonifractor*, and decreased abundance of the remaining 15 genera (Fig. 2). Generally, the 20 distinct genera mainly belonged to the phyla *Firmicutes* (9/20, 45%) and *Bacteroidetes* (5/20, 25%). Notably, the relative abundance of *Fusobacterium*, which increased approximately 10-fold in PSCI relative to that in non-PSCI (0.26% versus 2.51%, $p<0.01$), demonstrated the strongest relation with PSCI, whereas the relative abundance of SCFAs-producing genera decreased significantly in PSCI group compared to that in non-PSCI group, such as *Oscillibacter*, *Ruminococcus*, *Gemmiger*, and *Coprococcus* (Fig. 1C, E).

Altered short-chain fatty acids in PSCI subjects

Based on LEfSe, we found that numerous genera, which were capable to produce SCFAs by fermentation of dietary fiber, decreased significantly in PSCI subjects. Emerging evidence has confirmed SCFAs play an important role in brain health, such as affection and cognition [23]. In this study, 7 dominant kinds of SCFAs were tested by targeted

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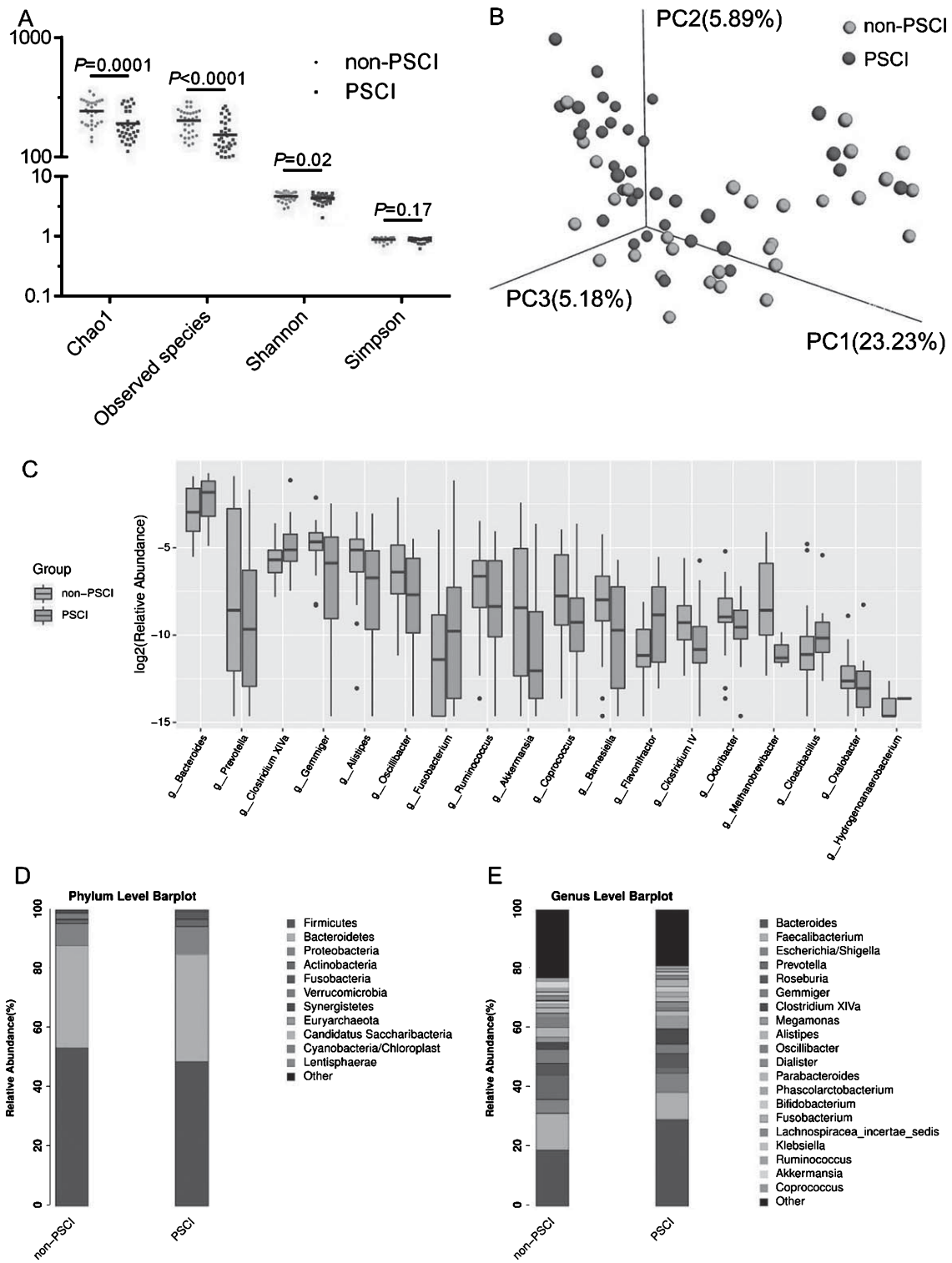


Fig. 1. Diversity and structure of gut microbiota in AIS patients with or without PSCI. A) Significantly distinct alpha diversity between the both groups. B) Unweighted PCoA was used to illustrate bacterial 3D clustering. C) The different relative abundance of genera identified by Wilcoxon rank-sum test. Component proportion of bacteria in phylum (D) and genus (E) level between both groups was showed.

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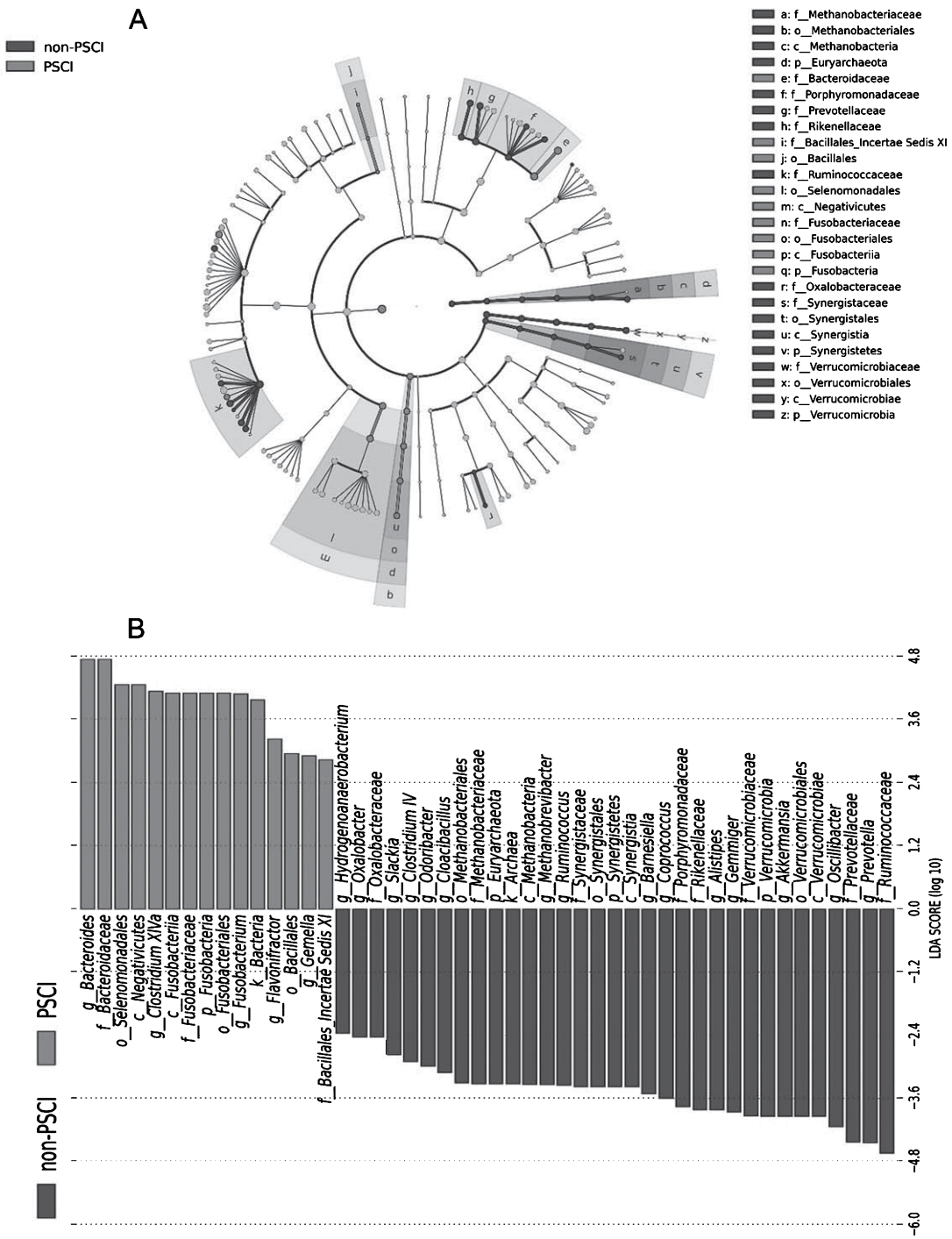


Fig. 2. Linear discriminant analysis integrated with effect size (LEfSe). Cladogram (A) and histogram (B) illustrated the phylogenetic distribution of gut microbiota related to both groups.

metabolomics, including acetic acid, propionic acid, isobutyric acid, butanoic acid, isovaleric acid, valeric acid, and hexanoic acid. Notably, Mann-Whitney U test identified the significantly decreased content of SCFAs in PSCI group, especially hexanoic acid (Fig. 3A–G). Correlation analysis showed that SCFAs were positively associated with SCFAs-producing bacteria, suggesting lack of SCFAs is probably due

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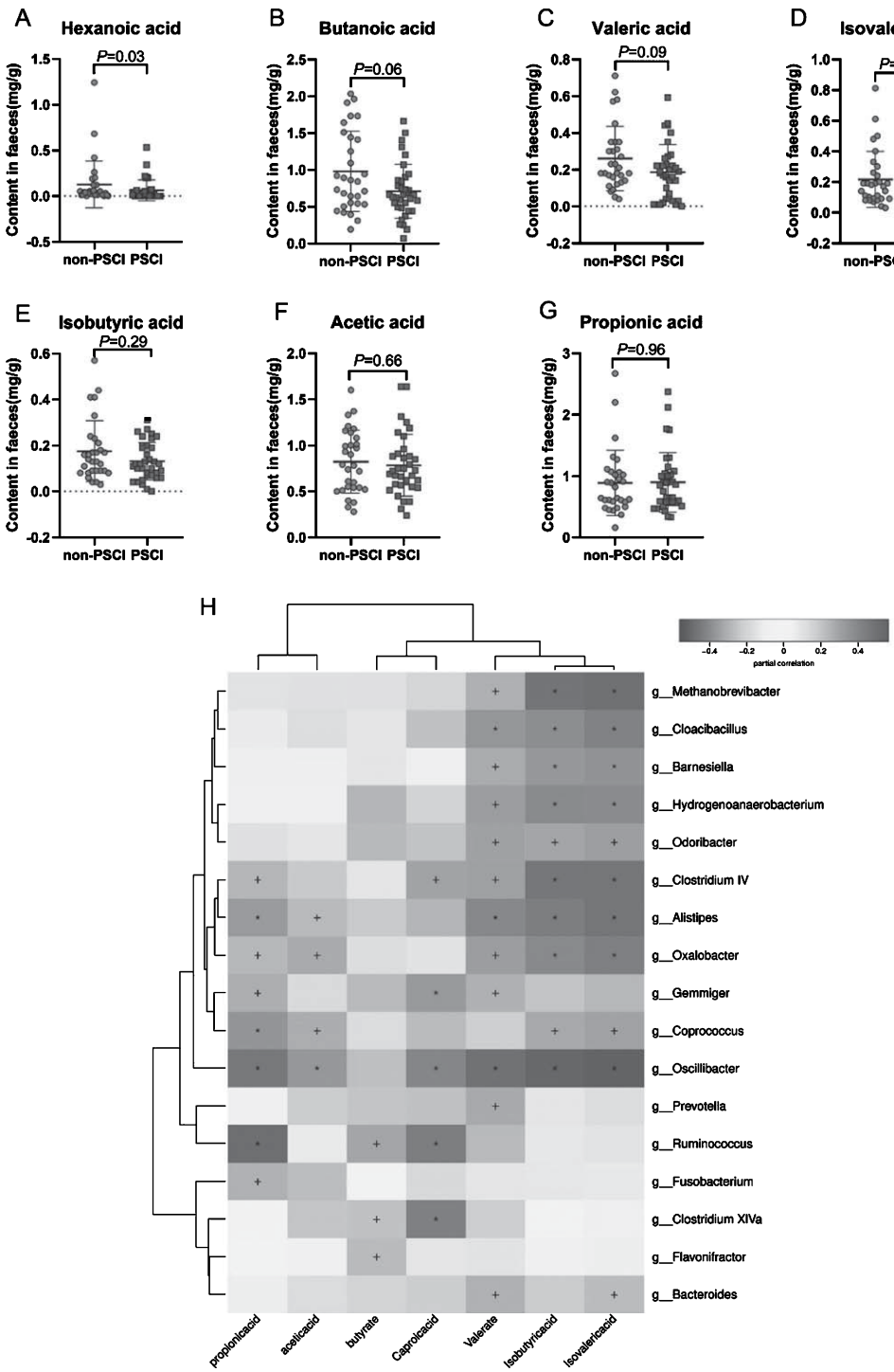


Fig. 3. Comparison of SCFAs between AIS patients with or without PSCI. 7 main kinds of SCFAs were denoted in the form of boxplot (A–G). H, the heat map illustrated the correlation analysis of SCFAs and gut microbiota. $^{+}p < 0.05$; $^{*}p < 0.01$.

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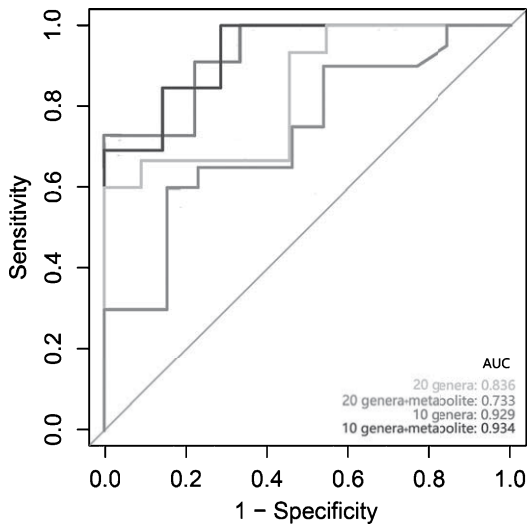


Fig. 4. Predictive models based on microbiota and metabolites for early diagnosis of PSCI.

to decreased abundance of SCFAs-producing bacteria in AIS patients (Fig. 3H).

Gut microbiota and SCFAs-based model discriminated PSCI

To assess the potential capability of gut microbiota and SCFAs as biomarkers for early diagnosis of PSCI, receiver operating characteristic curve (ROC) was performed. Utilizing different 20 genera found in LEfSe generated an area under curve (AUC) of 0.836. Further, we found the predictive performance could be improved significantly with an AUC of 0.934 by the most distinct 10 genera combined with hexanoic acid, which primarily belonged to *Fusobacteria*, *Firmicutes*, and *Bacteroidetes* (Fig. 4).

16S functional prediction and correlation analysis

Based on the Kyoto Encyclopedia of Genes and Genomes databases, we used PICRUSt to predict the function of gut microbiota. The significantly changed functions of gut microbiota were the upregulation of carbohydrate metabolism and immune system diseases in PSCI group. Remarkably, we identified the overexpression of utilization of glucose in L3 category in PSCI group, including fructose and mannose metabolism, galactose metabolism, and pentose phosphate pathway. In addition, amino acid metabolism and other iron coupled transporters pathway were also found to be significantly abundance in PSCI group (Supplementary Figure 3). The microbiota-microbiota correlation analysis of all AIS patients demonstrated that *Fusobacterium*, *Flavonifractor*, *Bacteroides,* and *Clostridium XlVa* were positively correlated with each other, whereas they were negatively associated with some SCFAs-producing or common bacteria, such as: *Gemmiger*, *Barnesiella*, *Coprococcus*, and *Ruminococcus* (Supplementary Figure 4). To determine the association between gut microbiota and clinical parameters of AIS patients, we performed correlation analysis of MoCA, clinical variables, and gut microbiota. Notably, *Fusobacterium* had the strongest negative correlation with MoCA score, whereas SCFAs-producing bacteria were positively related to MoCA score. Additionally, *Fusobacterium* was positively related to TG, which might be a potential pathway that *Fusobacterium* influences PSCI. Additionally, homocysteine, a cognition-related clinical variable, was positively related to *Alistipes* (Fig. 5).

The effect of probiotics intervention on PSCI

Next, we recruited 18 newly diagnosed AIS patients, whose treatment was supplemented by probiotics. The assessment 3 months later showed that compared to the 65 subjects without probiotic treatment mentioned above, the 18 probiotics-treated subjects had better mood, including depression and anxiety (assessed by HAMD and HAMA), whereas no statistical difference was found in the proportion of PSCI and average scores between both groups (assessed by MoCA and MMSE) (Table 3).

DISCUSSION

After the proposal of the "brain-gut-microbiota axis", microbiota-associated studies have paid great attention to the alteration of composition, function, and metabolome of gut microbiota in diseases of the central nervous system. Accumulating evidence has confirmed that gut microbiota and its metabolome were associated with cognitive function [24]. Therefore, utilizing gut microbiota and its metabolites as new screening tools for early recognition of PSCI is promising. We revealed for the first time the hallmark of gut microbiota and its metabolites SCFAs in AIS patients with PSCI in this study, following 16S sequencing and targeted metabolome analysis. We also established a gut microbiota and SCFAs-based model that had the power for early diagnosis of PSCI. Moreover, we found that traditional probiotics

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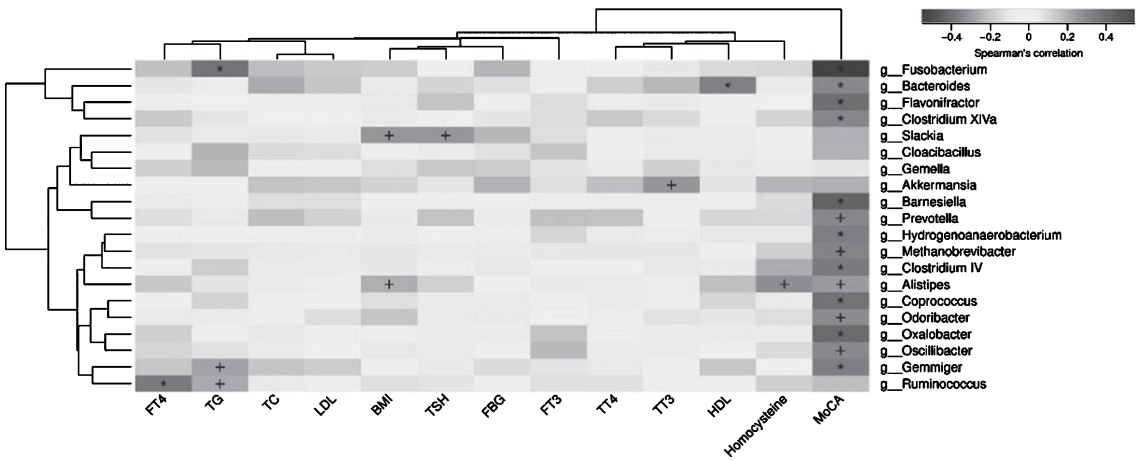


Fig. 5. Spearman's correlation analysis of gut microbiota, MoCA as well as clinical variables. $^{+}p < 0.05$; $^{*}p < 0.01$.

Table 3
MoCA scores in probiotics-treated subjects versus controls

	PSCI	non-PSCI	Average score
Probiotics	12 (66.67%)	6 (33.33%)	23.11 ± 3.46
Controls	35 (53.85%)	30 (46.15%)	21.82 ± 5.69

intervention could improve AIS patients' mood, but it had no effect on AIS patients' cognition.

It was exhibited that PSCI subjects had significantly decreased diversity of gut microbiota relative to subjects without PSCI, suggesting a high microbial diversity might reflect the relatively healthy status after acute ischemic stroke. Similar decrease of microbial diversity was also observed in other cognitive impairment-related diseases from humans and rodents, including Alzheimer's disease and streptozotocin-induced diabetes [25–27].

Consistent with the decreased diversity, we found the microbial composition of PSCI subjects was significantly different from that of non-PSCI subjects. Based on LEfSe and correlation analysis, it was found that *Fusobacterium* had the strongest correlation with PSCI. *Fusobacterium* is a gram-negative non-sporeforming pathogen, which could induce the dysbiosis of intestinal microenvironment, generate proinflammatory environment *in vivo*, and cause immune evasion of some pathogenic bacteria [28, 29]. Besides, our former studies discovered that *Fusobacterium nucleatum* enhanced intestinal tumor cell invasion and adhesion by activating Toll-like receptor and upregulating expression of microRNA-21 [30]. Hence, we speculated that *Fusobacterium* could aggravate cognitive impairment by promoting the inflammatory response of the neural system and activating specific receptors after stroke, which still needs future study to verify. Remarkably, emerging evidence demonstrated that *Fusobacterium* was enriched in some neural diseases, including autism spectrum disorder [31], mild cognitive impairment [32], and myasthenia gravis [33], hinting that *Fusobacterium* and its metabolites might be able to injure the neural system via direct or indirect involvement.

Moreover, the bacterial composition of PSCI subjects were also characterized by the lack of SCFAs-producing bacteria, such as *Oscillibacter*, *Ruminococcus*, *Gemmiger*, *Coprococcus*, and *Barnesiella*. SCFAs, the major metabolites from bacterial fermentation of dietary fiber, played an important role in the nervous system [34]. It was confirmed that SCFAs could cross the blood-brain barrier to the brain and their receptors were expressed in brain tissue and sympathetic ganglia [35]. Thereby, we tested the content of SCFAs from AIS patients' feces to explore the relationship between SCFAs and complete cognitive function. Consistent with the decreased SCFAs-producing bacteria, the content of main SCFAs in feces of PSCI subjects decreased significantly compared with that of non-PSCI subjects. Not only limited to PSCI, the decreased SCFAs were also found in Alzheimer's disease and Parkinson's disease [36, 37]. SCFAs play a critical role in microbiota-gut-brain axis by interacting with free fatty acid receptor, inhibiting histone deacetylases, and affecting microglia cell [23, 38, 39]. It has also been confirmed that SCFAs administration could ameliorate cognitive function in rodent researches and inhibit Aβ aggregation *in vitro* [36, 40, 41]. Taken

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together, the significantly increased *Fusobacterium* and decreased SCFAs induced severe immune disorders, which might be capable in explaining the occurrence and development of PSCI.

The probiotic intervention improved mood after stroke, including anxiety and depression, but it had no statistically significant effect on cognition. The probiotics used in this study were composed of *Lactobacillus* and *Bifidobacterium*, which were capable to produce SCFAs. *Lactobacillus* could produce SCFAs by fermentation of carbohydrates and by the phosphoketolase route in the hetero fermenting conditions [42]. *Bifidobacterium* uses the fermentation pathway to produce mainly acetate and formate under carbohydrates limitation, and acetate and lactate when carbohydrates are in excess [43]. Supplementation of *Lactobacillus* and *Bifidobacterium* increased the concentration of SCFAs in SHIME (for Simulator of Human Microbial Ecosystem) reactor [44]. Based on the research mentioned above, we tried to improve microbial structure and alleviate the lack of SCFAs in PSCI patients utilizing *Lactobacillus* and *Bifidobacterium*. The useful intervention for improving PSCI might need high-dose supplement of SCFAs or SCFAs-producing probiotics rather than traditional-dose *Lactobacillus* and *Bifidobacterium*. Additionally, fecal bacteria transplantation or supplement of new-type probiotics, such as *Akkermansia*, which decreased significantly in PSCI subjects might have potential for ameliorating PSCI.

Nonetheless, there were still some shortcomings. First, due to the simplicity of the research design, we only excluded patients with cognitive impairment before stroke and performed cognitive assessment 3 months after stroke. In future research, we will further explore the association between PSCI and microbiota, as well as its metabolized SCFAs, containing detailed and long-term follow-up of cognitive function. Secondly, we failed to collect fecal samples 3 months after stroke that should have been utilized to assess microbial structure and measure SCFAs concentration after taking probiotics.

The gut microbial signature and corresponding metabolites, as well as the diagnostic model associated with PSCI, still need a larger sample size of clinical studies to validate. We used 16S rRNA rather than metagenomic sequencing, which limited us to the finding of specific bacteria related to PSCI in the species level.

In brief, we found that PSCI was characterized by the anomalous gut microbial diversity, composition, and corresponding metabolites. PSCI subjects could be distinguished from non-PSCI subjects accurately, utilizing specific bacteria and metabolites. Traditional probiotics administration had beneficial effect on the control of depression and anxiety after stroke.

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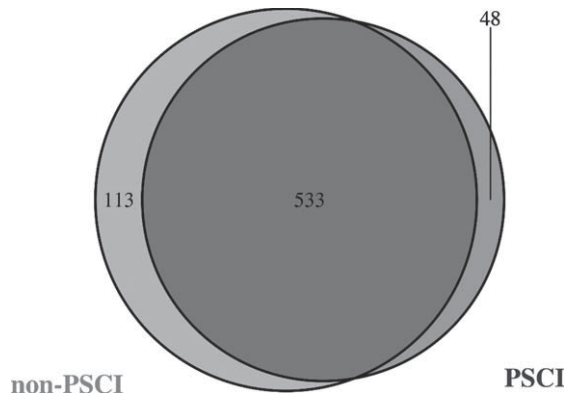
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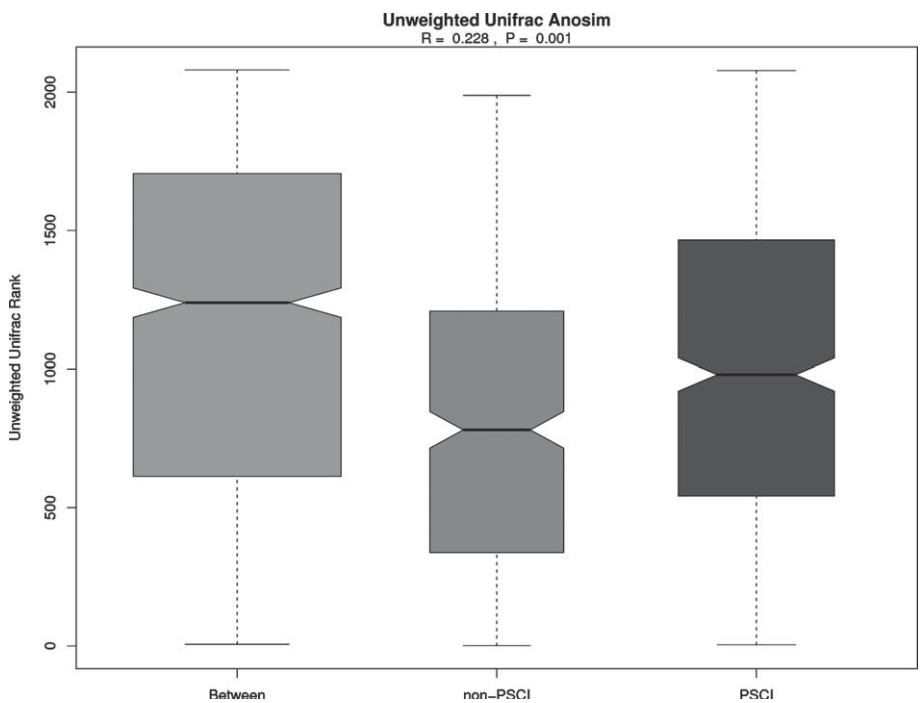
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SUPPLEMENTARY MATERIAL

The Association of Post-Stroke Cognitive Impairment and Gut Microbiota and its Corresponding Metabolites

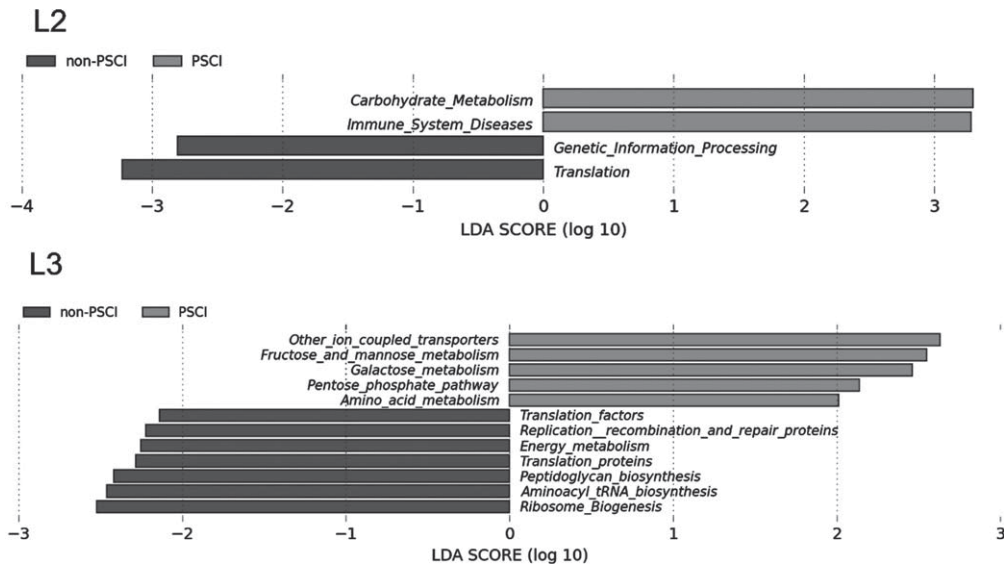


Supplementary Figure 1. Venn diagram between PSCI subjects and non-PSCI subjects.

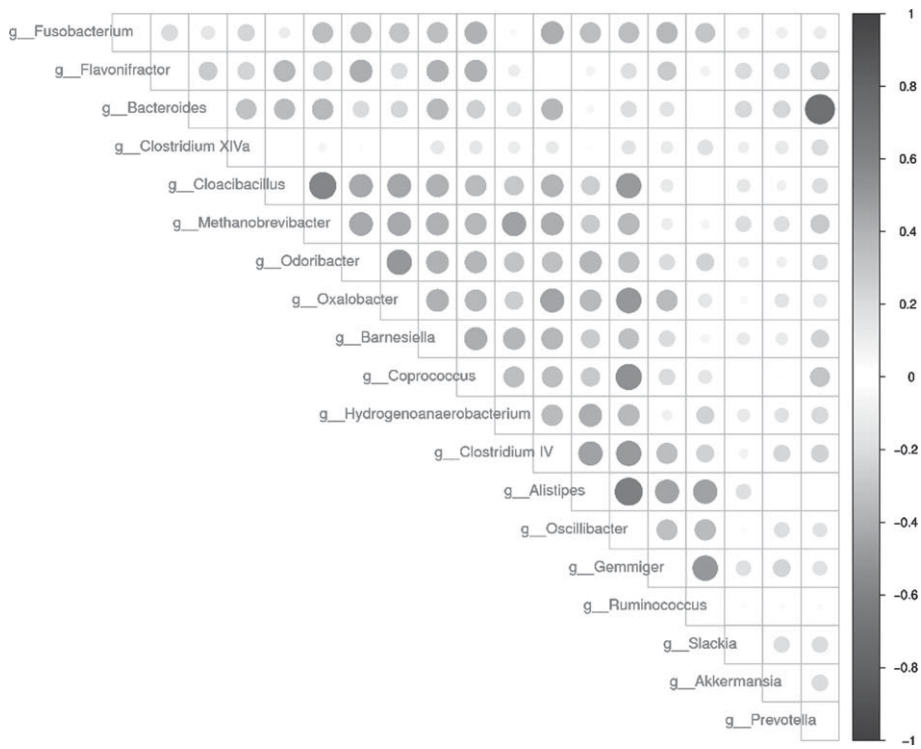


Supplementary Figure 2. Unweighted UniFrac Anosim between PSCI subjects and non-PSCI subjects.

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Supplementary Figure 3. Microbial 16S functional prediction of PSCI subjects and non-PSCI subjects.



Supplementary Figure 4. The microbiota-microbiota correlation analysis of all patients, including PSCI subjects and non-PSCI subjects.

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Effects of Probiotic Supplementation on Short Chain Fatty Acids in the App^{NL-G-F} Mouse Model of Alzheimer's Disease

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Abstract.
Background: The intestinal microbiota and its metabolites, particularly short-chain fatty acids (SCFAs), have been implicated in immune function, host metabolism, and even behavior.
Objective: This study was performed to investigate whether probiotic administration influences levels of intestinal microbiota and their metabolites in a fashion that may attenuate brain changes in a mouse model of Alzheimer's disease (AD).
Methods: C57BL/6 wild-type (WT) mice were compared to App^{NL-G-F} mice. The animals were treated with either vehicle or probiotic (VSL#3) for 8 weeks. Fecal microbiome analysis along with Aβ, GFAP, Iba-1, c-Fos, and Ki-67 immunohistochemistry was done. SCFAs were analyzed in serum and brains using UPLC-MS/MS.
Results: Probiotic (VSL#3) supplementation for 2 months resulted in altered microbiota in both WT and App^{NL-G-F} mice. An increase in serum SCFAs acetate, butyrate, and lactate were found in both genotypes following VSL#3 treatment. Propionate and isobutyrate were only increased in App^{NL-G-F} mice. Surprisingly, VSL#3 only increased lactate and acetate in brains of App^{NL-G-F} mice. No significant differences were observed between vehicle and VSL#3 fed App^{NL-G-F} hippocampal immunoreactivities of Aβ, GFAP, Iba-1, and Ki-67. However, hippocampal c-Fos staining increased in VSL#3 fed App^{NL-G-F} mice.
Conclusion: These data demonstrate intestinal dysbiosis in the App^{NL-G-F} mouse model of AD. Probiotic VSL#3 feeding altered both serum and brain levels of lactate and acetate in App^{NL-G-F} mice correlating with increased expression of the neuronal activity marker, c-Fos.

Keywords: Alzheimer's disease, butyrate, gliosis, microbiota, plaques, probiotics, short chain fatty acids

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative condition hypothesized to develop due to dysfunction of two major proteins, amyloid-β (Aβ) and microtubule-associated protein, tau [1]. Multiple

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factors are suggested to contribute to the etiology of AD including, but not limited to, aging, genetics, head injury, and exposure to certain chemicals, compounds, and metals including iron, cadmium, etc. [2–4]. Recent evidence supports a positive connection between AD and gut microbiota [5, 6]. The cellular millieu in the gut includes the microbiota, which refers to the bacteria, viruses, and fungi. Changes in gut microbial composition have been found in AD patients that correlate with the levels of certain AD biomarkers in cerebrospinal fluid. These results support a role for the health of the gastrointestinal tract and the enteric nervous system in the pathophysiology of AD [7–9].

The intestinal microbiota not only plays an important part in digestion but also supports overall homeostasis by influencing metabolism, growth, development, immune function, and protection from pathogens [10–12]. A number of factors can affect the intestinal microbiota composition such as dietary changes, antibiotic exposure, and infection leading to the loss of bacterial homeostasis implicated in gastrointestinal and neurological disorders [13, 14]. Recent studies continue to address how microbial metabolism may influence health outcomes associated with extra-intestinal disorders including allergy, metabolic syndrome, cardiovascular disease, and obesity [15–17]. However, the mechanisms by which specific microbes and their metabolites contribute to disease prevention or progression remain largely unknown.

It has been demonstrated that intestinal microbes can affect several host metabolic pathways, many of which are involved with carbohydrate or amino acid metabolism [18]. Short chain fatty acids (SCFAs) are a primary class of signaling molecules including isobutyric acid and butyric acid (C4), propionic acid (C3), acetic acid (C2), and formic acid (C1) that are produced by intestinal bacteria during metabolism of dietary carbohydrates and fibers in the colon [19–21]. There is a scarcity of data describing whether, and how, intestinal bacteria derived SCFA metabolites influence the brain in AD.

Therefore, this study was performed to investigate the role of intestinal microbiota and their metabolites, specifically SCFAs, on AD pathology using the App^{NL-G-F} mouse model of disease. To understand this, we manipulated the gut bacteria using probiotic, VSL#3, which is a combination of eight different lactic acid producing bacteria. We hypothesized that a disease or probiotic-associated shift in intestinal microbiota might result in a corresponding shift in levels of bacterial metabolic products, e.g., SCFA, that could influence the presentation of disease in the brain.

Our data supports the idea that the intestinal microbiota is altered during AD resulting in a change in serum and brain SCFA metabolite levels in the AD mice. Moreover, modulating the intestinal microbiota using a probiotic feeding paradigm altered select SCFA levels correlating with a marker of neuronal activity in the brain.

MATERIALS AND METHODS

Animals

App^{NL-G-F} mice were obtained from Dr. Takaomi C. Saido, Laboratory for Proteolytic Neuroscience, RIKEN Center for Brain Science, Japan. These mice were selected for use as these mice do not over express APP and expression is under endogenous promoter control. However, Aβ is elevated due to the combined effects of three mutations associated with familial AD. An APP construct containing a humanized Aβ region, includes the Swedish "NL", the Iberian "F", and the Arctic "G" mutations was used [22]. Fifteen female App^{NL-G-F} and 15 C57BL/6 (wild type) control mice were used at 6–8 months of age. Both genotypes were bred and housed under the same conditions (room, diet, bedding, lighting, etc.) to avoid potential artifacts due to the environment that are relevant in microbiome studies. Mice were weaned and used at 6–8 months age to ensure acclimation to the environment at the start of the treatment regimen. Animal use was approved by the University of North Dakota Institutional Animal Care and Use Committee (IACUC) protocol. The animals were provided food and water *ad libitum* and housed in a specific pathogen-free room with a 12 h light/dark cycle.

Animal groups and probiotic (VSL#3) dosing

Animals were randomly assigned into four groups: 1) wild type supplemented with vehicle (WT Vehicle), 2) wild type supplemented with VSL#3 (WT VSL), 3) App^{NL-G-F} supplemented with vehicle (App^{NL-G-F} Vehicle), and 4) App^{NL-G-F} supplemented with VSL#3 (App^{NL-G-F} VSL#3). The vehicle used was MediGel® (Clear H_2O, Portland, ME, USA) and VSL#3 was dissolved in the vehicle and administered to animals daily at estimated mean water consumption of 5 mL/25 g mouse/day.

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The treatment was given for eight weeks and the dose of probiotic VSL#3 (0.32×10^9 CFU bacteria/25 g mice) was calculated based on the body surface area normalization method from the recommended human dose of VSL#3 [23]. VSL#3 is a commercially available probiotic cocktail (Leadiant Biosciences, Inc.., Gaithersburg, MD, USA) of eight strains of lactic acid-producing bacteria: *Lactobacillus plantarum, Lactobacillus delbrueckii subsp. Bulgaricus, Lactobacillus paracasei, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, and Streptococcus salivarius subspecies, thermophilus.* VSL#3 is a well-known medical food that has been shown to improve disease symptoms in the treatment of ulcerative colitis and pouchitis, an inflammation of the ileal pouch in colectomy patients, liver cirrhosis and hepatic encephalopathy [24, 25].

Antibodies and reagents

Antibodies against GFAP and Aβ were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-c-Fos antibody was purchased from Abcam (Cambridge, MA, USA). Anti-Iba-1 antibody was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). Elite Vectastain ABC reagents, Vector VIP, biotinylated anti-rabbit, and anti-mouse antibodies were purchased from Vector Laboratories Inc (Burlingame, CA, USA). The $A\beta_{1-40}$ and $A\beta_{1-42}$ enzyme-linked immunosorbent assay (ELISA) kits were obtained from EMD Millipore (Burlington, MA). Standards used in mass spectroscopy analysis were purchased from Millipore-Sigma Burlington, MA, USA. Auto sampler vials were obtained from ThermoFisher Scientific, (Waltham, MA, USA), Silanized micro-vial inserts from Agilent (Santa Clara CA; part #5181-8872) and inserts were from VWR (Radnor, PA, USA).

Fecal sample collection and microbiome analysis

After 8 weeks of probiotic feeding, fecal samples were obtained by placing each mouse separately in a clean cage for 10–30 min and fecal pellets were collected in a sterile 1.5 mL Eppendorf tube. More than 90% of the mice excreted a fecal pellet within one min of being placed into a clean cage. In those instances, in which a mouse did not pass stool within 30 min, the mouse was gently picked up vertically by the tail for 20–30 s until a stool pellet was excreted and collected. Samples were stored at 4°C until all samples were collected and later stored at –80°C until microbiome analysis. The fecal DNA samples were sent to RTL Genomics (Research and Testing Laboratory, Lubbock, TX, USA) for 16S rRNA sequencing. Hypervariable regions V1 to V3 of 16S rRNA gene were amplified for sequencing at RTL Genomics in a reaction using HotStarTaq Master Mix Kit (Qiagen, Inc., Germantown, MD, USA) with the universal primer set 27F/519R.

Short chain fatty acid analysis

Carboxylic acids moiety of SCFAs were analyzed as nitrophenyl hydrazide derivatives (CANPD). Proteins from serum (10 μL) and hippocampus (10 mg) were precipitated after sonication with 280 μL of methanol containing butyrate-$^{13}C_4$, propionate-$^{13}C_3$, and lactate-$^{13}C_3$ (20 ng each) as internal standards. After centrifugation at 10,000 × g, the supernatant was subjected to derivatization to obtain carboxylic acids nitrophenyl hydrazides as described in [26] with modifications. Because ethanol showed high levels of short chain carboxylic acid contamination, it was substituted with methanol for the derivatization reaction. All reagents for derivatization were from Millipore-Sigma Burlington, MA, USA. Briefly, the supernatant volume was brought up to 300 μL with methanol, mixed with 300 μL of pyridine, 300 μL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (250 mM in methanol), 300 μL of 2-Nitrophenyl hydrazine hydrochloride (20 mM in methanol), and incubated at 60°C for 20 min. After quenching with 200 μL of potassium hydroxide (15% in 20% methanol) at 60°C for 20 min, 3 mL of Phosphoric acid (0.5 mM in water) were added to each sample. CANPD were extracted with 4 mL of ether and the extract was washed with 4 mL of water. Centrifugation at 1,500 × g was used to separate phases during extraction and washing steps.

An LC-MS method was used for CANPD quantification. CANPD extracts were evaporated under nitrogen stream, transferred to silanized microvial inserts (Agilent, Santa Clara CA; part #5181-8872) with 100 μL of ether, evaporated, re-dissolved in 20 μL of 50% methanol in water, and 10 μL was analyzed on UPLC-MS/MS in the selected ion monitoring mode.

UPLC separation was performed on a Waters ACUITY UPLC HSS T3 column (1.8 μM, 100 Å pore diameter, 2.1 × 150 mm, Waters, Milford, MA) with an ACUITY UPLC HSS T3 precolumn (1.8 μM,

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100 Å pore diameter, 2.1 × 5 mm, Waters) at a temperature of 55°C using a previously described method [2, 3]. The LC system consisted of a Waters ACUITY UPLC pump with a well plate autosampler at 8°C. Solvent A was water containing 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The flow rate was maintained at 0.45 mL/min. CANPD were eluted with increasing gradient of solvent B from 39% to 98% as we previously described [27]. A quadrupole time-of-flight mass spectrometer (Q-TOF, Synapt G2-S, Waters) was used for detection as described previously [28]. The electrospray ionization was set in negative ion mode with the cone voltage at 20 V, and the capillary voltage at 1.51 kV. The source and desolvation temperatures were 110°C and 350°C, respectively. MSE mode was used to collect data. Leucine enkephalin (400 pg/μL in ACN: water, 50:50 by volume) was used for mass correction. MassLynx V4.1 software (Waters) was used for instrument control, acquisition, and sample analysis. Nitrophenyl hydrazide derivatives of acetate (m/z = 194.0566), and propionate (m/z = 208.0722) were quantified against propionate-13C3(m/z = 211.0823), lactate (m/z = 224.0671) and pyruvate (m/z = 222.0515) were quantified against lactate-13C3,(m/z = 227.0772), and butyrate and isobutyrate (m/z = 222.0879) - against butyrate-13C4 (m/z = 226.1013).

Ki-67 stereology and counting in hippocampus

Immunopositive Ki-67 nuclei were counted in the subventricular zone (SVZ) using an Olympus BX51WI microscope with a motorized XYZ stage. Unbiased quantification of nuclei was conducted using the optional fractionator workflow in StereoInvestigator11.0 (Micro Brightfield, Inc., Williston, VT). For cell counting, a tight contour was outlined around the subvertical zone at the same region for all the treatment conditions. Positive nuclei were counted in every 24th section in systematically selected frames based on an optical dissector frame (50 × 50 μm) and a grid size (125 × 125 μm) that were empirically determined by initial oversampling. The section thickness was measured at each sampling site. The sampling and counting paradigm resulted in a Coefficient of Error (CE) less than 0.1 (10%) for all samples counted. The total numbers were estimated in Stereo Investigator with the optical fractionator formula (N = 1/ssf.1/asf.1/hsf.ΣQ–) where ssf = section sampling fraction (10), asf = area sampling fraction (area sampled/total area), hsf = height sampling fraction (counting frame height/30 μm), and ΣQ– (total particle count). Four sections (40μm) per brain were used at a counting interval of every 24th section and 7 brains were quantified per treatment condition. Cells in the dentate gyrus were counted manually due to the few Ki-67 positive cells being present in hippocampus.

Immunohistochemistry

Following the eight-week treatment paradigm, the left brain hemisphere of each animal was fixed in 4% paraformaldehyde and prepared for histologic analysis as described previously [29]. Briefly, paraformaldehyde-fixed tissue was embedded in a 15% gelatin (in 0.1 M phosphate buffer) matrix and immersed in a 4% paraformaldehyde for 2 days to harden the gelatin matrix followed by two changes of 30% sucrose for cryoprotection for two days each. The blocks were then flash frozen using dry ice/isomethylpentane, and 40μm serial sections were cut using a freezing microtome. Serial sections of 40μm were stored at 4°C in PBS with 0.1% sodium azide until immunostaining was performed. Anti-Aβ (1:1000), anti-GFAP (1:700), anti-Iba-1 (1:750), anti-cFos (1:2000), and anti-Ki-67 (1:500, used sodium citrate, heat mediated antigen retrieval) antibodies were used for immuno detection in sections followed by incubation with biotinylated secondary antibodies (1:2000 dilution; Vector Laboratories) and an avidin/biotin complex (Vector ABC kit). Immunoreactivity was visualized using Vector VIP as the chromogen. The stained sections were mounted onto gelatin-coated slides and cover slipped using Permount (Vector Laboratories) following a standard dehydrating procedure through a series of ethanol concentrations and Histo-Clear (National Diagnostics, Atlanta, GA, USA). A Leica DM1000 microscope and Leica DF320 digital camera system (Leica Microsystems Inc., Buffalo Grove, IL, USA) were used to take images and quantification of immuno staining was performed as described previously [30]. Briefly, optical densities from serial hippocampus sections from each condition were measured using Adobe Photoshop software (Adobe Systems). The average values for each condition were analyzed and plotted. This entailed 2 hippocampus sections per brain, 7 brains per condition, or 14 sections from each condition that were used for immunohistochemistry analysis.

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Enzyme linked immunosorbent assay (ELISA) for amyloid-β

Following the eight-weeks treatment paradigm, the animals were euthanized via CO_2 asphyxiation followed by cervical dislocation and cardiac exsanguination. The hippocampi of each animal were collected for biochemical procedures. The right hippocampus of each animal was collected, flash frozen in liquid nitrogen, and stored at –80°C for subsequent use. A small part of the hippocampus was weighed and lysed in ice cold radio immuno precipitation assay buffer (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM sodium chloride (NaCl), 1 mM sodium orthovanadate (Na_3VO_4), 10 mM sodium fluoride, 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM (ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid) EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), and 0.5% deoxycholate) with protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride 1 mM, aprotinin 0.8 μM, leupeptin 21 μM, bestatin 36 μM, pepstatin A 15 μM, E-64 14 μM). The samples in a Bullet Blender Storm homogenizer 24 (Next Advance, Inc. Troy, NY, USA) and later centrifuged to remove the insoluble content. The resulting clear tissue lysate (supernatant) was used for soluble $Aβ_{1–40}$ and $Aβ_{1–42}$ ELISAs. The pellet or insoluble content was re-suspended in 5 M guanidine HCl/50 mM Tris HCl, pH 8.0 and samples were again bullet blended and centrifuged (21,000 g, 4°C, 10 min) and the supernatant was removed and used to determine insoluble Aβ concentrations ($Aβ_{1–40}$ and $Aβ_{1–42}$ ELISA). The levels of soluble and insoluble $Aβ_{1–40}$ and $Aβ_{1–42}$ were quantified by using commercially available ELISA kits from Millipore Sigma. The levels of $Aβ_{1–40}$ and $Aβ_{1–42}$ (soluble and insoluble) were reported as pg/mL per mg protein derived from a standard curve established for each protein. Protein concentrations of cell lysates were determined using the bicinchoninic acid (BCA) protein determination assay (ThermoFisher Scientific).

Behavioral analysis

Light-dark box (Stoelting) testing was performed to assess anxiety-like behavior in the mice [31, 32]. The light/dark box consisted of two compartments, light and dark, connected by a door in the middle of the wall that separated the two compartments. Each animal was placed individually in the light compartment and allowed to move freely in the apparatus for 10 min. The movement was recorded and analyzed by Anymaze software (Stoelting Co., Wood Dale, IL). The total number of entries into the dark and light chamber, the total time spent in the light chamber (percentage of time spent in light) and latency to first enter the dark compartment were examined. After each trial, the chambers were cleaned, and the next mouse was placed for testing.

Statistical analyses

Statistical analyses were performed using Graphpad Prism (Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA). The tests for outliers (Grubb's test) and normality (Shapiro-Wilk test) were performed on each data set. If the data followed normal distribution a t-test (two tailed) was performed. If the data was not normally distributed, a nonparametric Mann Whitney test was performed to compare the two conditions. All plots in the manuscript were generated using GraphPad software. Results are presented as mean values ± the standard error of the mean (SEM). Differences were considered significant when $p < 0.05$ and indicated in the figure legend as appropriate.

RESULTS

Probiotic supplementation altered the fecal microbiome of both C57BL/6 (WT) and App^{NL-G-F} mice

To investigate the existence of gut dysbiosis and the effect of eight weeks of VSL#3 probiotic supplementation in AD mice, fecal samples of WT and App^{NL-G-F} (AD) mice were subjected to 16S rRNA sequencing. The relative abundance results showed a dramatic difference across treatments in control and AD mice (Fig. 1A). App^{NL-G-F} mice had a significant increase in *Clostridia, Lachnospiracea*, and *Akkermansia* genera and both an increase and a decrease in order *Bacteroidales* following probiotic treatment (Fig. 1B). While a decrease in *Lachnospiracea, Alistepes,* and *Oscillibacter* was observed in WT mice treated with probiotics (Fig. 1B).

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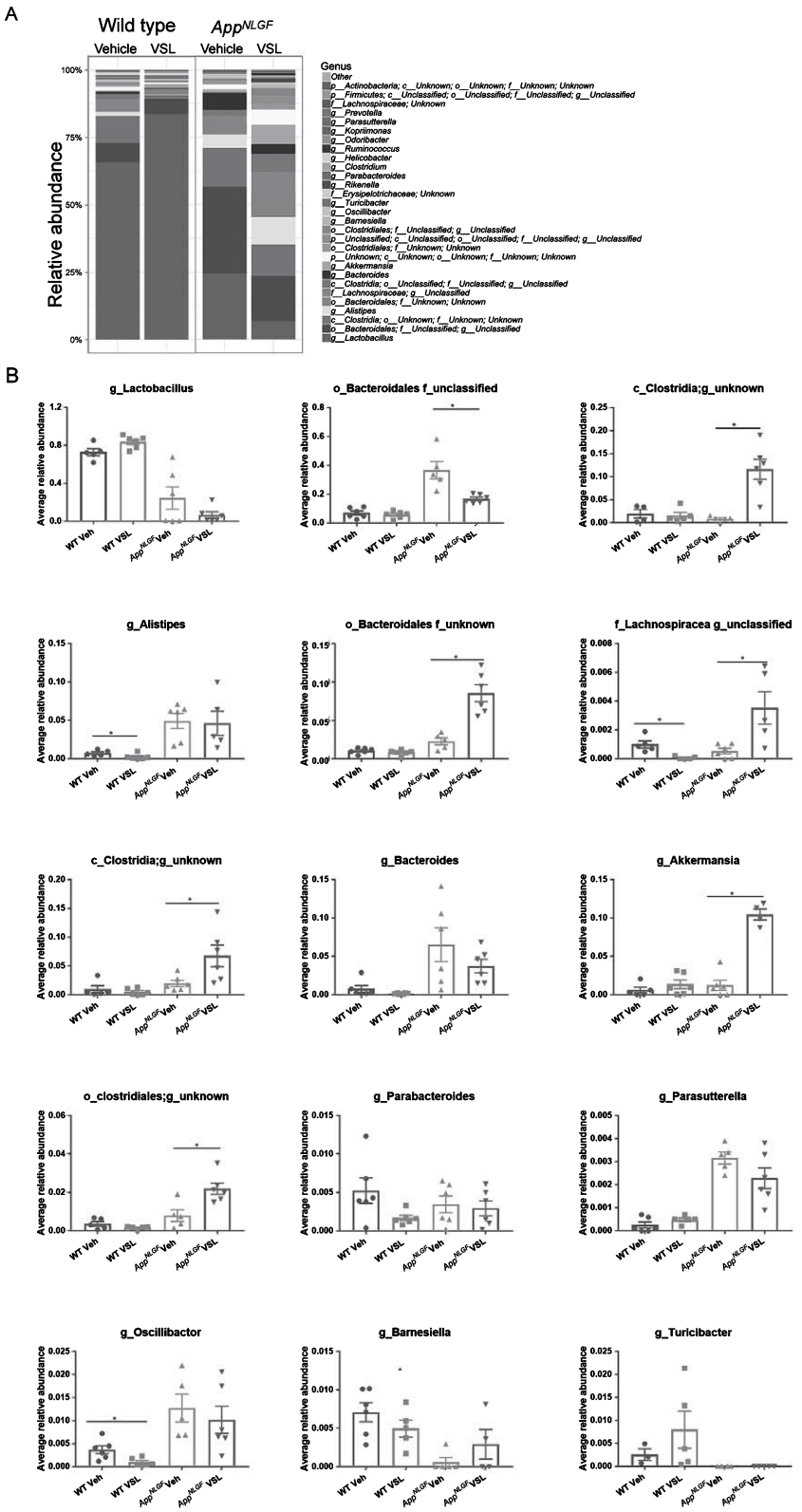


Fig. 1. (*Continued*)

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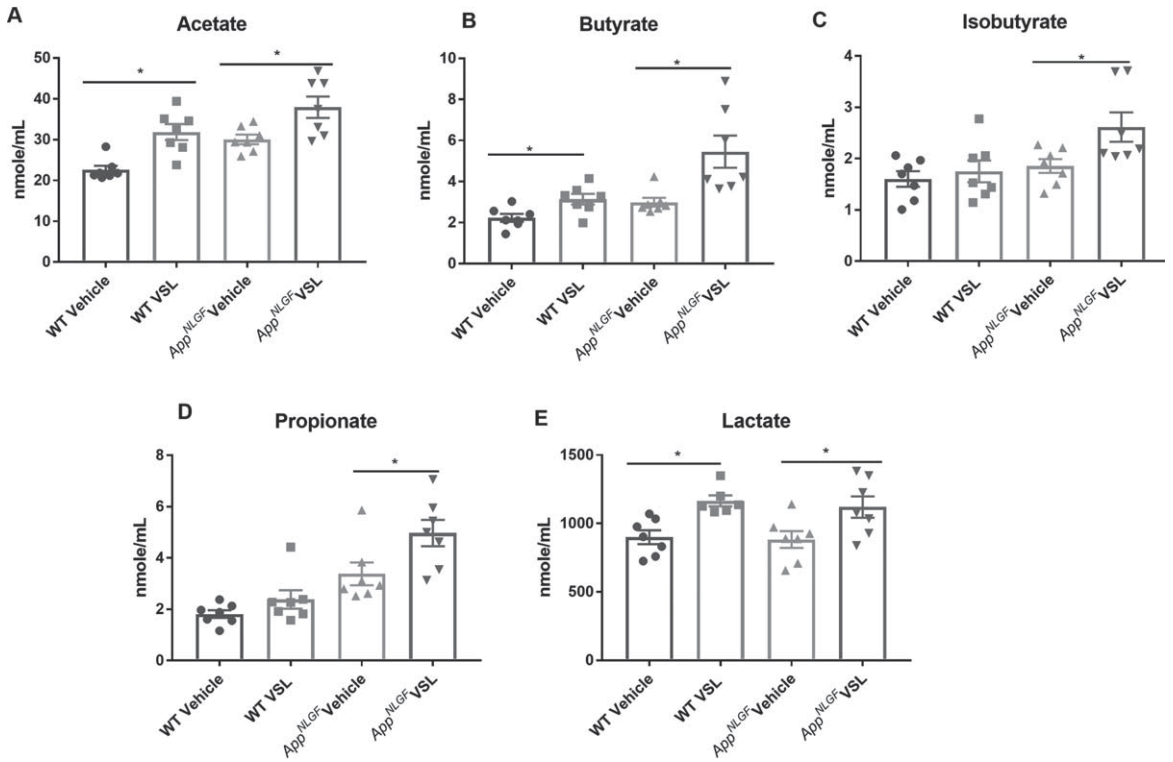


Fig. 2. Effects of VSL#3 supplementation on serum concentrations of short chain fatty acids (SCFAs) acetate (A), butyrate (B), isobutyrate (C), lactate (D), and propionate (E). Female C57BL/6 wild type and App^{NL-G-F} mice were provided normal drinking water or water supplemented with probiotic, VSL#3, *ad libitum* from 6–8 months of age. Blood was collected upon termination and centrifuged to collect serum for SCFA analysis performed using UPLC-MS/MS. Seven animals per group were examined. Data are presented as mean ± SEM. Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by *t*-test; $^{*}p < 0.05$.

Probiotic supplementation increased serum SCFAs inWT and App$^{\mathrm{NL-G-F}}$*mice*

In order to investigate the effect of altered gut bacterial composition on bacterial fermentation end-products, we elected to quantify levels of SCFAs, acetate, butyrate, isobutyrate, lactate, and propionate in serum from WT and App^{NL-G-F} mice. We also examined the impact of probiotic supplementation on the concentration of SCFAs in serum. Supplementation with probiotic significantly increased acetate, butyrate, lactate, isobutyrate, and propionate in the serum of VSL#3 fed App^{NL-G-F} mice compared to their vehicle controls (Fig. 2). The dietary response was highly variable between genotypes, as probiotic supplementation increased only acetate and lactate in WT mice (Fig. 2). To understand the association between alterations in serum SCFAs and changes in gut bacteria, a Pearson's correlation analysis was performed, and the results graphed as a correlation map (Fig. 3A) and as a *p* value map (Fig. 3B) for comparison. The results showed that an increase in serum propionate in vehicle fed App^{NL-G-F} mice correlated with a high abundance of *Clostridia* class

Fig. 1. Effects of VSL#3 probiotic supplementation on fecal bacterial genus profile in App^{NL-G-F} and wild type mice. Fecal samples were collected from female C57BL/6 wild type and App^{NL-G-F} mice with and without *ad libitum* VSL#3 in the drinking water from 6–8 months of age. Bacterial genera were determined from 16S rRNA sequencing. A) Relative abundances of the 30 most abundant genera in stool samples of wild type (WT) and App^{NL-G-F} mice are graphed. B) The relative abundances of the top 15 most abundant genera across the treatment groups were statistically compared. Operational taxonomic units (OTUs) of some samples lacked a taxonomy label at the genus level and are represented as order or family only. 6 animals per group were examined. Data are presented as mean values ± standard error of mean (SEM). Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by Mann–Whitney test, $^{*}p < 0.05$.

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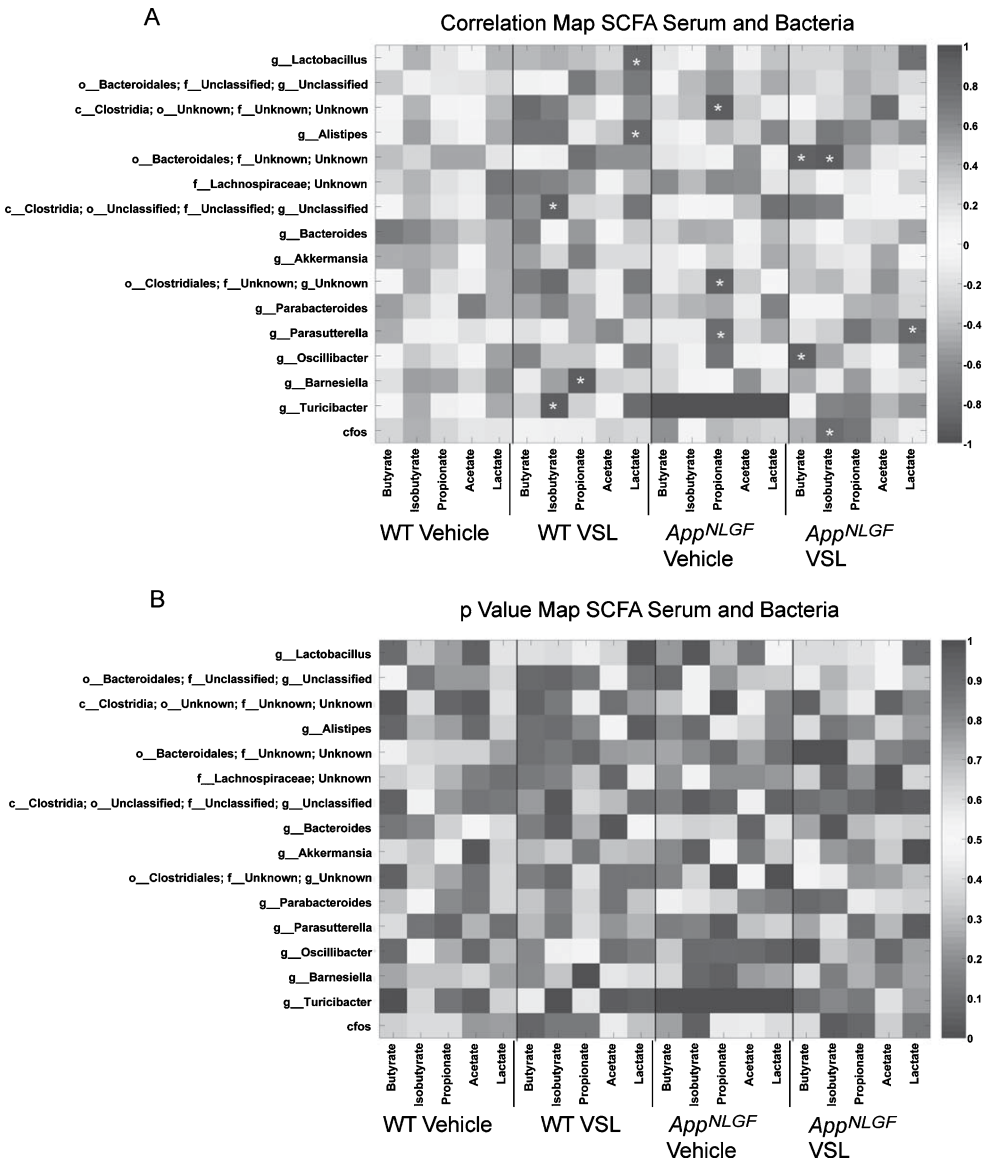


Fig. 3. Comparison of serum SCFAs and fecal microbiome bacteria in C57BL/6 (WT) and App^{NL-G-F} mice. Heat maps are presented of the Pearson correlation (A) and the p value (B) of serum SCFAs and fecal bacterial composition. Asterisks in (A) indicate statistically significant positive or negative correlation.

bacteria and a low abundance of the *Parasutterella* genus. However, there was no significant correlation between increased other serum SCFAs and any of the bacteria genera. Following probiotic supplementation, the order *Bacteroidales* positively correlated with an increase in serum butyrate and isobutyrate in App^{NL-G-F} mice (Fig. 3). A decrease in the genus *Parasutterella* negatively correlated with an increase in lactate in brains of App^{NL-G-F} mice treated with probiotics (Fig. 3).

Probiotic supplementation increased hippocampal SCFAs in $App^{\mathrm{NL-G-F}}$ *mice*

The impact of probiotic supplementation on levels of acetate, butyrate, isobutyrate, lactate, and propionate were assessed in hippocampi from WT and App^{NL-G-F} mice. Surprisingly, probiotic supplementation decreased acetate and propionate SCFAs in wild type mice (Fig. 4). Similar to the serum findings, elevated levels of acetate and lactate were observed

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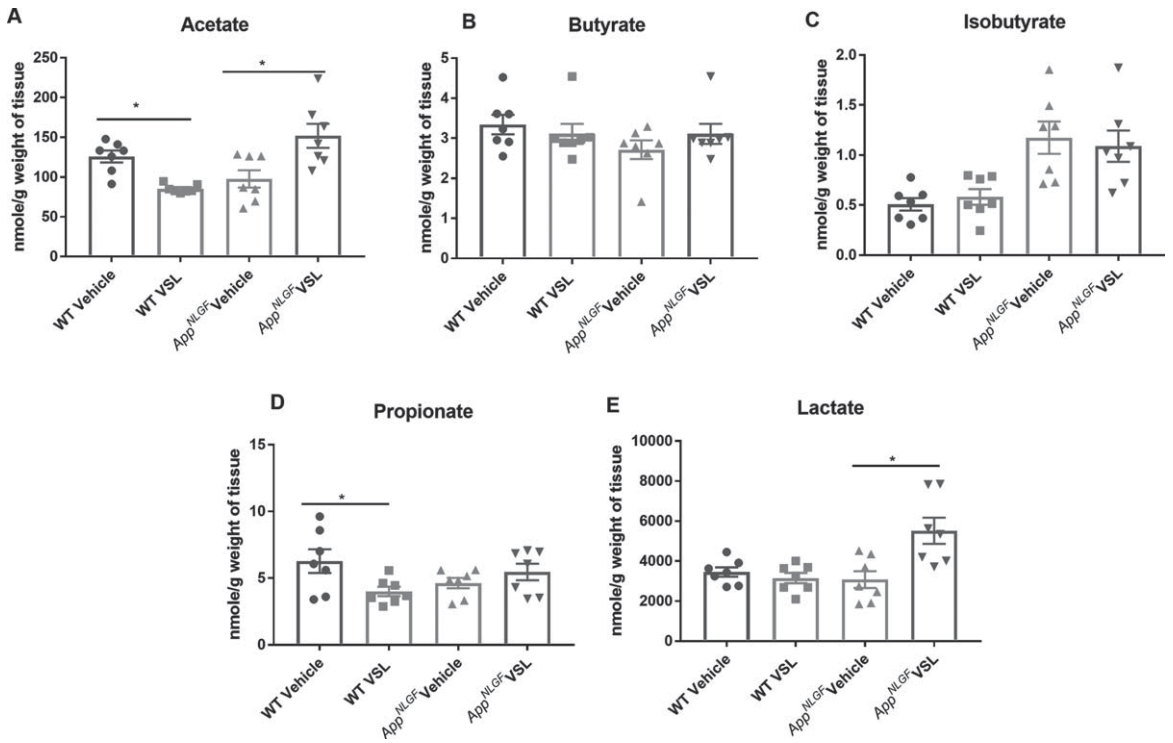


Fig. 4. Effect of VSL#3 supplementation on hippocampal concentrations of short chain fatty acids (SCFA) acetate (A), butyrate (B), isobutyrate (C), lactate (D), and propionate (E). Female C57BL/6 wild type and App^{NL-G-F} mice were provided normal drinking water or water supplemented with probiotic, VSL#3, *ad libitum* for four weeks from 6–8 months of age. Hippocampi were isolated, and concentrations of SCFA, were analyzed using UPLC-MS/MS. Seven animals per group were examined. Data are presented as mean ± SEM. Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by *t*-test; $*p < 0.05$.

in the hippocampi ofVSL#3 fed App^{NL-G-F} mice (Fig. 4). Correlation analysis was performed comparing fecal bacterial composition and brain SCFAs using Pearson's correlation and graphed as a correlation maps (Fig. 5A) or a *p* value map (Fig. 5B). The increase in hippocampal acetate positively correlated with the genus *Lactobacillus* and negatively correlated with the genus *Parasutterella* in App^{NL-G-F} mice treated with probiotics (Fig. 5). The VSL#3-induced increase in hippocampal lactate levels negatively correlated with class *Clostridia* (Fig. 5).

Probiotic supplementation had no effect on Ki-67 immunoreactivity in App^{NL-G-F} *mice*

Recently, gut microbiota have been reported to influence neurogenesis in the brain[33]. Using Ki-67 immunoreactivity as a maker of proliferation and a surrogate for possible neurogenesis changes, we investigated whether altered gut microbiota in App^{NL-G-F} mice had any effect on proliferation in the dentate gyrus and subventricular zone. Although there appeared to be less overall Ki-67 immunoreactivity in App^{NL-G-F} dentate gyrus compared to WT, the probiotic supplementation for eight weeks showed no effect on Ki-67 immunoreactivity in either the dentate gyrus or the subventricular zone ofApp^{NL-G-F} or WT mice (Fig.6).

Probiotic supplementation increased c-Fos immunoreactivity in App^{NL-G-F} *mice*

As a measure of assessing probiotic supplementation effects on neuronal activity, c-Fos immunostaining was performed on brain sections. c-Fos is commonly used as a functional marker of activity in neurons [34]. Eight weeks of probiotic supplementation resulted in a significant increase in c-Fos immunoreactivity in App^{NL-G-F} mice treated with VSL#3 with no change in WT mice (Fig. 7).

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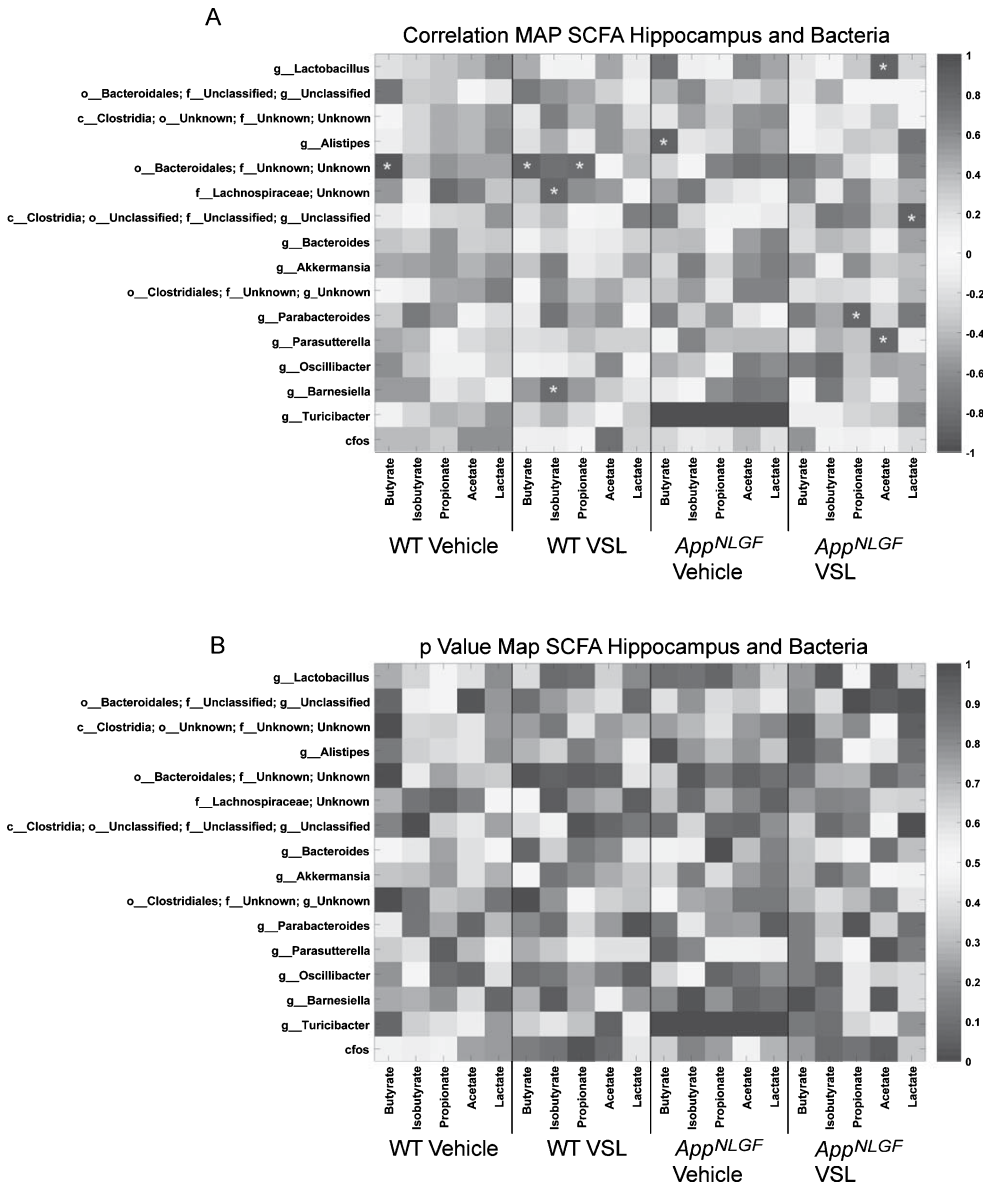


Fig. 5. Comparison of hippocampal SCFAs and fecal microbiome bacteria in C57BL/6 (WT) and App^{NL-G-F} mice. Heat maps are presented of the Pearson correlation of (A) and the *p* value for hippocampal SCFAs and fecal bacterial composition. Asterisks in (A) indicate statistically significant positive or negative correlation.

Probiotic supplementation had no effect on Aβ, GFAP, and Iba-1 immunoreactivityin App^{NL-G-F} mice

Since we observed are markable difference in serum and brain SCFA levels following probiotic supplementation, we examined whether changes occurred in plaque load and gliosis in App^{NL-G-F} mice. Immunohistochemistry for Aβ, GFAP, and Iba-1 was performed using brain sections from WT and App^{NL-G-F} mice. Surprisingly, probiotic supplementation did not alter microgliosis, astrogliosis, or Aβ plaque load in App^{NL-G-F} mice (Fig. 8).

Probiotic feeding had no effect on Aβ levels in the hippocampus App^{NL-G-F} mice

In order to provide a more quantifiable assessment of brain Aβ levels, ELISAs were next performed. The levels of $A\beta_{1–40}$ and $A\beta_{1–42}$, both soluble and insol-

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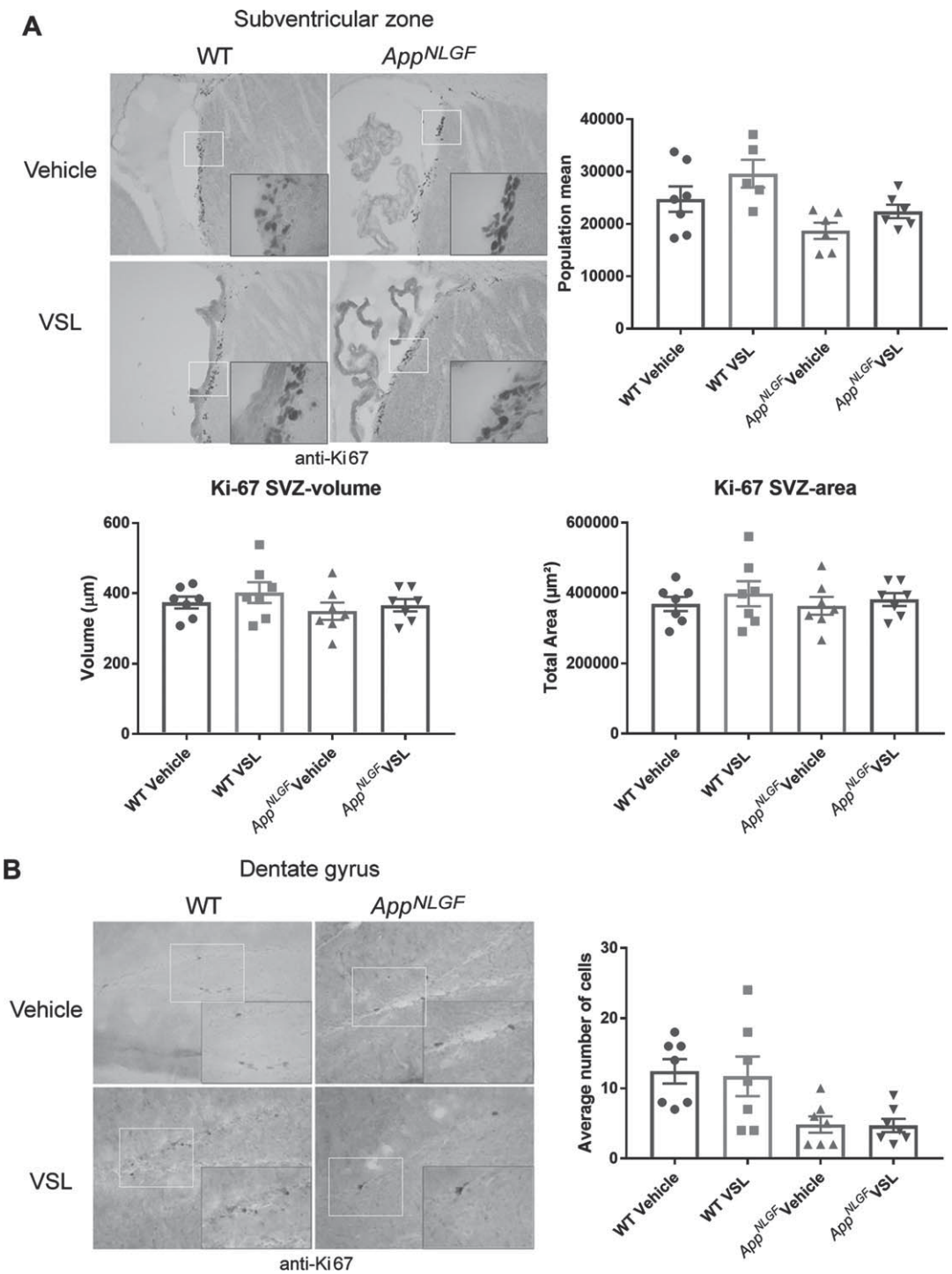


Fig. 6. Effect of VSL#3 supplementation on neurogenesis in the subventricular zone (SVZ) and dentate gyrus of the hippocampus. Brain sections from female C57BL/6 wild type and App^{NL-G-F} mice with and without VSL#3 supplementation from 6–8 months were stained with Ki-67 antibody. Seven animals per group were examined and Ki-67 positive cells were counted in 4 sections from each animal. Stereological quantification was done in the (A) SVZ region to count Ki-67 positive cells. B) However, manual counting was done in the dentate gyrus due to the limited numbers of detectable Ki-67 positive cells. Representative images are shown, and data are presented as mean ± SEM. Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by *t*-test; * $p < 0.05$.

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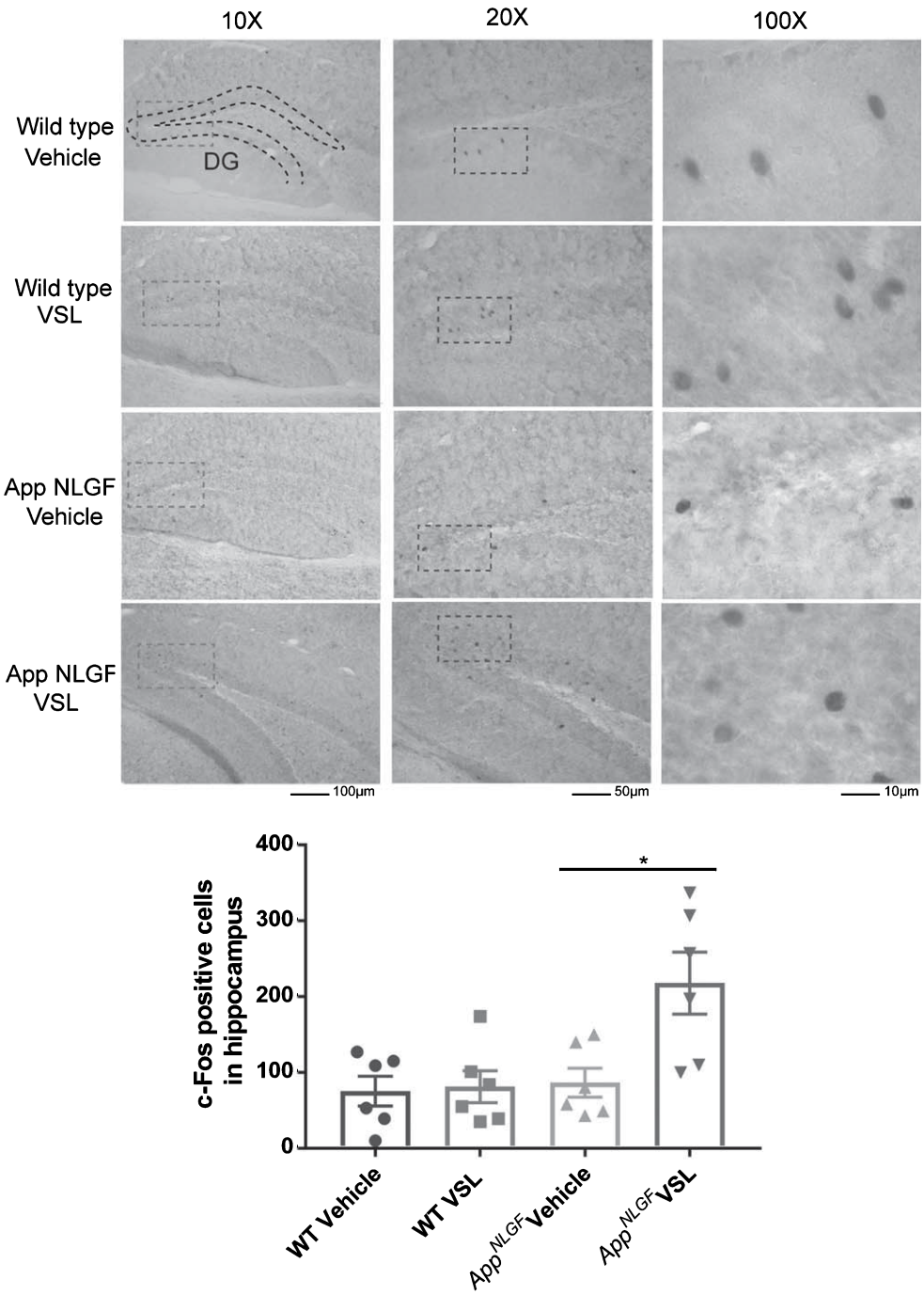


Fig. 7. Effect of VSL#3 dietary supplementation on hippocampal c-Fos immunoreactivity. Female C57BL/6 wild type and App^{NL-G-F} mice were provided normal drinking water or water supplemented with probiotic, VSL#3, *ad libitum* from 6–8 months of age. Brain sections from each treatment group were stained with anti-c-Fos antibody (purple labeling). Representative images of dentate gyrus (DG) of hippocampi are shown at 10X, 20X, and 100X magnification with the positive cells clearly visible at the higher magnifications and the anatomical references points indicated for dentate gyrus. The positive cells were counted and values were averaged from 3 sections per brain. Six animals per group were examined and the data are presented as the mean ± SEM. Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by *t*-test; $^*p < 0.05$.

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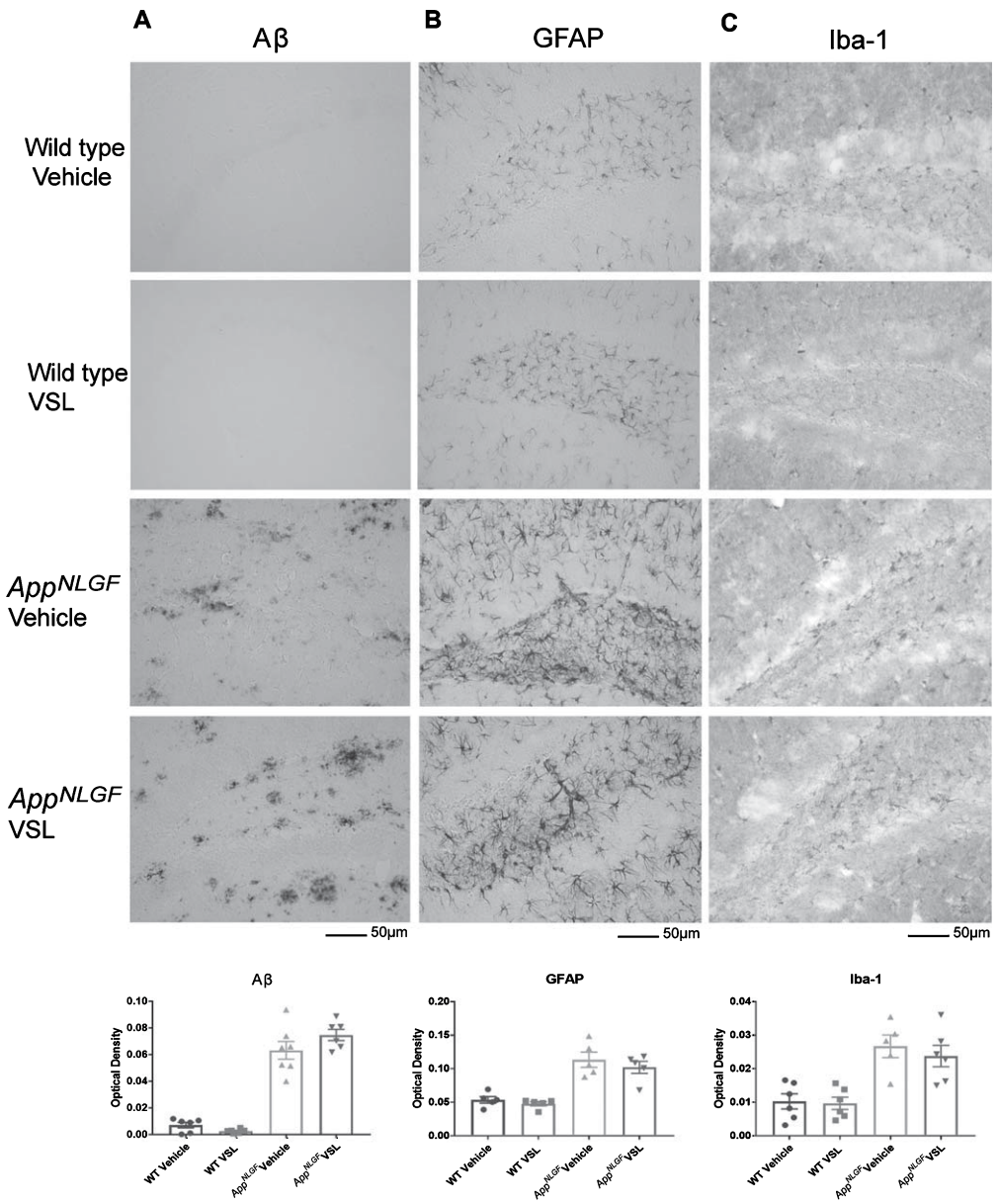


Fig. 8. Effect of VSL#3 supplementation on Aβ accumulation and gliosis in brains of C57BL/6 (WT) and App^{NL-G-F} mice. Female C57BL/6 wild type and App^{NL-G-F} mice were provided normal drinking water or water supplemented with probiotic, VSL#3, *ad libitum* from 6–8 months of age. Brain sections were immunolabeled for Aβ (A), GFAP (B), and Iba-1 (C). Representative images are shown from the hippocampus region and were taken at 20X. Quantitation of immunolabeling was performed using optical density values and the data are presented as mean ± SEM. Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by *t*-test; * $p < 0.05$ ($n = 7$). Scale bar is 50 μm.

uble, were assessed from the hippocampi of WT and App^{NL-G-F} mice. As expected, App^{NL-G-F} mice demonstrated high levels of soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ (Fig. 9). However, probiotic supplementation of App^{NL-G-F} animals had no effect on Aβ levels, consistent with our immunolabeling findings (Fig. 9).

Probiotic supplementation altered anxiety-like behavior in App^{NL-G-F} mice

Even though we did not observe a change in plaque load or gliosis in VSL#3 fed App^{NL-G-F} mice, the increased c-Fos immunoreactivity and changes in brain SCFA levels demonstrated clear brain effects.

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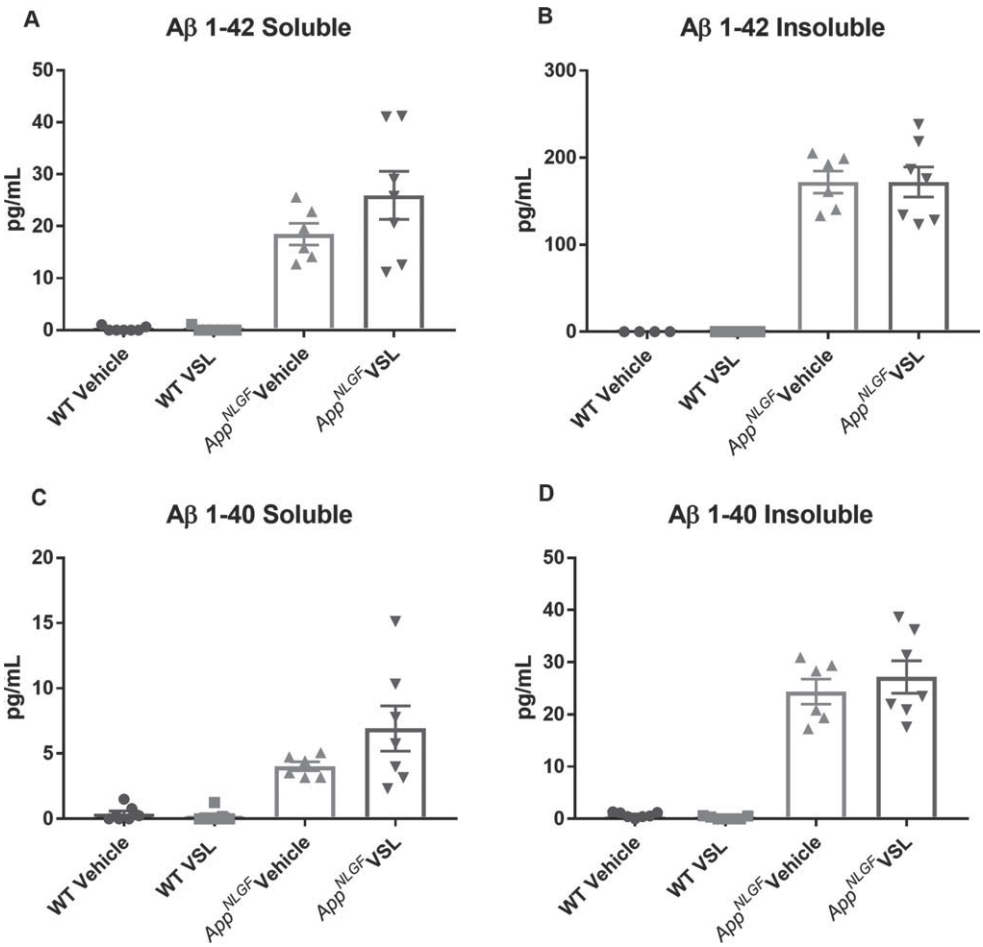


Fig. 9. Effect of VSL#3 dietary supplementation on hippocampal Aβ levels. Female C57BL/6 wild type and App^{NL-G-F} mice were provided normal drinking water or water supplemented with probiotic, VSL#3, *ad libitum* from 6–8 months of age. Hippocampi were isolated, lysed, and levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, both soluble and insoluble, were quantified by ELISA. Seven animals per group were examined and the data are presented as mean ± SEM. Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by t-test; *$p < 0.05$.

Since we previously did not observe probiotic feeding effects on memory in App^{NL-G-F} mice [35], we elected to quantify whether VSL#3 feeding might influence a distinct behavioral dysfunction associated with AD, anxiety. Anxiety-like behavior was quantified using a light-dark apparatus. The total number of entries into the dark chamber, total time spent in the light chamber (percentage of time spent in light) and latency to first enter the dark compartment were examined. VSL#3 supplementation of the App^{NL-G-F} mice decreased the number of entries into the dark and normalized the latency to first enter the dark to WT levels demonstrating an anxiolytic effect of the probiotic feeding (Fig. 10).

DISCUSSION

In this study we assessed potential beneficial effects of modulating gut bacteria using the probiotic VSL#3 in an amyloidosis model of AD, App^{NL-G-F} mice. We found that VSL#3 had a differential effect in WT and App^{NL-G-F} mice which could be due the difference in the microbiota composition between two genotypes. App^{NL-G-F} mice had a distinct microbiota when compared to WT mice which was associated with several differences in both serum and brain SCFA concentrations between WT and App^{NL-G-F} mice with and without the probiotic supplementation. Probiotic feeding and SCFA changes did not correlate with increased

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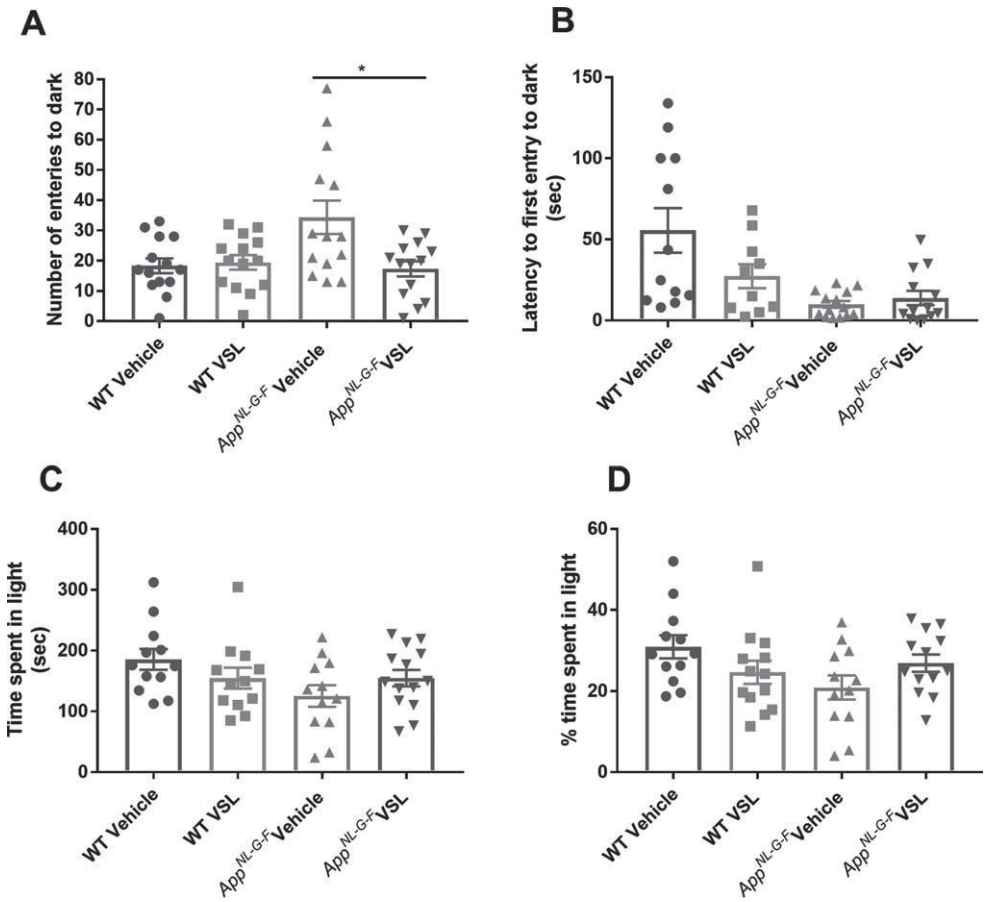


Fig. 10. Effect of VSL#3 supplementation on anxiety in C57BL/6 (WT) and App^{NL-G-F} mice. Female C57BL/6 wild type and App^{NL-G-F} mice were provided normal drinking water or water supplemented with probiotic, VSL#3, *ad libitum* from 6–8 months of age. All mice were subjected to light-dark test after 8 weeks of treatment and (A) total number of entries into the dark compartment, (B) latency of first entry into the dark compartment, (C) time spent in the light compartment, and (D) the percentage of time spent in the light were determined. Data are represented as the mean ± SEM ($n = 14$). Significant differences between vehicle and VSL#3 treated animals in each genotype were determined by *t*-test; * $p < 0.05$.

neurogenesis-like proliferation in the App^{NL-G-F} mice or alter plaque load or gliosis. However, VSL#3 supplementation significantly increased hippocampal c-Fos staining in App^{NL-G-F} mice that correlated with increases in brain lactate and acetate levels and reduced anxiety-like behavior.

Our fecal microbiome analysis revealed comparatively low levels of genera *Lactobacillus* and *Barnesiella* and elevated amounts of *Parasutterella* and *Alistipes* in App^{NL-G-F} mice. VSL#3 feeding increased *Clostridia, Lachnospiracea*, and *Akkermansia* genera in the App^{NL-G-F} mice. Gut bacterial metabolites are considered important for neurological function. The chief inhibitory neurotransmitter in the CNS, γ- Aminobutyric acid (GABA), can be produced by *Lactobacillus* and *Bifidobacterium* in the gut. It has been shown that decreased *Lactobacillus* results in a decrease in GABA in the intestines as well as CNS and correlates with suppression of inflammatory immune response [36]. A recent study suggests an association of *Parasutterella* with increased intestinal inflammation and development of irritable bowel syndrome in humans and mice [37]. Additional work supports this indicating that increased abundance of *Parasutterella* is associated with dysbiosis [38]. Therefore, the *Parasutterella* in App^{NL-G-F} mice could be contributing to their intestinal dysbiosis and increased inflammation. Increased levels of *Alistipes* and *Enterobacteriaceae* in conjunction with reduced *Faecalibacterium* have been found in patients with depressive disorders [39]. On the other hand, the Akkermansia-induced increase with VSL#3 feeding corresponds well with its role as a probiotic and

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well characterized immunomodulatory, metabolic, and intestinal protective benefits [40–42].

Alterations in gut microbiota often produce changes in microbial metabolites including SCFAs. Microbiota-derived SCFAs cross the blood-brain barrier and play an important role in gut-brain signaling mechanisms. Acetate, propionate, and butyrate are produced in the colon by bacterial fermentation of dietary fibers and are known to have a role in brain function and pathophysiology [43]. For example, changes in plasma as well as fecal SCFA concentrations have been associated with several metabolic disorders [44–46]. We observed a VSL#3-dependent increase in levels of acetate, butyrate, lactate, isobutyrate, and propionate in the serum of App^{NL-G-F} mice with only increases in acetate, lactate, and butyrate in WT mice. However, only acetate and lactate were elevated in brains of App^{NL-G-F} mice. The high levels of *Bacteroidetes*, known producers of acetate and propionate, in App^{NL-G-F} mice could explain the elevated levels observed in App^{NL-G-F} serum [47]. VSL#3 supplementation produced genotype selective effects resulting in decreased *Alistipes, Lachnospiraceae,* and *Oscillibactor* in WT mice and an increase in several genera including *Lachnospiraceae*, *Clostridia*, *Akkermansia*, and a decrease as well as increase in order *Bacteroidales* in App^{NL-G-F} mice. *Lactobacillus* are producers of acetate and lactate [48] consistent with the high levels of this genus observed in WT mice and the elevations of these SCFAs in serum of WT animals treated with probiotics. *Akkermansia muciniphila* produce both propionate and acetate suggesting the increased abundance in probiotic fed App^{NL-G-F} mice may contribute to the observed elevations in serum and brain [49–51]. A recent human study also showed the effect of altered gut microbiota composition on gut metabolites or tryptophan metabolism in probiotic supplemented AD patients [52]. Although the changes in serum and brain SCFAs were expected based upon prior study of VSL#3 feeding [53], further work is required to determine whether particular SCFAs are responsible for the behavioral or cellular changes we observed in the brain.

For example, butyrate is the main energy source for colonocytes and it is known that most of the butyrate-producing bacteria belong to *Clostridium clusters IV and XIVa*, including *Ruminococcaceae*, *Lachnospiraceae*, and *Butyricicoccus* [54]. We observed an increase in serum butyrate in App^{NL-G-F} mice fed with VSL#3 corresponding with increased *Lachnospiraceae* and *Clostridium* species. However, no change in butyrate was observed in VSL#3 treated WT mice in agreement with no change in levels of *Lachnospiraceae* and *Clostridia*. Again, this demonstrated a genotype-selective effect of probiotic feeding. Since SCFAs, including butyrate, reportedly increase the growth rate of human neural progenitor cells and increase neurogenesis[55–57], we assessed whether VSL#3 feeding affected neurogenesis in the App^{NL-G-F} mice. Indeed, VSL#3 feeding reportedly increases synaptogenesis and neurogenesis in mice [58, 59] similar to effects seen with intravenous or intraperitoneal administration of sodium butyrate [60, 61]. Although we assessed reduced neurogenesis-associated proliferation in App^{NL-G-F} mice, we observed no benefit from probiotic feeding. This may be due to the fact that VSL#3 feeding only enhanced serum levels of butyrate with no changes in the brain. Future efforts with prolonged or higher dosage probiotic administration well as improved methods for quantifying neurogenesis are required to fully explore any protective effects on elevating brain butyrate levels and enhancing neurogenesis.

Interestingly, a related branched-chain fatty acid, isobutyrate, solely produced by gut bacteria, was increased in the serum of VSL#3 fed App^{NL-G-F} mice. It is unclear why this change was specific to the App^{NL-G-F} mice and not also present in the brain. Gut microbes can use the branched-chain amino acids valine, isoleucine, and leucine to generate branched-chain fatty acids such as isobutyrate, 2-methylbutyrate, and isovalerate [62]. A role of isobutyrate on brain function is unknown and determining whether it has a role in the AD-related changes in App^{NL-G-F} mice requires further work. However, previous reports suggests that it, along with other SCFAs, could affect the pathophysiology of AD by affecting gliosis, inflammation, and Aβ/tau aggregation [63].

Although we observed no changes in neurogenesis correlating with brain SCFA levels after probiotic feeding, a recent study showed the capability of SCFAs to potently inhibit Aβ aggregation *in vitro* [64]. Based upon this we assessed possible effects of probiotic feeding on App^{NL-G-F} plaque load. Surprisingly, VSL#3 treatment resulted in no changes in Aβ levels, plaque load or gliosis. Never the less, longer treatment periods or increased dosage of VS#3 in future work may produce effects on Aβ deposition and subsequent gliotic and inflammatory changes.

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In spite of the lack of change in plaques or gliosis, VSL#3 feeding significantly and selectively increased neuronal c-Fos staining only in App^{NL-G-F}. c-Fos is a transcription factor widely used as a functional marker of activity in neurons and neuronal circuitry after a variety of stimuli [65]. Increased lactate levels could be involved in modulating the neuronal activity selectively in the App^{NL-G-F} mice. Lactate can modulate neuronal excitability via ATP-dependent mechanisms involving modulation of K_{ATP} channels [66]. Lactate has also been shown to induce expression of synaptic plasticity-related genes such as Arc, c-Fos, and Zif268 in neurons through a mechanism involving N-methyl-D-aspartate (NMDA) receptor activity and its downstream signaling cascade mediated by Extracellular signal-regulated kinase (ERK) 1/2 [67]. Alternatively, enhanced neuronal excitability in App^{NL-G-F} mice treated with VSL#3 might be due to altered neurotransmitter levels in the brain following probiotic treatment. Gut bacteria produce and/or consume a wide range of neurotransmitters, including dopamine, norepinephrine, serotonin, and GABA [68]. c-Fos and other immediate early genes are known to be highly responsive to extracellular stimulation by neurotransmitters, membrane depolarization, and trophic substances [69, 70]. The mechanism or consequences of elevated c-Fos expression and presumed neuronal activity in VSL#3 fed App^{NL-G-F} mice likely involve complex changes such as epigenetic modifications and will require additional effort to define [71].

In summary, our study demonstrates that the probiotic, VSL#3, affects control and diseased mice by uniquely induced differences in gut bacterial composition and SCFA concentration changes in both serum and brain. App^{NL-G-F} mice had a disease-, but not treatment-, specific elevation of brain isobutyrate levels. Although probiotic treatment did not alter plaque load or gliosis, it did increase brain lactate levels in App^{NL-G-F} mice correlating with increased c-Fos inferred neuronal activity and reduced anxiety-like behavior. Further effort with increased dose/duration or younger age of treatment onset using probiotics such as VSL#3 may provide robust effects on brain presentation of AD.

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High Salt Elicits Brain Inflammation and Cognitive Dysfunction, Accompanied by Alternations in the Gut Microbiota and Decreased SCFA Production

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Abstract.
Background: Excessive salt intake is considered as an important risk factor for cognitive impairment, which might be the consequence of imbalanced intestinal homeostasis.
Objective: To investigate the effects of dietary salt on the gut microbiota and cognitive performance and the underlying mechanisms.
Methods: Adult female C57BL/6 mice were maintained on either normal chow (control group, CON) or sodium-rich chow containing 8% NaCl (high-salt diet, HSD) for 8 weeks. Spatial learning and memory ability, short-chain fatty acids (SCFAs) concentrations, gut bacterial flora composition, blood-brain barrier permeability, and proinflammatory cytokine levels and apoptosis in the brain were evaluated.
Results: The mice fed a HSD for 8 weeks displayed impaired learning and memory abilities. HSD significantly reduced the proportions of Bacteroidetes (S24-7 and Alloprevotella) and Proteobacteria and increased that of Firmicutes (Lachnospiraceae and Ruminococcaceae). SCFA concentrations decreased in the absolute concentrations of acetate, propionate, and butyrate in the fecal samples from the HSD-fed mice. The HSD induced both BBB dysfunction and microglial activation in the mouse brain, and increased the IL-1β, IL-6, and TNF-α expression levels in the cortex. More importantly, the degree of apoptosis was higher in the cortex and hippocampus region of mice fed the HSD, and this effect was accompanied by significantly higher expression of cleaved caspase-3, caspase-3, and caspase-1.
Conclusion: The HSD directly causes cognitive dysfunction in mice by eliciting an inflammatory environment and triggering apoptosis in the brain, and these effects are accompanied by gut dysbiosis, particularly reduced SCFA production.

Keywords: Blood-brain barrier, cognition, gut microbiota, high-salt diet, microglia, short-chain fatty acids

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INTRODUCTION

High salt is thought to promote cognitive decline via hypertension [1, 2], but excessive salt can also impact brain function without inducing a marked increase in the blood pressure [3, 4]. A recent study showed that a high-salt diet (HSD) reduces the resting blood flow to the brain and caused dementia in mice [5]. In addition, an increase in the gut proportion of Th17 cells, which produce interleukin-17 (IL-17) to induce immune regulation and proinflammatory effects [6, 7], impair the synaptic plasticity, and increase oxidative stress [8], was also found to be involved in HSD-related cognitive dysfunction, which indicates the intricate effects of high salt on cognition.

The human gastrointestinal tract harbors trillions of microorganisms, which include at least 1000 different species of known bacteria with more than 3 million genes, and these microorganisms are termed the gut microbiota (GM) [9]. The GM and its metabolites can modulate not only gastrointestinal function but also enter the circulation to affect the brain. The complex interplay between the GM and the brain is denoted as the "gut-brain axis" [10], and this bidirectional interaction appears to act via neural, endocrine, immune, and humoral links. More than 90% of the important brain neurotransmitter 5-HT is synthesized in the gut [11], and at least 20% of the small molecules in human blood are products of the microbiota [12]. An important set of metabolite of carbohydrates produced by the microbiota in the lower gut, namely, short-chain fatty acids (SCFAs), which are the main energy supply material of the colon and small intestine epithelial cells, modulates microglia development and function through undetermined mechanisms [13, 14].

Because sodium added or inherent to food contributes to nearly 99% of the total sodium intake [9], the effects of dietary salt on the GM and brain function need to be elucidated. A recent study demonstrated that an HSD exerts a certain effect on protein digestion and the composition of the GM [15, 16]. To explore the association between the GM and cognitive performance induced by dietary salt, we used a mouse model fed an HSD for 8 weeks and found cognitive dysfunction, alteration of the GM, decreases in the concentrations of fecal SCFAs and increases in the blood-brain barrier (BBB) permeability.

MATERIAL AND METHODS

Animals and treatments

The adult female C57BL/6 mice (8–10 weeks, 20–24 g) used in the study were purchased from the Nanjing Biomedical Research Institute of Nanjing University and housed in the Animal Care Facility at Guangdong Medical University. The mice were maintained in a pathogen-free environment on a 12 h light/12 h dark cycle. The mice were randomly grouped after one week of acclimatization and were supplied water and chow ad libitum. The mice received normal chow and tap water (control group) or sodium-rich chow containing 8% NaCl (high-salt diet, HSD group). After 8 weeks of treatment, the mice were subjected to the Morris water maze (MWM). The mice were subsequently anaesthetized and killed via the inhalation of isoflurane. Brain tissues were collected and stored at –80°C for further analyses as detailed below. The animal protocol was approved by the Ethics Committee of Guangdong Medical University in accordance with the guidelines of the National Institutes of Health (NIH) on the care and use of animals.

Morris water maze

The MWM task was used to assess the spatial learning and memory ability of the mice as described previously. Briefly, a round pool with a diameter of 1.2 m was filled with water (22 ± 1°C) that was made opaque with a nontoxic white pigment, and a circular platform (10 cm in diameter) was submerged ~1 cm beneath the surface of the water and placed in the center of one quadrant of the pool. The platform remained in the same position throughout the learning trials and was removed from the pool during the probe test. Several additional maze cues were placed around the pool and remained in the same position throughout the training and testing periods. For training, the mice were subjected to four trials per day for 5 consecutive days (at 30 min intervals). The data obtained each day were averaged across the four trials. A probe trial was conducted on day 6; the hidden platform was removed, and the mice were placed in the quadrant opposite to the target quadrant and allowed to swim for 90 s. The number of platform crossings was scored. During the training period (days 1–5), the time that it took the mice to find the platform was recorded as the escape latency, and an

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escape latency of 90 s was recorded if the animal did not find the platform. The data were analyzed by two-way repeated-measures ANOVA with the group as a between-subject factor and the trial day as a within-subject factor for the training trials. For the probe trials, the quadrant or platform location was used as the within-subject factor.

DNA extraction from mouse feces and microbiota analysis

Individual fecal pellets from each mouse were collected and pooled in separate autoclaved cages within 30 min. The microbial DNA from the fecal samples was extracted using the QIAamp® PowerFecal DNA Kit (QIAGEN GmbH, Germany) according to the manufacturer's recommended protocols. The final DNA concentration and purification were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the DNA quality was checked by 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primers 338F (5'- ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') and a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR programs were as follows: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s of annealing at 55°C, and 45 s of elongation at 72°C; and a final extension at 72°C for 10 min. The PCRs were performed in triplicate using 20 μL reaction volumes consisting of 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel, further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's recommended protocol. An Illumina MiSeq was run for 250 cycles to produce paired-end reads. The data were analyzed using the free online Majorbio I-Sanger Cloud Platform (http://www.i-sanger.com).

Histology

From each mouse, the right half of the brain was removed and fixed in 4% buffered formalin. The brain tissue was dehydrated in gradient sucrose solution and then embedded in OCT. For Iba1 immunohistochemistry, 15 μm coronal cryosections were prepared and stained with the anti-Iba1 antibody (1 : 100 dilution, cat. no. ab143696, Abcam) for 24 h at 4°C and then with the secondary antibody (Alexa Flour 488-conjugated anti-rabbit IgG, 1 : 800) at RT. For the assessment of apoptotic cells in the mouse cortex and hippocampus, the brain sections were stained with Hoechst 33258 as described previously [17] and according to the manufacturer's instructions (Beyotime Biotechnology Inc, China).

Fecal SCFA analyses

The fecal concentrations of SCFAs (acetate, propionate, and butyrate) were determined by gas chromatography (GC) as described previously [18]. Briefly, 500 mg of feces was homogenized with 1 mL of phosphate buffer (pH 7.3) and centrifuged at 4°C and 16,000×g for 15 min. The supernatants were filtered through a 0.45 μm nylon filter (EMD Millipore). For acidification, an aliquot (200 μL) of the supernatant was added to 50 μL of 50% (v/v) sulfuric acid. The mixture was vortex and allowed to stand for 2 min, and the organic acids were extracted by adding 0.4 mL of diethyl ether. The supernatants were measured with a GC-2010 gas chromatograph (Shimadzu Japan) equipped with flame ionization using the autosampler AOC-20s/I (Shimadzu Japan), an injected sample volume of 1 μL, thermal conductivity detectors, capillary columns and GC ChemStation software. Nitrogen was supplied as the carrier gas at an initial flow rate of 1.3 mL/min. The oven temperature was initially set to 100°C, maintained at 100°C for 0.5 min, increased to 180°C at 8°C/min, maintained at 180°C for 1.0 min, increased to 200°C at 20°C/min, and maintained at 200°C for 5.0 min. The temperatures of the detector and the injection port were set to 250°C and 240°C, respectively. An external standard (Supelco™ WSFA-1 Mix, Supelco Sigma-Aldrich Co., Bellefonte, PA, USA) was used for the quantification of SCFAs. The GC solution Chromatography Data System (Shimadzu Deutschland GmbH) was used for data processing.

Evaluation of BBB disruption

The BBB permeability was determined by measuring the amount of Evans blue dye (EB). The extravasation of EB was performed as described previously [19, 20]. Briefly, 2% EB (3 mL/kg body weight, Sigma, USA) was slowly administered intravenously to the mice in the control and HSD groups.

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One hour after injection, the mice were perfused with saline to wash any remaining intravascular EB dye. After decapitation, the brain was rapidly removed and dissected to obtain the left and right hemispheres. The left hemisphere was weighed and homogenized in a 2-fold volume of dimethylformamide. After incubation at 60°C for 72 h, the samples were centrifuged for 10 min at 1500 r/min, and the supernatants were used to measure the absorbance of EB using a spectrophotometer (BioTek, USA) at 635 nm. The amount of EB was determined using a standard curve and is expressed as micrograms per gram of brain tissue.

Immunoblotting and western blot analysis

The brain tissues were lysed by ultrasonication in lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS, 1% Protease Inhibitor Cocktail, and/or Phosphatase Inhibitor (Roche)] and centrifuged. The protein concentrations were determined using the BCA method (Thermo Scientific). Equal volumes of tissue lysates (50 μL of protein) were mixed with SDS loading buffer, and the mixtures were boiled, separated on 8–15% SDS polyacrylamide gels and then transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk in TBS/0.1% Tween-20 (TBST) for 1 h, membranes were incubated with primary antibodies against Iba1 (1 : 1000; ab153696, Abcam), claudin-5 (1 : 500; ab15106, Abcam), caspase-3 (1 : 1000, cat. #9662, Cell Signaling Technology), cleaved caspase-3 (1 : 1000, cat. #9661, Cell Signaling Technology), caspase-1 (1 : 1000, cat. #2225, Cell Signaling Technology), β-tubulin III (1 : 1000, T8578, Sigma) and beta-actin (1 : 1000, cat. #8457, Cell Signaling Technology) overnight at 4°C. The membranes were washed in TBST, incubated with the secondary antibody (1 : 5000 in TBST) for 1 h, and visualized using enhanced chemiluminescence (ECL) (Thermo Fisher Scientific), and the densitometric results were analyzed using ImageJ software. The signal intensity of the proteins of interest was normalized to that of beta-actin or β-tubulin III.

Quantification of cytokines

The concentration of cytokines in mouse cortex lysates was measured by enzyme-linked immunosorbent assay (ELISA) according to the instructions provided by the manufacturer (Beijing 4A Biotech Co., Ltd., China). The absorbance at 450 nm was quantified using an Epoch Microplate Spectrophotometer (BioTek).

Statistical analysis

All the values are expressed as the means ± SEMs. The data were analyzed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA). The data from the MWM and SCFA assays were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. The parametric data were analyzed by unpaired *t* tests. $p < 0.05$ was considered significant.

RESULTS

HSD impairs the spatial learning and memory ability of mice

The spatial learning and memory ability of the mice were assessed using the MWM test. The amount of time that elapsed until the animal climbed onto the platform to escape the water (escape latency) and the number of platform crossings were recorded and analyzed. The mice fed the normal diet or HSD learned to find the platform, as indicated by a reduction in the escape latency over the 5-day training period (Fig. 1A). As demonstrated by repeated-measures two-way ANOVA, the mice in the HSD group spent more time trying to find the platform than those in the control group, and significant differences were found on days 4 and 5. During the probe trial (day 6), the number of platform crossings obtained for the HSD group was significantly lower than that found for the control group, which suggested a deficit in spatial learning memory. Taken together, the results indicated that the spatial learning and memory abilities of the HSD group were significantly damaged.

HSD induces alterations in the GM

To analyze the effects of the HSD on the microbiota composition, we performed 16S ribosomal (rRNA) gene sequencing of the mouse fecal contents. The sequence analysis of the V3–V4 hypervariable regions of the 16S rRNA gene revealed that the microbiota composition was considerably altered by the consumption of the HSD for 8 weeks. The phylum-level analysis showed that the HSD significantly reduced the proportions of Bacteroidetes (50.53 ± 10.75%) and Proteobacteria

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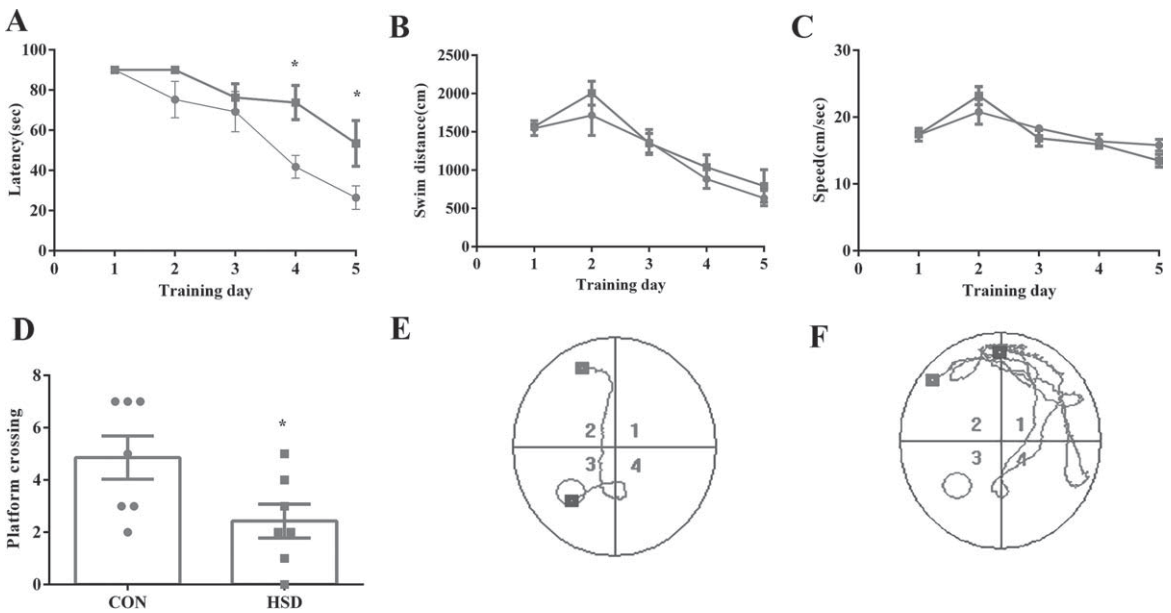


Fig. 1. Effects of the high-salt diet (HSD) on the learning and memory ability of mice. These abilities were assessed using the Morris water maze. A) The mice in the HSD group spent more time searching for the platform during the navigation test period than the control mice, as demonstrated by an analysis of the average escape latency calculated each day, and this difference was significant on days 4 and 5. B, C) Total travel distance and average swim speed of the mice on the training days. D) Number of platform crossings during the probe trial. E, F) Representative tracks on day 5 obtained for the mice in the control and HSD groups during the navigation test. $n = 7$ in each group. The data are expressed as the means ± SEMs. $^*p < 0.05$.

(2.96 ± 0.54%) and increased that of Firmicutes (42.77 ± 10.88%) compared with the control levels (70.73 ± 7.52%, 6.69 ± 3.50%, and 19.9 ± 5.81%, respectively, Fig. 2A). The family-level analysis showed that the HSD mice presented significantly reduced sequence proportions of *S24-7* and *Alloprevotella* (phylum Bacteroidetes) and higher sequence proportions of *Lachnospiraceae* and *Ruminococcaceae* (phylum *Firmicutes*; Fig. 2D). More details are available in the Supplementary Material.

HSD decreases the fecal SCFA concentrations in mice

The SCFA levels were measured by GC. The quantitative analysis of the SCFA concentrations in the fecal samples revealed notable decreases in the absolute concentrations of acetate, propionate, and butyrate and a significant reduction in the acetate concentration (Fig. 3A) in the HSD-fed mice compared with the control mice. The lowered fecal SCFA concentrations suggested that the HSD inhibited bacterial fermentation and decreased the SCFA availability in the lower gut.

HSD induces BBB dysfunction in mice

The BBB permeability was determined by measuring the amount of Evans blue dye in the brain 3 h after tail vein injection. Marked increases in the Evans blue content were found in the brain of the HSD-fed mice rain compared with those of the normal diet-fed mice (17.5 ± 4.9 versus 4.6 ± 0.6 μg/ml [$F_{(3,16)} = 10.4$, $p < 0.01$], see Fig. 3B). To examine the expression of tight junction proteins in the mouse brain, we measured the expression of claudin-5 by western blotting. The protein level of claudin-5 in the hippocampus of the HSD-fed mice was significantly lower than the level found in the control mouse brain (Fig. 3C).

HSD increases Iba1 expression in the hippocampus

To examine the activation of microglia in the mouse brain, we measured the expression of Iba1 by western blotting and immunostaining. The western blotting analysis showed increased expression of Iba1 in the hippocampus of the HSD-fed mice, which suggested increased microglia activation (Fig. 3D).

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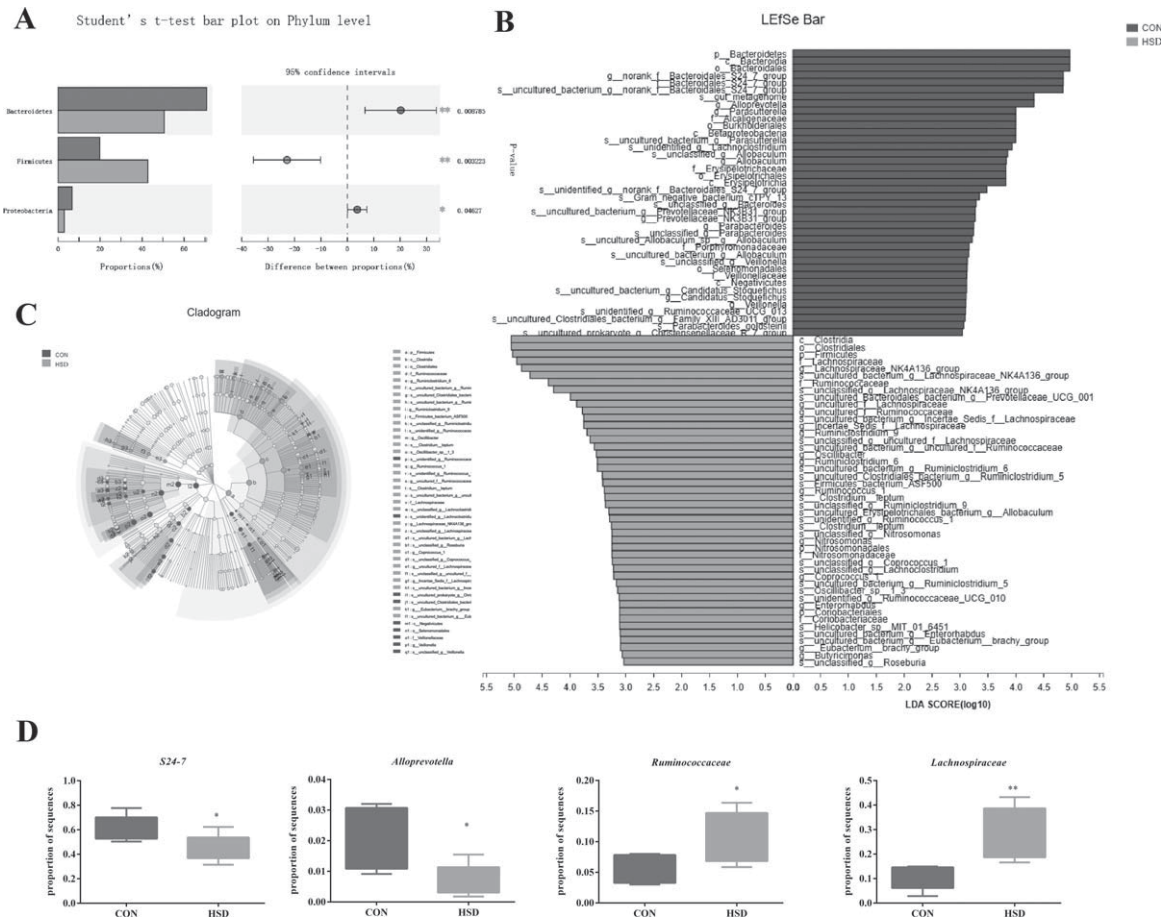


Fig. 2. Effects of the high-salt diet (HSD) on the mouse gut microbiota composition. A) Differences between two groups at the phylum level. Four primary phyla were identified. The proportion of Firmicutes was significantly increased in the HSD group, whereas the proportion of Bacteroidetes and Proteobacteria was decreased significantly in this group. B) Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) at the species level. Differences with p values <0.05 and score $\geq$2.0 are shown. C) Taxonomic cladogram representing the taxa with differential abundance between the two groups. The bacterial taxa enriched in the normal diet (CON) and HSD groups are shown in green and red, respectively. The size of each dot is proportional to the taxon abundance. D) Relative abundances of *S24-7*, *Alloprevotella*, *Ruminococcaceae* and *Lactobacillaceae* in the CON and HSD groups. $n = 5$ in each group. The data are expressed as the mean $\pm$ SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

The immunostaining images showed a considerable increase in Iba1 in the hippocampus of the HSD-fed mice (Fig. 3E). These data suggested that a high-salt diet induced microglial activation in the mouse brain.

HSD increases apoptosis and the expression of cleaved caspase-3, caspase-3, and caspase-1 in the mouse cortex

To investigate the brain damage induced by the HSD, we first examined whether HSD induces apoptosis in the hippocampus and cortex by Hoechst staining. In the HSD-fed mice, the percentage of apoptotic cells was significantly higher in the area of the cortex adjacent to the hippocampus (Fig. 4A, B). These results provide evidence showing that HSD directly induces brain injury. Furthermore, the expression of cleaved caspase-3, caspase-3 and caspase-1 in the cortex was significantly higher in the cortex of the HSD-fed mice (Fig. 4C, D), which was consistent with the degree of apoptosis in the cortex of HSD-fed mice.

HSD increases the IL-1β, IL-6, and TNF-α levels in mouse brains

Because the HSD could induce apoptosis and microglial activation in the brain, we also examined the levels of proinflammatory cytokines in the cortex by ELISA. We found that the levels of IL-1β,

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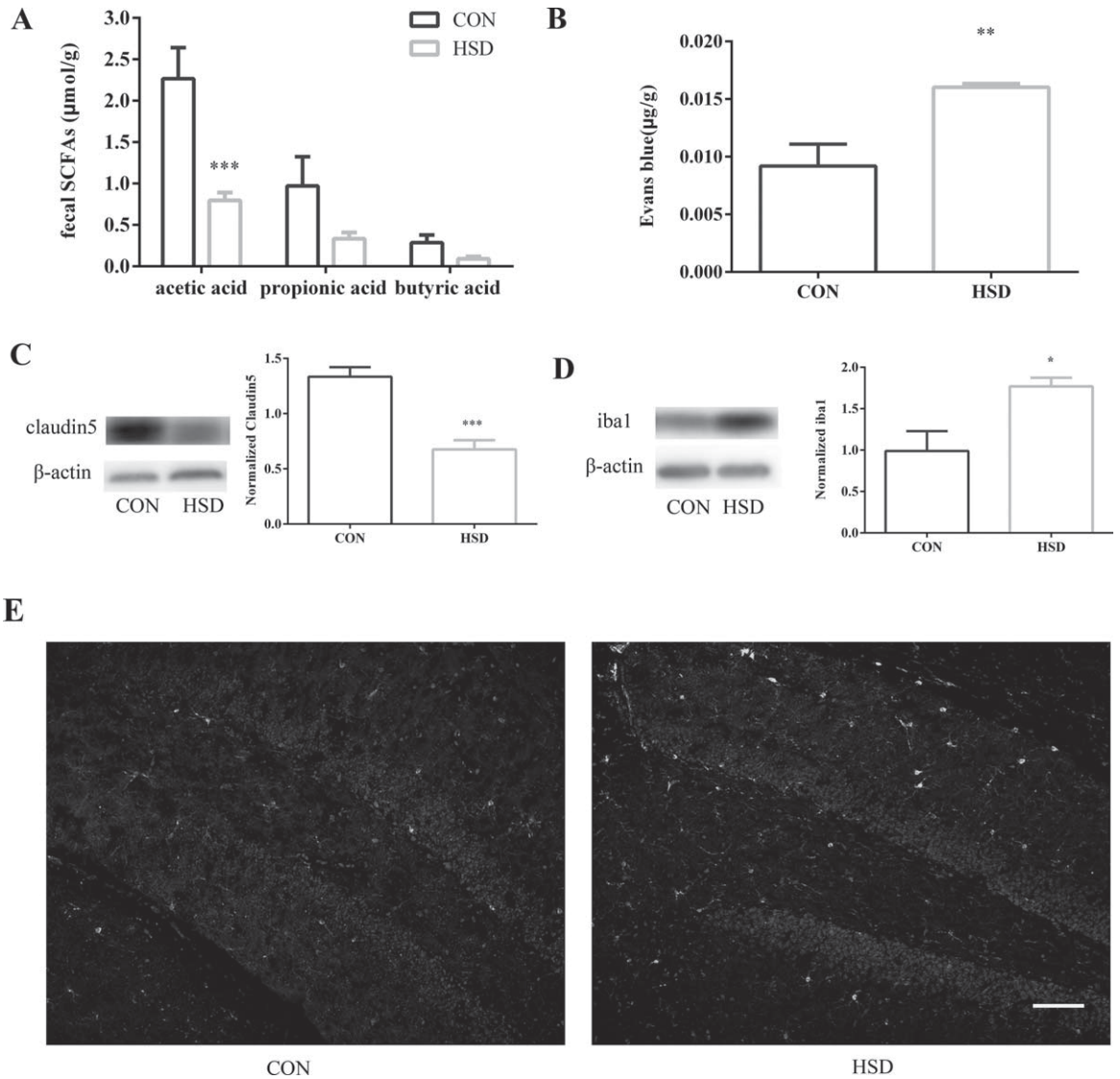


Fig. 3. Effects of the high-salt diet (HSD) on the fecal short-chain fatty acid (SCFA) contents, blood-brain barrier (BBB) integrity, microglial activation, and caspase expression. A) The HSD significantly decreased the fecal acetate, propionate and butyrate contents ($n = 4$ in each group). B) The HSD significantly increased the BBB permeability, as indicated by an increased brain Evans blue dye content (CON, $n = 3$; HSD, $n = 4$). C, The HSD decreased the claudin-5 expression level and increased the Iba1 expression level in the hippocampus. The expression levels of hippocampus claudin-5 and Iba1 are relative to those of β-actin ($n = 5$ in each group). D, E) The HSD significantly increased the expression of cleaved caspase-3, caspase-3, and caspase-1 in the cortex ($n = 5$ in each group). The data are expressed as the means ± SEMs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

IL-6, and TNF-α in the HSD group were significantly higher than those in the control group (Fig. 4E).

DISCUSSION

Our results indicate the following: 1) the HSD elicits considerable transformation in the GM as well as cognitive dysfunction; 2) the HSD directly induces apoptosis in the brain, and this effect is accompanied by increases in the levels of proinflammatory cytokines and caspases; and 3) decreases in fecal SCFA concentrations along with increases in microglia activation and BBB permeability might be attributable to the above-described phenomenon, which is consistent with the accumulating knowledge of the GM and its metabolites. In the present study, brain injury was observed following consumption of

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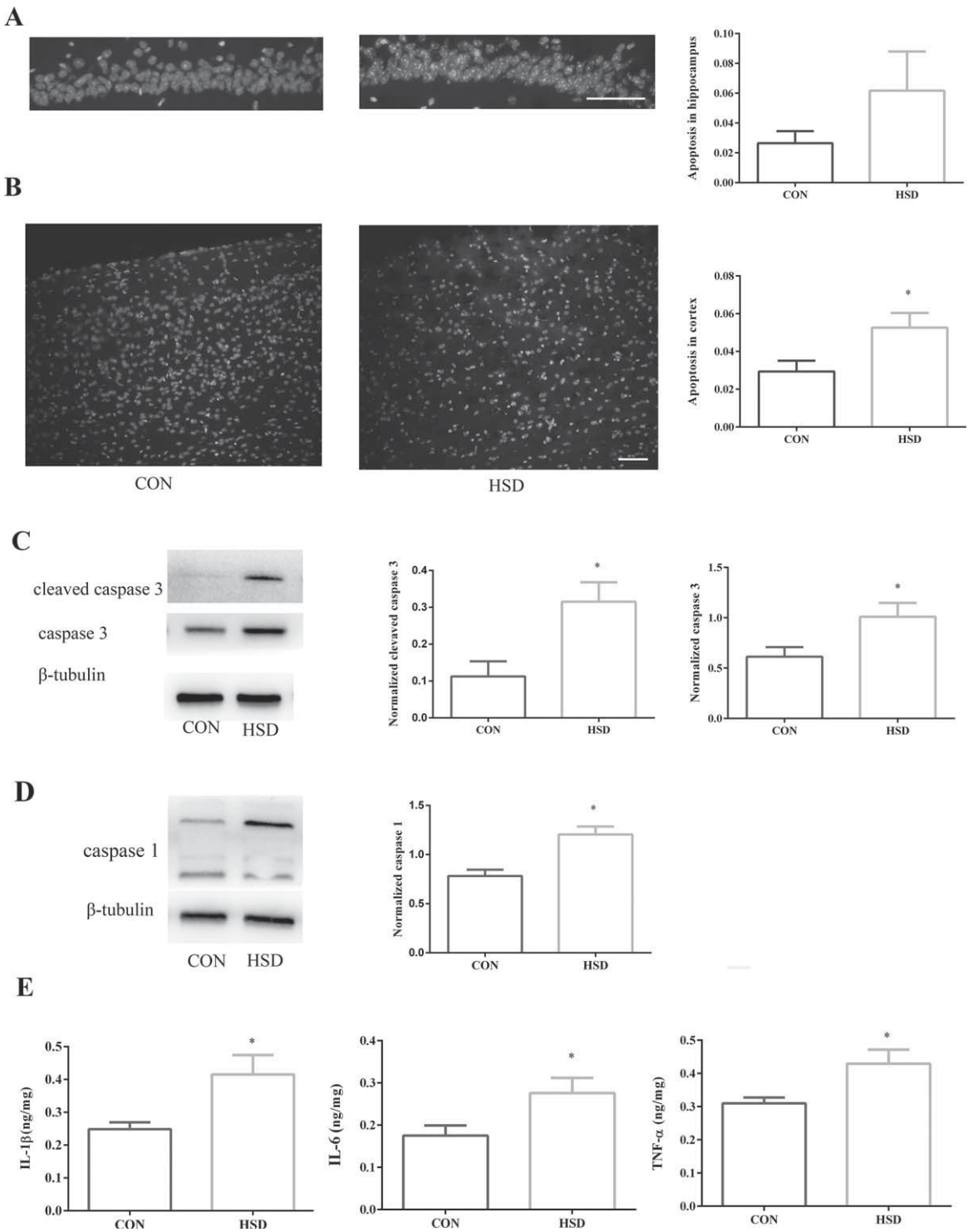


Fig. 4. The high-salt diet (HSD) induced apoptosis in the hippocampus and cortex. The nuclei were stained with Hoechst 33258, and apoptotic cells are characterized by nuclear condensation and diffraction. A, B) Hoechst staining showing HSD-induced apoptosis in the hippocampal CA1 region (A) and cortex (B) adjacent to the hippocampus (scale bars, 100 μm; $n = 3$ in each group). C) The mice fed the HSD exhibited a significantly higher apoptosis rate in the cortex. D) The HSD significantly increased the IL-1β, IL-6, and TNF-α levels in the mouse cortex ($n = 7$ in each group). The data are expressed as the means ± SEMs. *$p < 0.05$.

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the HSD, and this injury was indicated by apoptosis and high protein levels of caspases, IL-1β, IL-6, and TNF-α in the brain (Fig. 4). In addition, the HSD did not influence the body weight and food intake but significantly increased the water intake and urine excretion (Supplementary Figure 1). These results provide a conceptual framework for elucidating the GM alterations and cognitive dysfunction induced by an HSD.

High salt is recognized as one of the most common risk factors for hypertension and cardiovascular disease. One long-term cohort study suggests that a reduction in the sodium intake, particularly in older adults with low physical activity, might further improve the brain health later in life [2]. Another recent trial found that a reduced sodium intake combined with aerobic exercise is associated with improvements in executive function in adults with cognitive impairments but no dementia or risk factors for cardiovascular disease [21]. Accumulating lines of evidence show that high salt can affect cognitive function via multiple mechanisms beyond its impacts on hypertension [2–4, 8, 22–24]. For example, salt exerts a pro-inflammatory effect and boosts the activation of classical LPS-induced macrophages [7, 25, 26]. In the present study, we found that the HSD significantly activated microglia, which are the resident macrophages in the brain, and significantly increased the levels of proinflammatory cytokines in the cortex, which could be produced by excessive activated microglia.

The mechanisms through which the HSD activate microglia in the brain remain unclear. Accumulating lines of evidence link GM dysbiosis to several neural diseases, such as autism spectrum disorder [27, 28], major depression [29], Parkinson's disease [18], Alzheimer's disease (AD) [30, 31], and multiple sclerosis [32]. A maternal high-fat diet induces social deficits and GM dysbiosis in offspring [33]. Coincidentally, studies using animal models have shown that probiotics, such as *Lactobacillus* and *Bifidobacteria*, restore the memory impairments induced by stress [34, 35]. More importantly, the consumption of probiotics for 12 weeks exerts positive effects on cognitive function and some metabolic statuses in patients with AD [36]. A recent study showed that a high-salt intake alters the gut microbiome in mice and a human cohort, particularly by depleting *Lactobacillus* spp. [6], which indicates that pivotal bacterial genera or probiotics might be therapeutic methods for cognitive dysfunction associated with dietary salt. Therefore, the inflammatory response elicited by redundant dietary salt has been implied in a broad spectrum of neural diseases, and elucidating the triangular relationship among dietary salt, cognition, and GM would be meaningful. A recent study found that dietary salt promotes cognitive impairment through tau phosphorylation [37]. Interestingly, previous studies have found that the level of trimethylamine N-oxide (TMAO) in cerebrospinal fluid is higher in individuals with mild cognitive impairment and AD dementia. TMAO is produced by the GM, and elevated TMAO in cerebrospinal fluid is associated with biomarkers of AD pathology (phosphorylated tau and phosphorylated tau/$A\beta_{42}$) and neuronal degeneration (total tau and neurofilament light chain protein) [38]. These findings further indicate that the cognitive impairment caused by high salt is closely related to the GM.

The switch in the GM induced by long-term HSD consumption found in this study, such as an increased Firmicute/Bacteroidetes ratio, was similar to that observed in aged mice [41]. Members of the *S24-7* and *Rikenellaceae* families were the most abundant representatives of the Bacteroidetes phylum, the abundance of which was significantly decreased by the HSD in the present study. The abundance of S24-7, a butyrate-producing bacterium, is reduced by a high-fat diet and increased following exercise and in lean mice compared with the levels in obese mice [39]. Several immune-related peptidases are also secreted by members of the *S24-7* family [40]. The other genus decreased by the HSD, *Alloprevotella*, reportedly produces moderate amounts of acetic acid and major amounts of succinic acid as end-products of the fermentation process [41], and species of this genus are through to induce protection against cardiovascular diseases [42]. In contrast, an HSD induces blooms in some of the major constituent groups within Firmicutes, such as *Lactobacillaceae*, *Lachnospiraceae*, and *Ruminococcaceae*. Interestingly, the trends in the alterations in the abundances of *Lachnospiraceae* NK4A136 and *Ruminococcaceae* were opposite to those found in a previous study [15].

We also found that the HSD significantly reduced the production of SCFAs in the gut. SCFAs mainly consist of acetic acid, propionic acid and butyric acid, which account for 90% to 95% of the total amount of SCFAs. SCFAs regulate the permeability of the intestinal epithelial barrier and BBB by upregulating and reorganizing tight junctions or by promoting epithelial cell differentiation [43]. Physiological concentrations of SCFAs immediately promote epithelial barrier function in the large intestine [44]. An HSD

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significantly damages the ischemic brain by increasing BBB disruption due to the loss of tight junctions without any apparent alteration in the blood pressure [4]. Furthermore, SCFAs can enter the brain and promote full maturation of microglia and their anti-inflammatory capabilities [14], which results in the alleviation of BBB damage in germ-free mice by restoring tight junctions [45]. The intravenous or intraperitoneal administration of sodium butyrate facilitates long-term memory consolidation [46] and promotes angiogenesis and neurogenesis [47]. Acetate is known to improve intestinal defense responses mediated by epithelial cells and thereby protects the host from lethal infection [48]. The decreased SCFA concentrations observed in the current study might account for the increase in BBB permeability and the excessive activation of microglia induced by long-term HSD consumption. Moreover, the protein levels of IL-1β, IL-6, and TNF-α were significantly higher in the brains of HSD-fed mice. These results suggest that HSD induces GM dysbiosis and an inflammatory environment in the mouse brain, which ultimately resulted in the loss of learning and memory abilities.

Our results showed that the HSD can affect cognitive function in many ways, including but not limited to reducing the expression of tight junction proteins and increasing the BBB permeability. An increased BBB permeability might allow higher levels of harmful substances, such as bacterial-related LPS and metabolites, to enter the brain, which causes inflammation in the brain. These complex factors contribute to salt-related cognitive impairment. We also found that the HSD reduced the abundances of SCFA-producing bacteria and increased the activation of microglia in the brain. SCFAs exert anti-inflammatory effects, and whether SCFAs can affect the high-salt-induced inflammatory environment requires further exploration. In contrast, more studies are needed to identify the pivotal role of the GM in dietary sodium-associated brain dysfunction.

Conclusion

Our study shows that excessive salt intake directly elicits brain injury and cognitive dysfunction in mice, which might be the consequence of the GM dysbiosis induced by an HSD, as indicated by alterations in the GM composition and decreased fecal SCFA concentrations. Certain bacterial strains could account for the increased BBB permeability, and SCFAs might be targeted to improve high salt-associated cognitive decline, but further investigations are needed to confirm these findings.

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AVAILABILITY OF DATA AND MATERIALS

The dataset analyzed in the current study is available from the corresponding author upon request.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/JAD-200035.

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Gut Microbiome Features of Chinese Patients Newly Diagnosed with Alzheimer's Disease or Mild Cognitive Impairment

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Abstract.
Background: Patients with Alzheimer's disease (AD) have gut microbiome alterations compared with healthy controls. However, previous studies often assess AD patients who have been on medications or other interventions for the disease. Also, simultaneous determination of gut microbiome in patients with mild cognitive impairment (MCI) or AD in a study is rare.
Objective: To determine whether there was a gut microbiome alteration in patients newly diagnosed with AD or MCI and whether the degree of gut microbiome alteration was more severe in patients with AD than patients with MCI.
Methods: Fecal samples of 18 patients with AD, 20 patients with MCI, and 18 age-matched healthy controls were collected in the morning for 16S ribosomal RNA sequencing. No patient had medications or interventions for AD or MCI before the samples were collected.
Results: Although there was no difference in the microbial α-diversity among the three groups, patients with AD or MCI had increased β-diversity compared with healthy controls. Patients with AD had decreased *Bacteroides, Lachnospira*, and *Ruminiclostridium_9* and increased *Prevotella* at the genus level compared with healthy controls. The changing direction of these genera in patients with MCI was the same as patients with AD. However, *Lachnospira* was the only genus whose abundance in patients with MCI was statistically significantly lower than healthy controls. *Bacteroides, Lachnospira*, and *Ruminiclostridium_9* were positively associated with better cognitive functions whereas *Prevotella* was on the contrary when subjects of all three groups were considered. The negative correlation of *Prevotella* with cognitive functions remained among patients with MCI.
Conclusion: Patients newly diagnosed with AD or MCI have gut dysbiosis that includes the decrease of potentially protective microbiome, such as *Bacteroides*, and the increase of microbiome that can promote inflammation, such as *Prevotella*. Our results support a novel idea that the degree of gut dysbiosis is worsened with the disease stage from MCI to AD.

Keywords: Alzheimer's disease, Chinese, gut microbiome, mild cognitive impairment

INTRODUCTION

The possible role of gut microbiome-brain axis in the development of Alzheimer's disease (AD), the most common neurodegenerative disease in the elderly, has been proposed in recent years [1–3]. This proposal is supported by the findings that patients with AD have gut microbiome features that are different from mentally healthy people, gut microbiome that is linked to inflammation is increased in AD patients, and some bacteria that are amyloid generating are increased in those patients [4, 5]. Animals with

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AD-like brain changes have altered gut microbiome that increases inflammatory responses [6]. Replacing gut microbiome of AD-like mice with that of healthy mice attenuates AD-like brain and behavior changes [7].

The complex pathological changes including amyloid-β peptide accumulation and deposition, activation of inflammatory responses and oxidative stress, which induce cell injury and dysfunction, ultimately lead to the presentation of AD [8]. Gut microbiome can alter gut mucosal permeability, activate gut immune cells, and potentially induce systemic and neural inflammation [4, 5]. These processes may be the pathological mechanisms for the involvement of gut microbiome in the development of AD.

One of the significant questions is whether gut microbiome alteration occurs before the presentation of AD symptoms. If so, this alteration may contribute to the development of AD symptoms. If the alteration occurs after the presentation of AD, gut microbiome may contribute to the worsening of AD presentation. Thus, it is necessary to determine whether there is a gut microbiome change at a stage before the full presentation of AD. Mild cognitive impairment (MCI) is considered as a prodromal stage of AD [9]. Although some patients with MCI may not progress to AD and MCI in some patients may not be the results of AD-like neuropathology, amnestic MCI has a conversion rate of 10 to 15% per year to probable AD [10]. Thus, determining the changes of gut microbiome in patients with MCI may provide an indication for the role of gut microbiome in MCI and AD presentation. Three studies published in 2019 have investigated the gut microbiome in patients with MCI. One study showed that patients with MCI had gut microbiome similar to that of patients with AD [4]. Another study showed that gut microbiome was different among healthy controls and patients with MCI or AD. It was possible to distinguish healthy controls from patients with MCI or AD or patients with AD from patients with MCI [11]. The third study showed that patients with MCI had increased *Bacteroides* [12]. These results suggest gut microbiome has changed already even in patients with MCI. However, two of these three studies clearly included patients on medications, such as acetylcholinesterase inhibitors, to improve symptoms [4, 12] and the other study did not provide this information [11]. Although the effects of these medications on gut microbiome are not known, the potential effects of these agents on gut microbiome through gut motility and neuro-endocrinal influence cannot be totally excluded. Thus, studying newly diagnosed patients is necessary to limit the effects of drugs and interventions physicians or the families of patients may have initiated.

We hypothesize that patients with newly diagnosed MCI or AD have similar gut microbiome that is different from that of healthy controls and that the gut dysbiosis in patients with MCI is less severe than patients with AD. To test these hypotheses, fecal microbiome of patients with newly diagnosed AD or MCI and age-matched healthy controls were determined.

MATERIALS AND METHODS

Participants

This study was conducted according to the principle of the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University [protocol number: SYSEC-KY-KS-2018-040]. A total of 60 participants who were 60 years old or older were recruited between August 14, 2018 to April 19, 2019 from the neurology and rehabilitation clinics of Sun Yat-sen Memorial Hospital. All participants were not diagnosed with AD or MCI before, and had never taken acetylcholinesterase inhibitors, such as donepezil, and/or memantine. Among them, there were 20 subjects in each group of cognitive normal control or patients with AD or MCI. All participants enrolled in the study signed the written informed consent.

The clinical assessment and diagnosis of AD dementia and MCI were made by experienced neurologists independent from the study, according to the 2018 National Institute of Aging and Alzheimer's Association (NIA-AA) guidelines. Briefly, their cognitive and functional status was assessed based on a neuropsychological battery including the Chinese version of the Mini-Mental State Examination (MMSE), Montreal Cognitive Assessment (MoCA), Instrumental Activities of Daily Living (ADL), Clinical Dementia Rating (CDR), and Alzheimer's Disease Assessment Scale-Cognitive section (ADAS-Cog). Hachinski ischemic score and Hamilton depression rating scale were assessed to help exclude diseases of severe neural tissue ischemia, depression, or anxiety. If the neuropsychological measurement results indicated the participants having MCI or probable AD, blood was obtained to test thyroid function, homocysteine, vitamin B-12, folic acid, blood glucose, glycosylated

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hemoglobin, blood lipid, and syphilis. Brain magnetic resonance spectroscopy was performed as well. These tests and examinations were used to exclude other diseases that may cause cognitive decline.

The exclusion criteria for participating in this study included: 1) a family history of dementia; 2) any other neurodegenerative diseases, such as Parkinson's disease and amyotrophic lateral sclerosis; 3) enduring mental illness, such as schizophrenia; 4) severe cardiac, pulmonary, hepatic or renal diseases, or any tumor; 5) a history of taking antibiotics or glucocorticoid within six months, and 6) intestinal diseases, such as irritable bowel syndrome.

Fecal sample collection and DNA isolation

Fecal samples were collected in the morning from recruited subjects newly diagnosed with AD or MCI and age-matched healthy controls. Microbial genomic DNA was extracted from fecal pellets using the PowerSoil DNA Isolation Kit (MOBIO Laboratories) according to the manufacturer's protocols and was stored at –80° for further testing. DNA quality and quantity were assessed by the ratios of 260 nm/280 nm and 260 nm/230 nm, respectively.

16S RNA gene sequencing and analysis

The V3-V4 sequenced regions of the 16S rRNA gene were amplified by PCR. The PCR amplified product was purified, quantified, and normalized to form a sequencing library. The constructed library was first subjected to library quality inspection. Two samples from the AD group and two samples from the healthy control group failed to pass the library quality inspection and were then excluded. Thus, a total of 56 quality-qualified libraries were sequenced using Illumina Miseq/Microseq (Illumina, San Diego, CA, USA). Afterwards, the raw image data files obtained by high-throughput sequencing were converted into the original sequenced reads by base calling analysis and the results were stored in the FASTQ file format that contained sequence information of reads and its corresponding sequencing quality information followed by data processing according to the overlapping relationship among paired-end (PE) reads and the PE sequence of Tags. The quality of reads and the effect of merge were filtered through three steps. Step 1) PE reads splicing: splicing the reads of each sample through overlap using FLASH v1.2.7 software, the obtained splicing sequence was the raw tags data; Step 2) Tags filtering: the raw tags obtained by splicing were filtered by Trimmomatic v0.33 software to get high quality tags data (i.e., clean tags); Step 3) Removal of chimeras: The chimeric sequences were identified and removed using UCHIME v4.2 software to obtain the effect tags. Clustering of Tags at 97% sequence similarity level using UCLUST in QIIME2 software to obtain operational taxonomic unit (OTU) and the OTU was taxonomically annotated based on the Greengene (Release 13.8). 0.005% of all sequence numbers as a threshold was used to filter OTU. The OTU representative sequence was compared with the microbial reference database to obtain the species classification information corresponding to each OTU and then the composition of each sample was counted at each level (i.e., phylum, class, order, family, genus and species). On this basis, the QIIME2 software was used to generate the species abundance table at different classification levels and R package was used to map the community structure plot under the taxonomic level of the sample. Species annotation was performed using the RDP Classifier (version 2.2) with a confidence threshold of 0.8. α-diversity of samples (evenness, faith pd and the Shannon index) and β-diversity [Bray Curtis, unweighted unifrac, weighted unifrac, and principal component analysis (PCA)] were investigated with QIIME2 and R software to compare the similarity of species diversity in different samples. Linear discriminant analysis with effect size (LEfSe) was performed online based on the taxonomic files obtained from the QIIME2 analysis.

Statistical analysis

Continuous parameters, including age, years of education, scores of cognitive tests, and relative abundance of taxa were presented as mean ± standard deviation (S.D.). Categorical factors, such as gender and rates of participants with hypertension or diabetes, were presented as percentage (%). Differences among groups were tested with either Chi-square test for category variable or one-way analysis of variance (ANOVA) for continuous variables. One-way ANOVA was followed by post hoc Dunn's multiple comparison tests for parametric data or Kruskal-Wallis sum-rank test was followed by Dunn's multiple for non-parametric data. Spearman correlation coefficients were used to detect the relationships between the taxa and clinical data. The Chi-square test, ANOVA and Spearman correlation coefficient were performed by SPSS version 25.0 (SPSS Inc., Chicago, IL) with a $p < 0.05$ as the significance level.

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Table 1
General characteristics of participants

Characteristics	AD patients ($n=18$)	MCI patients ($n=20$)	Normal control ($n=18$)	p
Age (SD)	63.5 (4.7)	64.5 (4.5)	64.2 (4.7)	0.8
Male (%)	2 (11)	4 (20)	4 (22)	0.798
BMI (SD)	21.9 (2.4)	21.8 (2.0)	21.1 (2.2)	0.83
Diabetes (%)	2 (11)	2 (11)	1 (6)	0.825
Hypertension (%)	2 (11)	3 (15)	4 (22)	0.654
Education, y (SD)	7.7 (3.7)	9.7 (3.9)	11.5 (4.2)	0.843
MMSE score, mean (SD)	14.3 (6.9)*#	24.7 (2.2)*	28.3 (1.3)	<0.001
MoCA score, mean (SD)	9.8 (5.2)*#	20.5 (2.5)*	24.1 (2.6)	<0.001
ADAS-Cog score, mean (SD)	34.1 (22.3)*#	13.9 (3.5)*	9.4 (5.5)	<0.001

Results are means (S.D.) or number of individuals (%). *$p<0.05$ compared with healthy control group; #$p<0.05$ compared with the MCI group. AD, Alzheimer's disease; BMI, body mass index; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; ADAS-Cog, Alzheimer's Disease Assessment Scale-cognitive section.

RESULTS

Subject characteristics

The characteristics of AD, MCI patients and age- and gender-matched cognitively normal controls are shown in Table 1. AD patients had lower MMSE and MoCA scores than MCI patients and healthy controls. These scores of MCI group were also lower than those of the healthy control group. No significant difference was found in the duration of education and the number of patients with hypertension or diabetes among patients with AD or MCI and healthy controls. No subject in the three groups was obese (body mass index > 30).

α-diversity and β-diversity

All indices of α-diversity (evenness, faith pd, and Shannon diversity based on OTU levels) were similar among the three groups (Fig. 1A–C). Significant differences in β-diversity were observed based on the Bray curitis and weighted Unifrac analyses. The patients with AD or MCI had a higher β-diversity than healthy controls (Fig. 1D, E). PCA analysis showed that the healthy controls had microbial components different from those of patients with AD or MCI (Fig. 1F).

Composition of gut microbiota at the phylum and genus levels

At the phylum level, *Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia*, and *Fusobacteria* were the dominant bacteria (Fig. 2A). There was a difference in the abundance of *Protobacteria* among the three groups ($p=0.04$), while *Firmicutes, Bacteroidetes, Verrucomicrobia*, and *Fusobacteria* were not different among the three groups. At the genus level, *Bacteroides, Faecalibacterium, Prevotella_9, Megamonas*, and *Phascolarctobacterium* were the dominant genera. There were differences in the relative abundance of *Bacteroides* ($p=0.037$), *Blautia* ($p=0.041$), *Lachnospira* ($p=0.01$), and *Prevotella_9* ($p=0.0095$) among the groups (Fig. 2B).

Differences in microbiome of patients with MCI or AD

The LEfSe method was used to perform comparison among the AD, MCI, and healthy control groups. Considering the differences in the taxa at all levels with a logarithmic linear discriminant analysis score > 2.0 and a p value < 0.01, we found that the abundance of 39 taxa between AD and control group (Fig. 3A), 26 taxa between MCI and control group (Fig. 3B), and 48 taxa between AD and MCI group (Fig. 3C) were significantly different. Using the Venn diagram, 10 taxa were common in the result of AD versus control and MCI versus control, 14 taxa were common in the result of AD versus control and AD versus MCI and 9 taxa were common in the result of AD versus MCI and MCI versus control. These 10, 14 and 9 taxa belonged to 4, 3 and 2 genera, respectively (Fig. 3D).

As described above, *Firmicutes* and *Bacteroidetes* at the phylum level were not different among AD, MCI, and control groups (Fig. 4A, B). The ratio of *Firmicutes* to *Bacteroidetes* was not different among the three groups (Fig. 4C). We then focused on the taxa at the genus level. The abundance of *Prevotella_9*

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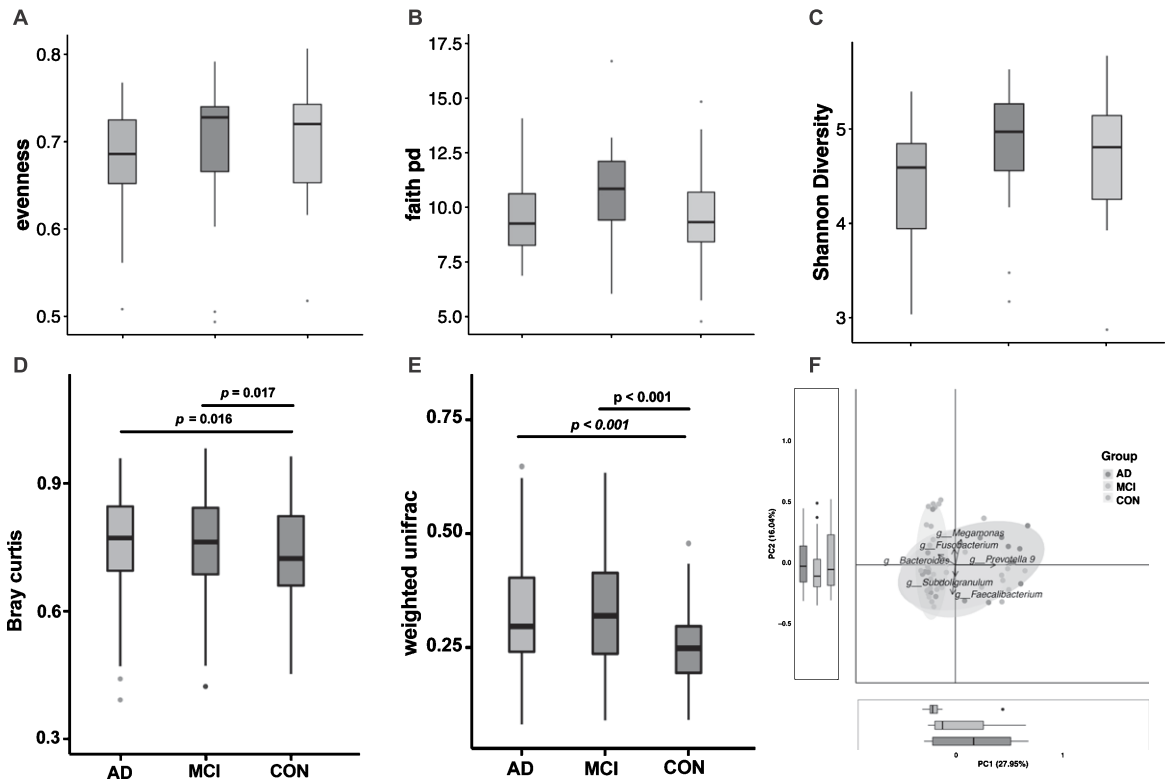


Fig. 1. The α-diversity and β-diversity analysis of the gut microbiota in patients with AD ($n = 18$) or MCI ($n = 20$), and the healthy controls ($n = 18$). The evenness (A), faith pd (B), and Shannon diversity (C) of α-diversity and Bray curtis (D), weighted unifrac (E), and PCA (F) of β-diversity analysis are presented. AD, Alzheimer's disease; MCI, mild cognitive impairment; CON, healthy controls; PCA, principal component analysis.

was significantly increased (Fig. 4D) and the abundance of *Bacteroides* was significantly decreased in the AD group (Fig. 4E) compared with healthy controls. However, the abundance of these two genera in the MCI group was not different from those in the AD group or healthy controls. Interestingly, the ratio of *Prevotalla_9 to Bacteroides* of the healthy controls was significantly lower than that of MCI group ($p = 0.04$) or AD group ($p = 0.006$) (Fig. 4F), indicating that it may be worthwhile to determine whether this ratio has a potential to discriminate individuals with AD or MCI from healthy controls before the intervention for AD or MCI has started. Compared to the healthy controls, the abundance of *Lachnospira* was significantly decreased in the MCI ($p = 0.006$) and AD ($p = 0.002$) group (Fig. 4G). Patients with AD but not those with MCI had a lower abundance of *Ruminiclostridium_9* than healthy controls (Fig. 4H). The abundance of *Eubacterium eligens* group of MCI group was significantly higher than that of AD group ($p = 0.0003$) or healthy controls ($p = 0.024$) (Fig. 4I). Further analysis at the species level showed that patients with MCI and AD had a lower abundance of *Bacteroides fragilis* than control group (Fig. 4J) and that patients with AD had increased *Prevotella_9; s_uncultured bacterium* compared with control group and patients with MCI (Fig. 4K).

Correlation between bacterial genera and clinical data

When all subjects from three groups were considered, the abundance of *Bacteroides, Lachnospira*, and *Ruminiclostridium_9* were positively correlated with MMSE and MoCA. The abundance of *Prevotella_9* and the ratio of *Prevotella_9 to Bacteroides* were negatively correlated with MMSE and MoCA scores and positively correlated with ADAS-Cog scores (Fig. 5). Our results showed no significant correlation between the above taxa and age or duration of education when all studied subjects were included (Fig. 5A). However, the abundance of *Ruminiclostridium_9* was positively correlated with age in patients with AD (Fig. 5B). The abundance of *Prevotella_9* and the

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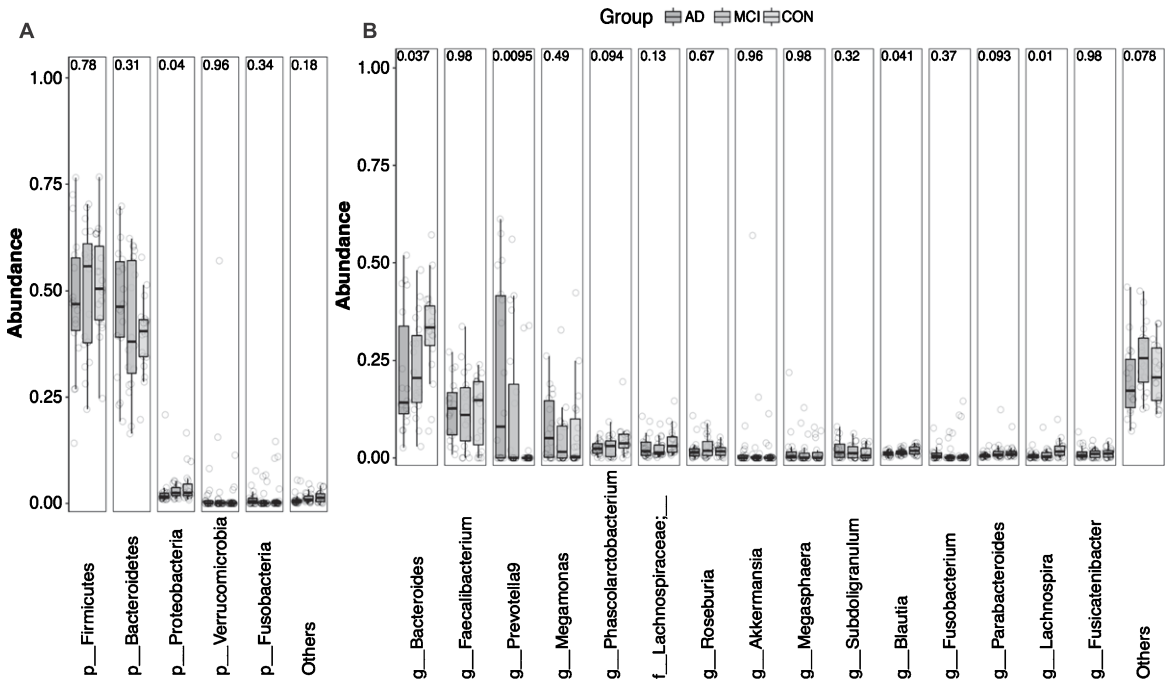


Fig. 2. Relative abundance of gut microbial taxa at phylum-level (A) and genus-level (B) in patients with AD ($n = 18$) or MCI ($n = 20$) and the normal controls ($n = 18$). AD, Alzheimer's disease; MCI, mild cognitive impairment, CON, healthy controls.

ratio of *Prevotella_9 to Bacteroides* were negatively correlated with MoCA scores in patients with MCI (Fig. 5C). Finally, the abundance of *Lachnospira* was positively correlated with MMSE in healthy controls (Fig. 5D).

DISCUSSION

We found that patients with MCI or AD had an increased β-diversity compared with healthy controls. Multiple genera including *Bacteroides, Prevotella_9*, and *Ruminiclostridium_9* had a similar direction of changes in patients with AD or MCI when compared with healthy controls. However, the changes in these genera reached significance only in patients with AD but not in patients with MCI. One genus, *Lachnospira,* was low in patients with AD or MCI compared with healthy controls. The third change pattern is that *Eubacterium eligens* group was increased in patients with MCI compared with healthy controls and patients with AD. These patterns of changes suggest that patients with MCI and AD have similar changes in gut microbiome but the gut dysbiosis in patients with MCI is less severe than that in patients with AD. Consistent with our findings, a previous study has shown a similar gut microbiome change in patients with AD or MCI [4]. Another study showed significant changes of gut microbiome in patients with MCI compared with healthy controls, but this change was distinguishable from patients with AD [11]. Results of these two previous studies and our study clearly indicate that gut dysbiosis occurs in patients with MCI, a disease stage before the full presentation of AD.

We propose that gut dysbiosis may interact with brain pathological process to form a viscous cycle to facilitate the presentation of AD. The occurrence of gut dysbiosis in patients with MCI cannot be used as evidence to support that gut dysbiosis works as an initiator for AD brain changes. It is known that AD neuropathological changes, such as amyloid-β accumulation, occur years before MCI [9]. Animal studies have shown AD-like mice have gut microbiome changes [6, 7, 13]. Thus, it is likely that the pre-symptomatic AD brain changes can alter gut microbiome. These lines of evidence would suggest that gut dysbiosis is a change subsequent to the AD neuropathology. However, transplantation of healthy mouse feces to AD-like mice improves the neuropathological and behavioral presentation of the AD-like mice [7]. Probiotics improve symptoms of AD patients [14]. These lines of evidence suggest that gut microbiome is important in modulating the pathological processes of AD. Those bacterial

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Fig. 3. Bacterial taxa with different abundances among three groups. A, B, and C panels are histogram of the LDA scores for genera that were different between the two comparing groups. Panel D is a Venn diagram showing the genera that were different among three groups. AD, Alzheimer's disease; MCI, mild cognitive impairment; CON, healthy controls; LDA, Linear discriminant analysis.

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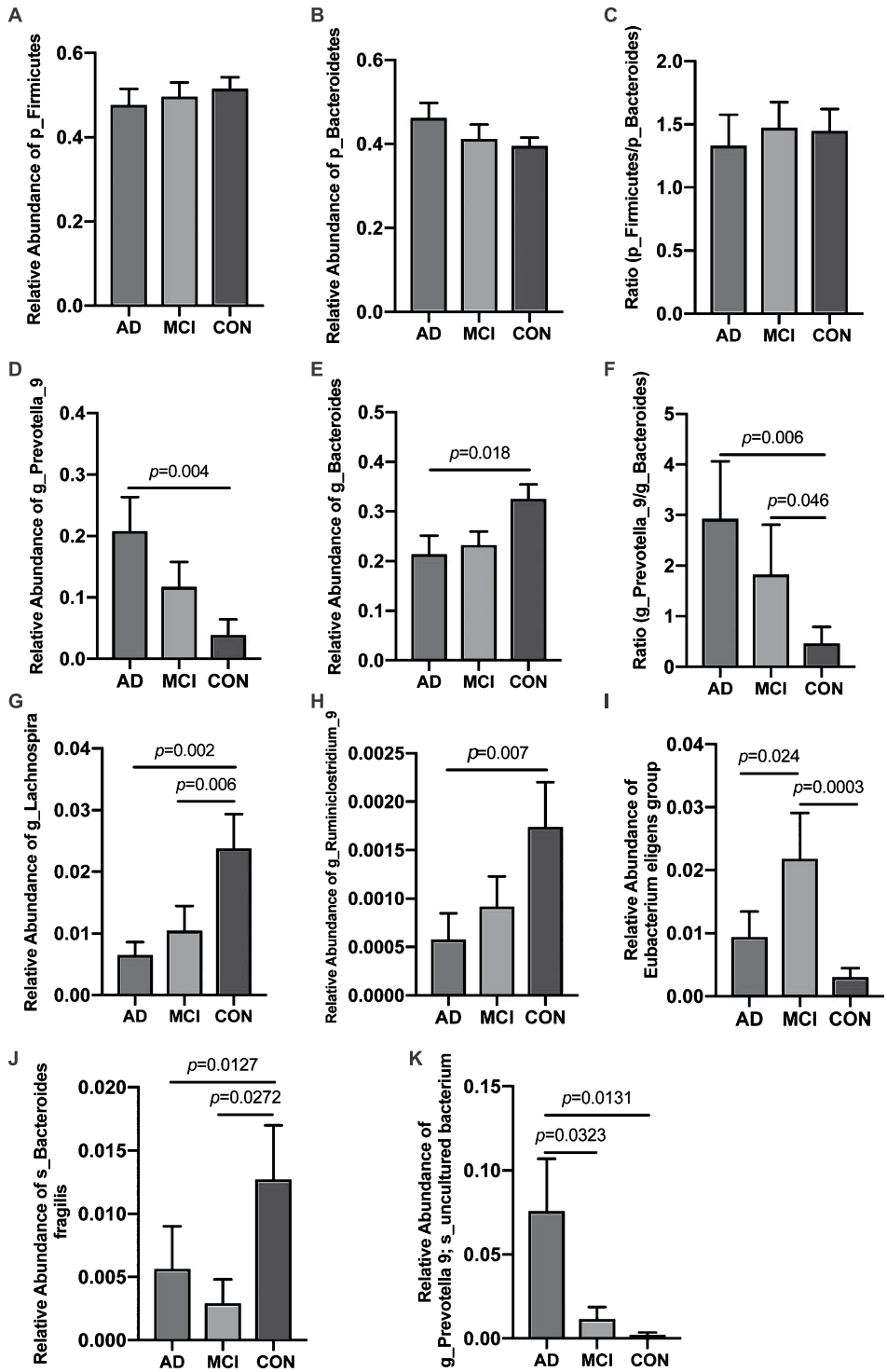


Fig. 4. Bacterial genera with different abundances among three groups. A-C) Relative abundance of *Firmicutes* and *Bacteroidetes* at the phylum level. C-H) Relative abundance of genera at genus level that were different among three groups. I, J) Relative abundance of genera at species level from *g_Bacteroides* and *g_Prevotella* that were different among three groups. AD, Alzheimer's disease; MCI, mild cognitive impairment; CON, healthy controls; LDA, Linear discriminant analysis.

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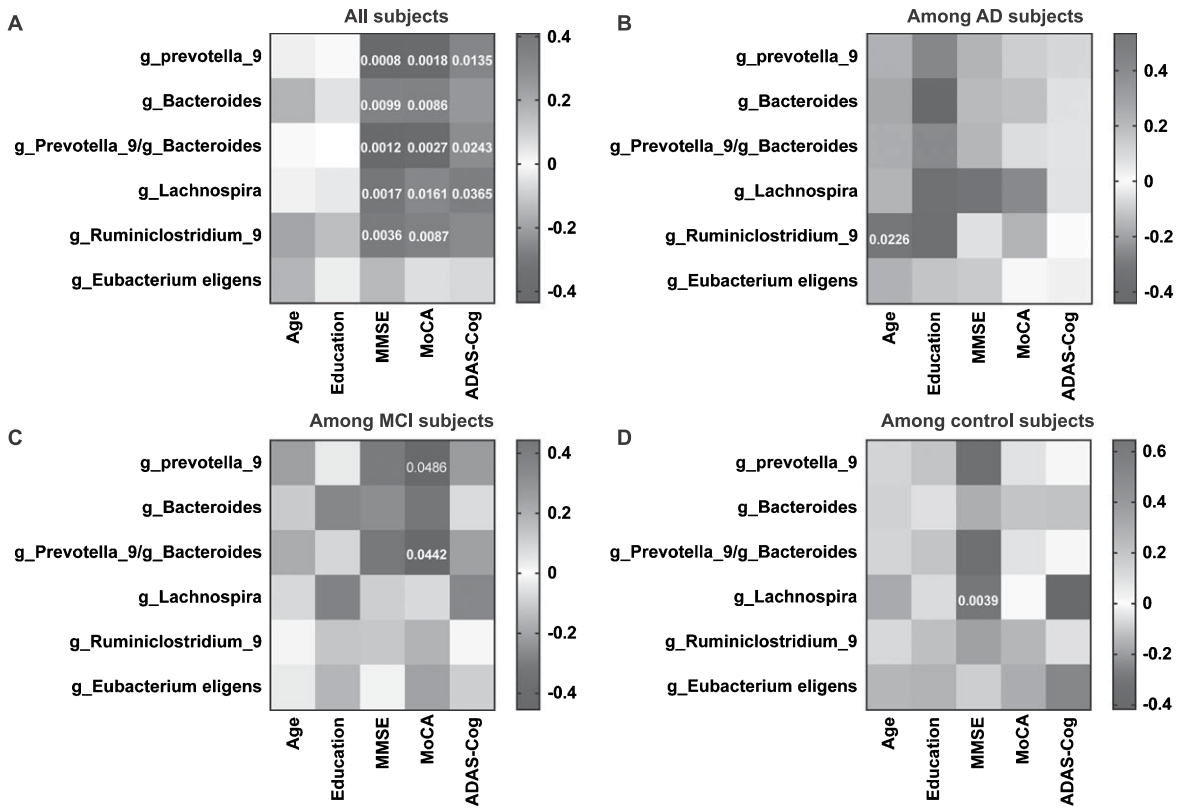


Fig. 5. Heatmaps showing correlations between microbiota genera and clinical characteristics. The scale bar showed the Spearman r value. The *p* values were shown in the blocks. A) All subjects from three groups were included. B) Subjects with AD were analyzed. C) Subjects with MCI were analyzed. D) Subjects of control group were analyzed. MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive subscale.

genera that have an altered abundance in patients with AD or MCI may be candidates that play a role in modulating the pathological processes of AD or whose change in abundance is simply caused by AD pathology. Although a previous study has attempted to link AD biomarkers, such as amyloid-β concentrations in the cerebrospinal fluid, to gut microbiome [15], there is not enough information in the literature to help us distinguish which role of the four genera whose abundances were altered in AD patients plays. Nevertheless, in our study, *Bacteroides* abundance was positively correlated with MMSE and MoCA. Similarly, *Lachnospira* was positively correlated with MMSE and MoCA and negatively correlated with ADAS-Cog. On the other hand, *Prevotella_9* was negatively correlated with MMSE and MoCA and positively correlated with ADAS-Cog. These results suggest a potential of using these genera as biomarkers for cognitive functions. Consistent with the findings when data from all subjects of three groups were analyzed, *Prevotella_9* was negatively correlated with MoCA in patients with MCI and *Lachnospira* was positively correlated with MMSE in control group. These results further suggest a role of these two genera in cognitive functions.

We did not find a difference in the abundance in *Firmicutes* and *Bacteroidetes* at the phylum level among the three groups of subjects. The ratio of *Firmicutes* to *Bacteroidetes* was also not different. Two previous studies have shown that patients with AD have decreased *Firmicutes* [11, 16]. One of these two studies also showed that patients with AD had increased *Bacteroidetes* [16] and the other one showed no change in *Bacteroidetes* in patients with AD [11]. Although the ratio of *Firmicutes* to *Bacteroidetes* was not calculated, this ratio shall be decreased in patients with AD in the study showing that these patients had reduced *Firmicutes* and increased *Bacteroidetes*. However, another study showed an increased ratio of *Firmicutes* to *Bacteroidetes* in patients with dementia compared with non-dementia subjects [17]. It appears that the majority of these non-dementia subjects in the

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study were MCI sufferers who had increased *Bacteroidetes* [12] and, therefore, had a decreased ratio of *Firmicutes* to *Bacteroidetes.* Thus, the ratio of *Firmicutes* to *Bacteroidetes* in patients with dementia may not be different from those in healthy controls in that study, a finding that is similar to ours.

Our results showed at the genus level that *Bacteroides* was decreased in AD patients, which is similar to a previous study [4]. However, *Bacteroides* was found to be increased in patients with AD or MCI in other studies [12, 16, 18]. Our study also showed that *Bacteroides fragilis* was decreased in patients with AD. This bacterial species is a protective bacterial species in one study [19]. However, this bacterium was associated with increased amyloid-β deposition in AD mouse brain in another study [20]. It has been recognized that *Bacteroides fragilis* can be beneficial or detrimental, depending on the environment [21]. Similar to the complex situation of *Bacteroides, Lachnospira* of *Firmicutes* was increased in patients with AD in a previous study [4], but we observed a decrease in *Lachnospira* in AD patients. The reason for these different findings is not known but our patients are newly diagnosed and have not been on any interventions for AD, which is different from the previous study [4]. Similar to our study, an earlier study showed that patients with AD had reduced *Lachnospira* [22]. Additional factors, such as dietary and age differences among the subject populations, may also have contributed to the different findings on gut microbiome from various studies.

We showed that the ratio of *Prevotella_9* to *Bacteroides* was increased in patients with AD and MCI. If this finding is validated by future studies, this ratio may be an indicator to discriminate patients with AD or MCI from healthy controls and may suggest that a balance between *Prevotella_9* and *Bacteroides* is important for maintaining a healthy status. This ratio has been used as an indicator of improved glucose metabolism but *Prevotella* can be pathobiontic for promoting inflammatory responses [23, 24]. Thus, the increase of *Prevotella* may contribute to the development of neuroinflammation in patients with AD. However, different species of *Prevotella* may have pro-inflammatory and anti-inflammatory effects [25, 26]. Our results showed that *Prevotella_9; s_uncultured bacterium* was increased in patients with AD. The functions of these bacteria have not been reported.

Patients with AD had decreased *Ruminiclostridium_9* that is a species of *Firmicutes* and a short-chain fatty acid producer [4]. Short chain fatty acids have been shown to be neuroprotective [27–29]. Thus, the decrease in *Ruminiclostridium_9* may increase the susceptibility of patients to various insults. Finally, patients with MCI had increased *Eubacterium eligens* group. The significance of this increase is not known. However, the *Eubacterium eligens* group is considered to be probiotic [30]. The increase of *Eubacterium eligens* group in patients with MCI may be protective and this protective strategy disappears in patients with AD, which may participate in worsening the progress of AD pathology.

Since metabolic disorders, such as diabetes, interact with gut microbiome and may be linked to AD [31, 32], the presence of these disorders can be confounding factors for the interpretation of our findings. However, only one or two subjects in each group had diabetes and none of the subjects had obesity. Thus, the influence of these metabolic disorders in our findings may be small.

One limitation of our study is that we do not know how long patients had suffered from AD before they came to our hospital for care. It is possible that the severity of gut dysbiosis may be different at different stages of AD. All of our patients were newly diagnosed but their duration with AD is difficult to estimate. Staging them based on their signs and symptoms may reduce the power of our study due to a relatively small sample size. Future studies are needed to determine whether gut dysbiosis is worsened with increased severity of AD. Also, all of our subjects in this study are Chinese. The other three studies on the gut microbiome in patients with MCI have subjects in China or Japan [4, 11, 12]. Since diet, geographic location and genetics can affect gut microbiome [33, 34], it is not appropriate to extrapolate our findings or the findings from the other three studies to patients of other origins or locations.

CONCLUSIONS

We have shown that patients newly diagnosed with AD or MCI have gut microbiome changes that include a decrease of potentially protective microbiome and an increase of microbiome that can promote inflammation. Our results support a novel idea that the degree of gut dysbiosis is worsened with the disease stage from MCI to AD.

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Gut Microbiota Alterations and Cognitive Impairment Are Sexually Dissociated in a Transgenic Mice Model of Alzheimer's Disease

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Abstract.
Background: Normal aging is accompanied by cognitive deficiencies, affecting women and men equally. Aging is the main risk factor for Alzheimer's disease (AD), with women having a higher risk. The higher prevalence of AD in women is associated with the abrupt hormonal decline seen after menopause. However, other factors may be involved in this sex-related cognitive decline. Alterations in gut microbiota (GM) and its bioproducts have been reported in AD subjects and transgenic (Tg) mice, having a direct impact on brain amyloid-β pathology in male (M), but not in female (F) mice.
Objective: The aim of this work was to determine GM composition and cognitive dysfunction in M and F wildtype (WT) and Tg mice, in a sex/genotype segregation design.
Methods: Anxiety, short term working-memory, spatial learning, and long-term spatial memory were evaluated in 6-month-old WT and Tg male mice. Fecal short chain fatty acids were determined by chromatography, and DNA sequencing and bioinformatic analyses were used to determine GM differences.
Results: We observed sex-dependent differences in cognitive skills in WT mice, favoring F mice. However, the cognitive advantage of females was lost in Tg mice. GM composition showed few sex-related differences in WT mice. Contrary, Tg-M mice presented a more severe dysbiosis than Tg-F mice. A decreased abundance of *Ruminococcaceae* was associated with cognitive deficits in Tg-F mice, while butyrate levels were positively associated with better working- and object recognition-memory in WT-F mice.
Conclusion: This report describes a sex-dependent association between GM alterations and cognitive impairment in a mice model of AD.

Keywords: Anxiety, APP/PS1 mice, dysbiosis, high-throughput DNA sequencing, short-chain fatty acids, spatial memory, wildtype littermates

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting around 50 million people worldwide [1]. This debilitating disorder is marked by the aggregation of two proteins: amyloid-β, which accumulates in plaques, and tau, which can become hyperphosphorylated and aggregates in neurofibrillary tangles [1]. Despite the recent advance in the understanding of AD pathology, the exact etiology continues to be debated, impeding further development of effective therapies to treat or prevent this neurodegenerative disease.

Several risk factors have been associated with AD, such as lack of primary education, metabolic alterations, smoking, depression, and *APOE* ε4 genotype, among others [2]. Women had an 1.6-fold increase in AD incidence than men [3, 4], and this higher prevalence has been linked to the abrupt decline in sex hormones after menopause, and length of estrogen exposure during life [5, 6].

Cognitive abilities showed a sex dimorphism in young and old healthy subjects [7] as women showed advantages in object location memory [8, 9] and visual memory for objects [10, 11] over men. Contrary to this observation, male cognitive advantage is found on tests of mental rotation of spatial stimuli [12, 13] compared to females. With aging, however, this cognitive dimorphism in healthy subjects seems to be reversed or lost [14–16], with a worst [17–20] or better cognitive performance in men than women [14], or equal cognitive decline between genders [21].

Those conflicting results can be harmonized if we take in account the task and the related memory that was assessed. In early stages of AD, women showed still a better cognitive performance than age-matched men, but at advance stages of the disease, this cognitive advantage is lost [15, 22–26]. The Alzheimer's Disease Neuroimaging Initiative (ADNI) study shows that during amnestic mild cognitive impairment (MCI), females performed better in verbal memory compared to males. However, high levels of amyloid burden associates with a loss of this female cognitive advantage [22–24], as no sex-related differences were observed on block design [27] and visuoconstructive tests [28] in AD patients. Notwithstanding, AD pathological hallmarks showed a sexual dimorphism that is present even in late stages of the disease, as AD women present more neuritic plaques and neurofibrillary tangles compared to aged-matched men [29]. In contrast, another study claimed that no difference in the prevalence of senile plaques or neurofibrillary tangles was found by gender [30]. The use of animal models may help to better elucidate the sex-related differences in AD pathology, as human data shows conflicting results. Age-matched female and male transgenic (Tg) mice have an identical genetic background; therefore, the pathological and cognitive differences may be associated only with sex. At 15–19 months of age, female Tg2576 mice present more amyloid plaques than their male counterparts [31]. Similarly, a stronger plaque burden was observed in female APP/PS1 Tg mice compared to males [32, 33]. Despite the greater amyloid burden developed in aged female Tg mice, no worst cognitive decline is observed compared to males [34]. Emerging research proposes that the gut microbiota (GM) could play a central role in the regulation of emotions and cognition [35, 36]. Alterations in the GM seems to accompany aging [37, 38], where a decreased bacteria diversity and a pro-inflammatory profile dominates GM in aged people [39, 40]. Moreover, recent evidence suggests that GM could play a major role in AD, as lower GM diversity is observed in AD patients compared to aged-matched healthy subjects [41–46]. So far, these studies have not discriminated the sex factor in their results, despite women's higher incidence of AD. Experiments carried out in AD mice models showed a reduction in amyloid pathology after antibiotic-treatment in APP/PS1 male mice, but not in female mice [47]. Females' GM antibiotic resistant effect deserves attention, as it may underlie important etiological factors associated with females' vulnerability to develop AD. Therefore, it is necessary to determine the differences in the GM presented in female and male Tg mice, as well as describe the possible interaction between microbial communities and cognition as possible factor associated with women's greater vulnerability to develop AD.

MATERIALS AND METHODS

Experimental design

Hemizygous APP/PS1 male (Tg-M) and female (Tg-F) mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) transgene on a C57BL/6 genetic background (Jackson Laboratory, Bar Harbor, ME, USA, RRID: MMRRC-34829-JAX) and their wild-type (WT) littermates (males, WT-M and females, WT-F) were used for the study. Animal management was supervised by licensed veterinarian care and approved by the Bioethics

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Committee of the Center for Research and Advanced Studies of the National Polytechnic Institute (Cinvestav) in accordance with the Mexican official standard NOM-062-ZOO-1999 and the principles set forth in the 8th edition of the National Institute of Health (NIH) guide for the care and use of laboratory animals and endorsed by the Institute for Laboratory Animal Research, the Division on Earth and Life Studies, and the National Research Council of the National Academies. Once animals reached 4 months of age, they were individually housed with *ad libitum* access to food (AIN-93 diet) [48] and water under optimal vivarium conditions (12 h/12 h light–dark cycle, 20°C, and 40–50% relative humidity) for 2 months.

Estrous cycle evaluation

Cognitive evaluations in female mice were done only during the metaestrous/diestrous, the reproductive stages with the lowest hormonal levels [49], as estradiol have a direct impact in cognition and memory [50]. Cytology and typification of the reproductive stage was based on previous descriptions [51, 52].

Cognitive assessment

One week before sacrifice, behavioral testing was performed. To habituate the animals for the behavioral tests, animals were placed in the behavioral room 2 days before start testing trials. Anxiety-related behaviors were assessed by the Elevated Plus Maze (EPM) [50]. Briefly, the 50 cm elevated plus-shaped maze was made of medium-density fiberboard with a matte black acrylic surface and four arms (50 cm long and 10 cm wide) where two arms were open, and two arms were enclosed by 30 cm height walls. The task consisted of one trial per animal, where the animals were placed in the center of the EPM facing an open arm. Usually, rodents avoid open spaces and spend most of the time in enclosed places, such as the closed arms of the EPM [50]. Entries into the open/close arms were recorded during 5 min, and the animal's trajectory was used to calculate percentage of time spent in each arm [50] (Fig. 1A, B).

Working memory was evaluated by the T-maze (TM) [53]. The TM is an appropriate test to evaluate hippocampal and PFC functions in rodents [54] and to examine cognitive deficits in early stages of AD pathology [55]. Briefly, a T-shaped maze apparatus of polymethylmethacrylate with starting arm ($8.5 \times 10.5 \times 33.0$ cm), and two choice arms ($8.5 \times 10.5 \times 30.0$ cm each, left and right) was used as previously described [56]. The TM task consisted of two phases: 1) Sample phase, animals were placed at the start arm and allowed to choose between left or right choice arms. A central divider was then inserted into the start arm to create a start box. Once animals have chosen an arm, they were confined there for 30 s, thereafter the door was reopened and allow mice to return to the start arm, where animals were removed and returned to their cage. 2) Choice phase, 2 min later, animals were placed again in the start arm without the central divider and allowed them to choose an arm. When animals faced the TM apparatus, they display a tendency to alternate and avoid the entry into the previously entered arm. If the animal chooses two different arms in both phases, this is counted as spontaneous alteration. Two hours later the procedure was repeated. Three trials were given on each day for 2 days, with a total of 6 trials per animal. Percentage of spontaneous alternations were calculated from total of correct arm entries (Fig. 1C).

Recognition memory was evaluated by the Novel Object Recognition task (NOR) [57]. NOR is an appropriate test to evaluate recognition memory related to hippocampus [58] and prefrontal cortex (PFC) [59] functions, both regions heavily affected in AD [56]. This task is based on the natural tendency of rodents to explore a novel object more than a familiar one [57]. NOR test was carried out in a 40 cm^2 open field arena with 40 cm height walls of plywood surrounding the floor. NOR task consisted of three phases: 1) Habituation phase: animals explored the open field for 5 min without objects in the box; 2) Training phase: Twenty-four hours after habituation phase, animals were placed for 5 min in the box from the same starting position, and with two objects with identical features (weight, size, texture, shape and color) positioned in adjacent corners at 10 cm from the walls; 3) Trial phase: The recognition memory test was carried out 1.5 h after the training trial with the mice exploring the open field for 5 min in the presence of one familiar and one novel object, this last one different features than the former. A discrimination index (DI) was calculated for each animal after the test session (Fig. 1D) as DI = (time with novel object — time with familiar object) / (time with novel object+time with familiar object). Between all testing trials, all surfaces and objects were washed with 40 % ethanol solution. Exploration was defined as smelling and/or touching the object with the nose and/or forepaws [57].

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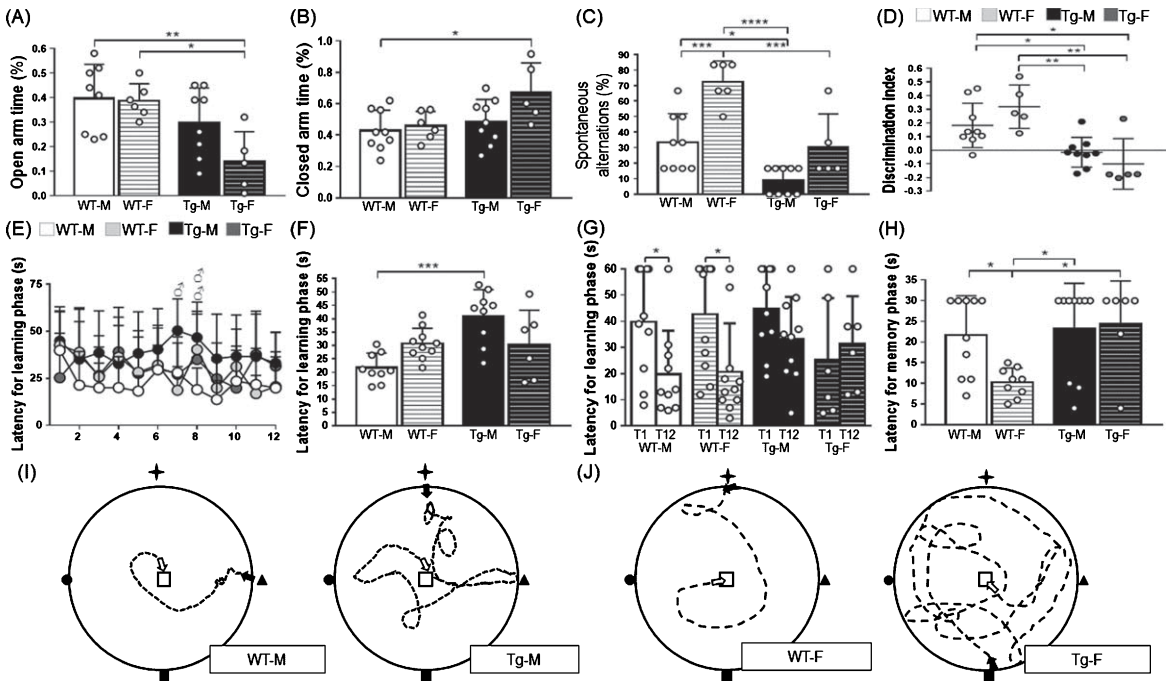


Fig. 1. Effect of gender and genotype in anxiety and memory performance. A) Anxiety was inversely proportional to the time spent in the open arm [F (1, 23) = 2.35, 3.03 and 12.42 for interaction, row and column factor respectively] and B) directly proportional to the time spent in the closed arms [F (1, 25) = 2.15, 4.07 and 6.30 for interaction, row and column factor respectively] in the elevated plus maze. C) Working memory was directly proportional to the spontaneous alternations [F (1, 25) = 2.25, 24.31 and 30.05 for interaction, row and column factor respectively] in the T-maze. D) Recognition memory was directly proportional to the discrimination index [F (1, 24) = 3.50, 0.18 and 26.80 for interaction, row and column factor respectively] in the novel object recognition test. E) Spatial learning curves show the latency to find the hidden platform during learning phase [F (33, 396) = 1.03, F (11, 396) = 1.69 and F (3, 396) = 14.68 for interaction, row and column factor respectively] in the WM. X axis corresponds to the 12 consecutive learning assays. F) Spatial learning was inversely proportional to the latency to find the platform during learning phase [F (1, 30) = 10.95, 0.09 and 10.07 for interaction, row and column factor respectively] in the water maze. G) Learning performance was compared between trial 1 and 12 during learning phase in the WM. H) Spatial memory was inversely proportional to the latency to find the place where the platform was originally located [F (1, 31) = 4.02, 2.80 and 6.34 for interaction, row and column factor respectively] during WM memory phase. I) Representative schemes of WT-M and Tg-M mice trajectory for spatial learning trials from the start point to the platform area. J) Representative schemes of WT-F and Tg-F mice trajectory for the spatial memory trial from the start point to the platform area. Cardinal points are marked in maze walls as a star-shaped polygon (north), a square (south), a triangle (west), and a circle (east). Black arrow indicates start point, white arrow indicates finish point, central square indicates platform area and dotted line indicates mouse trajectory in the WM test. For A, WT-M $n = 9$, WT-F $n = 7$, Tg-M $n = 9$, and Tg-F $n = 6$. For B and C, WT-M $n = 10$, WT-F $n = 7$, Tg-M $n = 10$, and Tg-F $n = 6$. For D, WT-M $n = 10$, WT-F $n = 6$, Tg-M $n = 10$, and Tg-F $n = 6$. For E, F, and G, WT-M $n = 10$, WT-F $n = 11$, Tg-M $n = 10$, and Tg-F $n = 6$. For H, WT-M $n = 10$, WT-F $n = 9$, Tg-M $n = 10$, and Tg-F $n = 6$. For A-C and F-H, white color circles within bars indicate experimental subjects. For D and E, white, grey and black circles indicate experimental groups. Data are shown as mean ± SD bars. For A-F and H, two-way ANOVA with Tukey *post-hoc* correction was performed comparing experimental groups. For G, unpaired *t* tests were performed comparing learning assay no.1 with no. 12 for each experimental group. For A-F and H, data between square brackets indicate F (DFn, DFd) values of two-way ANOVA with Tukey *post-hoc* corrections. Statistical significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. For E, statistical significance between WT-M versus WT-F is shown by □, Tg-M versus Tg-F by ○, WT-M versus Tg-F by ⌂, WT-F versus Tg-M by #, WT-M versus Tg-M by ♂and WT-F versus Tg-F by ♀one symbol represents $p < 0.05$, two symbols $p < 0.01$, three symbols $p < 0.001$, and four symbols $p < 0.0001$. T1 = learning trial no. 1, T12 = learning trial no.12, F, female; M, male; Tg, transgenic; WT, wild type.

Spatial memory was assessed by Water Maze (WM) [60]. The WM is an appropriate test to evaluate spatial memory related to hippocampus [61] and PFC [62] functions. Briefly, the WM task was carried out in a 100 cm diameter and 30 cm height round pool half filled with 26°C white water and an escape platform located in the center, as previously described [60]. Spatial clues were posted on each wall of the room with paper figures of different shapes and colors. WM task consisted of three phases, followed by an adaptation phase that consisted of placing the mice for 20 s on the scape platform visible for the animal: 1) Training phase: mice were released and allowed to swim in order to reach the scape platform in a maximum time of 60 s. This phase was repeated three times, each starting from a different cardinal point.

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2) Learning phase: the animal was allowed to swim in order to find the hidden scape platform in a maximum time of 60 s for 12 consecutive trials, starting 3 non-consecutive times from different cardinal points. 3) Memory phase: immediately after all animals have completed the 12 consecutive learning trials, they performed 1 probe trial, in which the scape platform was removed from the pool. The animal was released from the north and allowed to swim to find the missing platform in a maximum time of 30 s. The time spent (latency) to find the hidden platform (learning phase) and the platform area (memory phase) in the pool was recorded and assigned as an inversely proportional measure to the spatial learning and memory respectively (Fig. 1E-H). Experiments were recorded with a Logitech DH Pro Webcam C920 camera connected to a computer equipped with Logitech Capture 1.10.110 with the Eniima software (inhouse made software) in order to track animal's behavior and trajectory (Fig. 1I, J).

Determination of short chain fatty acids in feces

Fecal samples were collected for each animal before the cognitive tests. Animals were placed for 1 h in a Super Mouse 750™ Micro-Isolator® system cage for housing mice (15 cm width x 30 cm long x 17 cm high) previously cleaned and disinfected. At least 100 mg of feces pellets were collected per animal, immediately frozen and store at –70°C until use, for short-chain fatty acids (SCFAs) determination or microbiota analysis.

For SCFAs determination, the collected fecal samples were dried to constant weight and subsequently processed using the solid-phase extraction (SPE) method to analyze via HPLC (PerkinElmer Series N3896). At least 50 mg of dried feces were suspended completely in 1 ml of deionized water by vigorous mixing in a vortex at maximum speed for 10 min. The suspension was centrifuged at 13000 rpm for 10 min, the supernatant was transferred to a fresh tube and passed through activated C-18 max 100 mg/mL GracePure™ Reversed-Phase SPE Columns according to the manufacturer's instructions as previously described [63]. The SCFAs were eluted with 1 mL of filtered water (Nylon Syr Filter, 13 mm, 0.22 μm) and analyzed via HPLC using 20:80 acetonitrile:NaH_2PO_4 (J.T. Baker No. 9012-03 and Sigma-Aldrich No. S8282 respectively) at a pH of 2.2 using phosphoric acid, (J.T. Baker No. 0260-05) as mobile phase [64]. Standard curves were prepared for acetate (Sigma-Aldrich No. 45754-100ML-F), propionate (Sigma-Aldrich No. P1386-500ML), and butyrate (Sigma-Aldrich No. B103500-500ML). SCFAs proportion was calculated taking the total amount of acetate + propionate + butyrate for each experimental subject as 100%.

Fecal microbiota analysis

Bacterial DNA was extracted using the Favor-Prep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corp, Cat. No: FASTI001-1) according to the manufacturer´s instructions. The purified DNA quantity was measured using 260/280 nm absorbance using a Nano Drop 2000 spectrophotometer (Thermo Scientific), and its quality was evaluated by 0.5% agarose gels electrophoresis. Genomic libraries of ∼281 base pairs (bp) 16S rDNA 3rd hypervariable region (V3) amplicons were generated for each experimental subject using specific V3-341F primers against V3 that also contained a different 12-bp Golay barcode (set of barcodes 1–100) complementary to position 340–356 of *Escherichia coli* 16S rRNA molecule *rrnB* GeneBank J01859.1 and an overhang adapter specified by Ion Torrent, and V3-518R reverse primers complementary to position 517–533 of the same molecule [65]. The amplicons of the V3 regions were generated by 1X 50 μl polymerase chain reactions (PCR) containing 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer (settings specified above), 0.25 μl of 5 U/ μ l high thermostable DNA recombinant polymerase (Thermo Scientific No. EP0402), 1 μl of 10 mM dNTP mix (Thermo Scientific No. R0191), 4 μl of 25 mM $MgCl_2$ (Thermo Scientific No. R0971), 5 μl of 10X taq buffer with KCl (100 mM Tris-HCl [pH = 8.8 at 25°C], 500 mM KCl and 0.8% volume/volume [% v/v] nonidet P40, Thermo Scientific No. B38) and 20 ng of genomic DNA in 37.75 μl of nuclease-free water (Thermo Scientific No. R0581) per reaction. Amplification was carried out using Applied Biosystem Veriti Thermal Cycler (Supplementary Table 1). For sequencing, equivalent amounts of 1 to 100 barcoded amplicons (∼10 μg each) were combined. Each mix was purified using E-Gel iBase Power System (Invitrogen). The libraries size and concentration were determined using an Agilent 2100 Bioanalyzer, and libraries for each run were adjusted to 26 pM prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies), according to the manufacturer´s instructions. Amplicon enrichment with ion spheres was done using Ion

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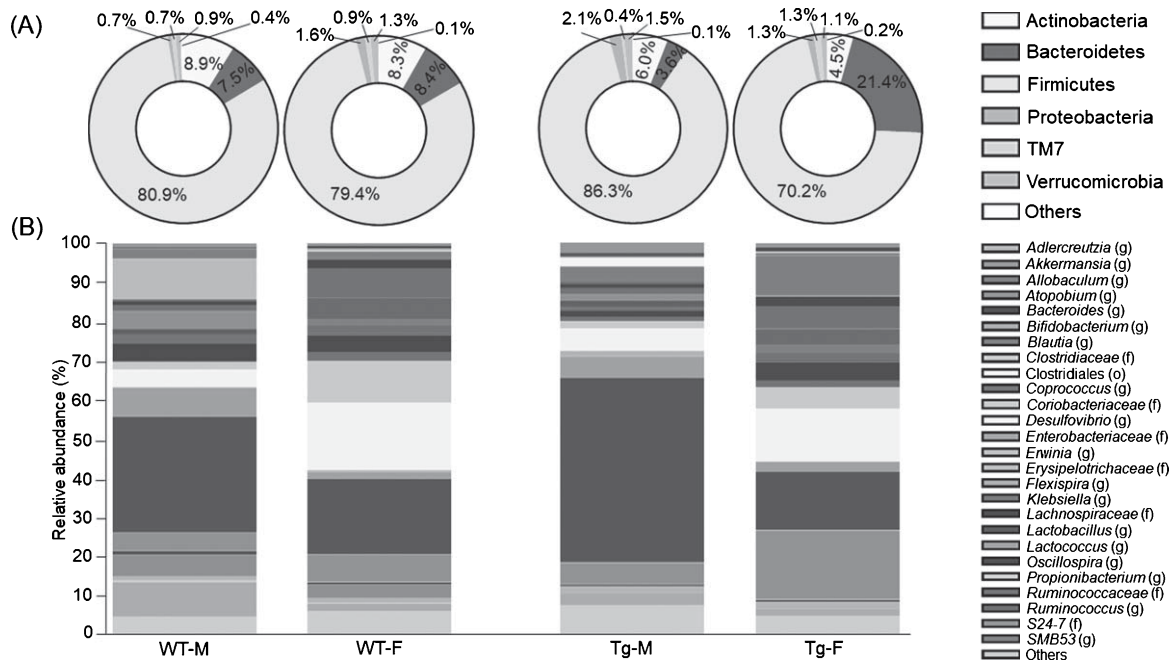


Fig. 2. Relative abundance of bacterial *taxa* in fecal samples. A) Pie charts of *phyla*'s relative abundance. The numbers on/beside charts indicate the percentage of abundance. For A, the color of each tag indicates bacterial *phyla*. Others refers to *phyla* abundances < 0.1% of relative abundance. B) Relative abundance of bacterial *taxa*. The color of each tag indicates order, family and genus bacterial *taxa*. Others refers to *taxa* <0.5% of relative abundance. WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. Data are shown as mean. Complete abundances and statistical significances are shown in Table 1. F, female; f, family; g, genus; M, male; o, order; Tg, transgenic; WT, wild type.

OneTouch ES. The sequencing was done using the Ion 316 Chip Kit v2 and the Ion Torrent Personal Genome Machine (PGM) System. After sequencing, reads were filtered by the PGM software to remove low quality and polyclonal sequences. During this process, sequences matching the 3´ adapters were automatically trimmed and filtered. Ion Torrent PGM software, Torrent Suite v4.0.2, was used to demultiplex the sequenced data based on their barcodes. Poor quality reads were eliminated from the datasets, i.e., quality score $\leq$20, containing homopolymers >6, length <200 nucleotides (nt), and containing errors in primers or barcodes. Filtered data were exported as FASTQ files. Demultiplexed sequencing data were analyzed using quantitative insights into microbial ecology (QIIME) software v1.9.0 pipeline [66]. FASTQ files were converted into FASTA files, and all demultiplexed files were concatenated into a single file. Closed reference operational taxonomic units (OTUs) were determined at the 97% similarity level using the UCLUST algorithm. Chimeras were detected and removed from the datasets using the ChimeraSlayer. Sequence alignments were done against the Greengenes 13.9 core set (sequencing summary is shown in Supplementary Table 2). Microbial sequence data were pooled for OTUs analysis and comparison of taxonomic relative abundance was obtained (Figs. 2 and 3, Supplementary Figure 1, Table 1, and Supplementary Table 3). Prior to calculate microbial diversity, the OTU table was rarefied at 8,700 sequences per sample (samples with <8,700 were omitted) using a "single_rarefaction.py" QIIME script for alpha diversity metrics. *Taxa* richness measurements (Observed and Chao1) and alpha diversity indexes (Shannon and Simpson) were calculated (Fig. 4A, Table 2, and Supplementary Figure 2). Weighted and unweighted UniFrac distances were used to perform the principal coordinate analysis (PCoA; Fig. 4B). Community diversity was determined by the number of OTUs and beta diversity, measured by UniFrac unweighted and weighted distance matrices in QIIME software v1.9.0. Analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (ADONIS), were used to determine statistically significant clustering of groups based upon microbiota structure distances. Furthermore, linear discriminant analysis (LDA) effect size (LefSe) program v1.0 was used to per-

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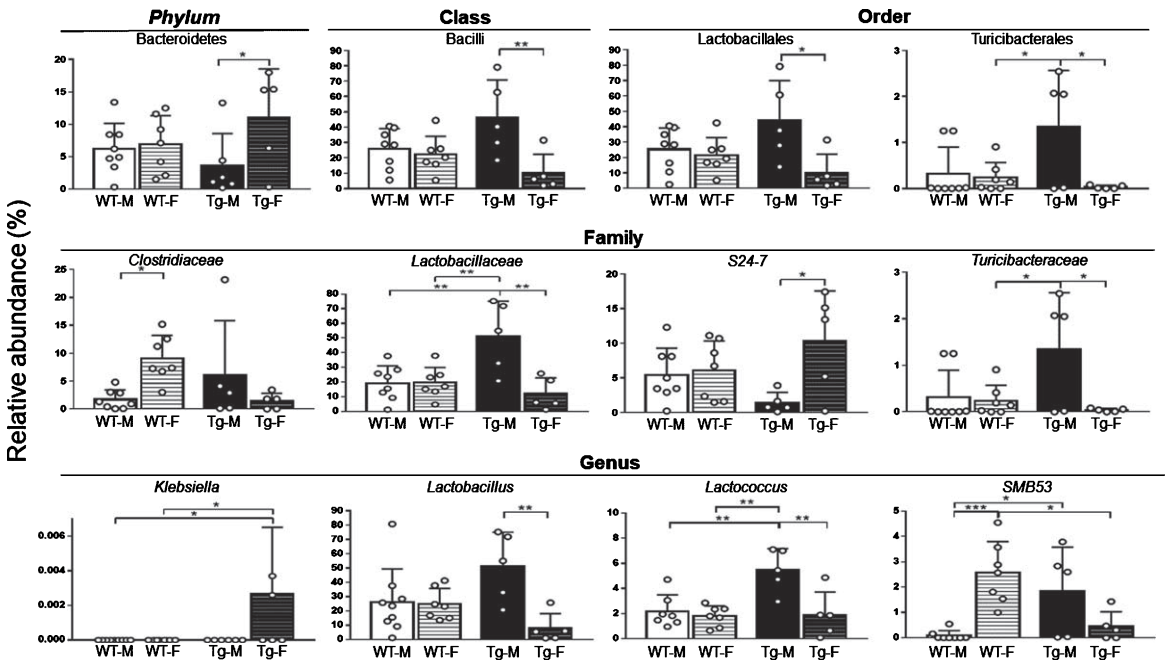


Fig. 3. Relative abundance of prominent bacterial *taxa* in fecal samples. Relative abundance values of Bacteroidetes [F (1, 21) = 5.20, 6.93 and 0.92 for interaction, row and column factor respectively], Bacilli [F (1, 21) = 6.38, 9.92 and 0.42 for interaction, row and column factor respectively], Lactobacillales [F (1, 21) = 5.24, 8.15 and 0.29 for interaction, row and column factor respectively], Turicibacterales [F (1, 21) = 5.27, 6.69 and 2.29 for interaction, row and column factor respectively], *Clostridiaceae* [F (1, 21) = 8.99, 0.44 and 0.64 for interaction, row and column factor respectively], *Lactobacillaceae* [F (1, 21) = 11.16, 10.98 and 4.26 for interaction, row and column factor respectively], *S24-7* [F (1, 21) = 4.96, 6.54 and 0.003 for interaction, row and column factor respectively], *Turicibacteraceae* [F (1, 21) = 5.20, 6.60 and 2.34 for interaction, row and column factor respectively], *Klebsiella* [F (1, 25) = 0.05 for interaction, row and column factor], *Lactobacillus* [F (1, 22) = 7.59, 8.67 and 0.30 for interaction, row and column factor respectively], *Lactococcus* [F (1, 21) = 7.50, 11.60 and 8.23 for interaction, row and column factor respectively] and *SMB53* [F (1, 21) = 20.96, 1.55 and 0.17 for interaction, row and column factor respectively] present in fecal samples are shown. WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. White color circles within bars indicate experimental subjects. Data are shown as mean. Data between square brackets indicate F (DFn, DFd) values of two-way ANOVA with Tukey *post-hoc* correction. Statistical significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Complete abundances and statistical significances are shown in Table 1 and Supplementary Table 3. F, female; M, male; Tg, transgenic; WT, wild type.

form LDA (scores $\geq$2; Fig. 5 and Supplementary Table 4) in order to estimate significant effect size of each bacterial *taxa* between experimental groups [67].

Metagenome prediction

We used Phylogenetics Investigation of Communities by Reconstruction of Unobserved States (PICRUSt v.1.1.1) in order to predict related metabolic profiles of the metagenomes from 16S rDNA gene data set by Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs classification database at hierarchy level 3 pathways. Statistical Analysis of Taxonomic and Function (STAMP v2.1.3) software was used to determinate significant differences in abundance of OTUs related to metabolic pathways (Fig. 6 and Supplementary Table 5) [68].

Correlations between determined parameters

Associations between cognition, SCFAs concentration and proportion, bacterial *taxa* relative abundance, microbial diversity, and proportion of predicted metabolic pathways-related sequences were assessed by Pearson's correlation coefficient (GraphPad Prism version 6) (Fig. 7).

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD). Open arm time, closed arms entries, spontaneous alternations, DI, WM latencies, SCFAs concentration and proportion, GM relative abundances, alpha diversity indexes, food intake, and body weight changes were analyzed by a two-way ANOVA followed by a Tukey *post-hoc* test. Beta diversity was analyzed by ANOSIM and ADONIS,

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Table 1
Fecal microbiota relative abundance (%) reported in Figures 2 and 3

Taxa	WT-M	WT-F	Tg-M	Tg-F
Firmicutes (p)	75.7119	81.8732	81.293	73.4341
Actinobacteria (p)	16.7785	7.4523	5.383	3.9604
Bacteroidetes (p)	5.7831	8.0194	6.4088	19.0961°
Verrucomicrobia (p)	1.1459	0.718	2.9396	1.048
Proteobacteria (p)	0.3247	1.2157	3.3628	1.2433
TM7 (p)	0.2041	0.6366	0.5097	1.0352
Others (p)	0.0513	0.0848	0.1027	0.1829
Clostridiales (o)	4.9036	18.3429	6.3821	14.2808
Erycipelotrichaceae (f)	11.022	0.11803	0.0232	0.18556
S24-7 (f)	5.0183	0.07403	5.8484	0.18384°
Lachnospiraceae (f)	4.8664	4.58625$^{\square}$	1.5317	4.88957
Clostridiaceae (f)	1.6447	11.1083$^{\square}$	1.7642	5.49927
Ruminococcaceae (f)	1.4862	7.66618	1.5629$^{\#}$	5.94374
Coriobacteriaceae (f)	0.4584	0.2753	0.0141	0.023
Enterobacteriaceae (f)	0.0247	0.0077	0.0293	0.0053
Lactobacillus (g)	30.9196	20.3754	50.7033$^{\male\male}$	15.5784$^{\circ\circ}$
Bifidobacterium (g)	9.2151	2.0181	3.2659	1.7438
Lactococcus (g)	7.7709	1.8941	5.8340$^{\male\male\#\#}$	2.6664$^{\circ\circ}$
Atopobium (g)	5.9431	3.7677	0.0155	0.0022
Ruminococcus (g)	4.6284	0.0108	1.7443	0.0109
Others (o, f and g)	4.0083	5.832632	7.0538	4.500245
Allobaculum (g)	2.2476	1.3244	3.9467	10.4922
Coprococcus (g)	2.1357	2.2622	0.978	1.9634
Akkermansia (g)	1.1459	0.718	2.9396	1.048
Adlercreutzia (g)	1.076	1.2847	1.9564	2.016
Oscillospira (g)	0.9632	2.4655	1.1927	2.5113
Bacteroides (g)	0.6825	0.4044	0.3086	0.4135
SMB53 (g)	0.4086	2.1813$^{\square\square\square}$	3.2852$^{\male}$	1.7301$^{\female}$
Blautia (g)	0.1732	0.4018	0.2543	0.4719
Desulfovibrio (g)	0.0804	0.2587	2.4434	0.1312
Propionibacterium (g)	0.0421	0.058	0.072	0.1056
Flexispira (g)	0.0351	0.5416	0.2183	0.2797
Erwinia (g)	0.008	0.0038	0.0022	0.0049
Klebsiella (g)	0	0	0	0.0027$^{\house\female}$

Fecal microbiota proportions are shown for bacterial *phyla*, orders, families, and genera in Figs. 3 and 4. WT-M $n=9$, WT-F $n=8$, Tg-M $n=6$, and Tg-F $n=6$. Data are shown as mean. Statistical significances (p values) were calculated by two-way ANOVA with Tukey *post-hoc* correction. Statistical significance between WT-M versus WT-F is shown by □, Tg-M versus Tg-F by ○, WT-M versus Tg-F by ⌂, WT-F versus Tg-M by #, WT-M versus Tg-M by ♂, and WT-F versus Tg-F by ♀; one symbol represents $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, and four symbols $p<0.0001$. f, family; g, genus; o, order; p, *phylum*; Tg-C, transgenic mice fed with control diet; Tg-F, transgenic mice fed with agave fructans supplemented diet; Tg-F-Abx, transgenic mice fed with agave fructans supplemented diet and treated with antibiotics as described in materials and methods; WT-C, wild type mice fed with control diet.

whereas differences for *taxa* enrichment, abundance of sequences related to predictive metabolic pathways and correlations between determined parameters were evidenced by LefSe, t- and one-tailed tests, respectively. All results were considered statistically significant at $p<0.05$.

RESULTS

Behavioral and cognitive performance

Behavioral tests in female mice were done only at metestrus-/diestrus phase of the estrous cycle. Anxiety was determined as the proportion of time spent in the open/closed arms in the EPM test. Tg-F mice spent significantly less time in the open arms compared to WT-F, whereas time spent in closed arms was higher in Tg-F compared to WT-M. This data indicates that Tg-F mice showed higher anxiety than their WT-counterpart, which was not observed in males (Fig. 1A, B). Working memory was assessed by the T-maze test. Percentage of spontaneous alternations (%) was higher in WT-F compared to WT-M, Tg-M, and Tg-F mice. WT-M also showed better working memory than Tg-M mice (Fig. 1C). Object

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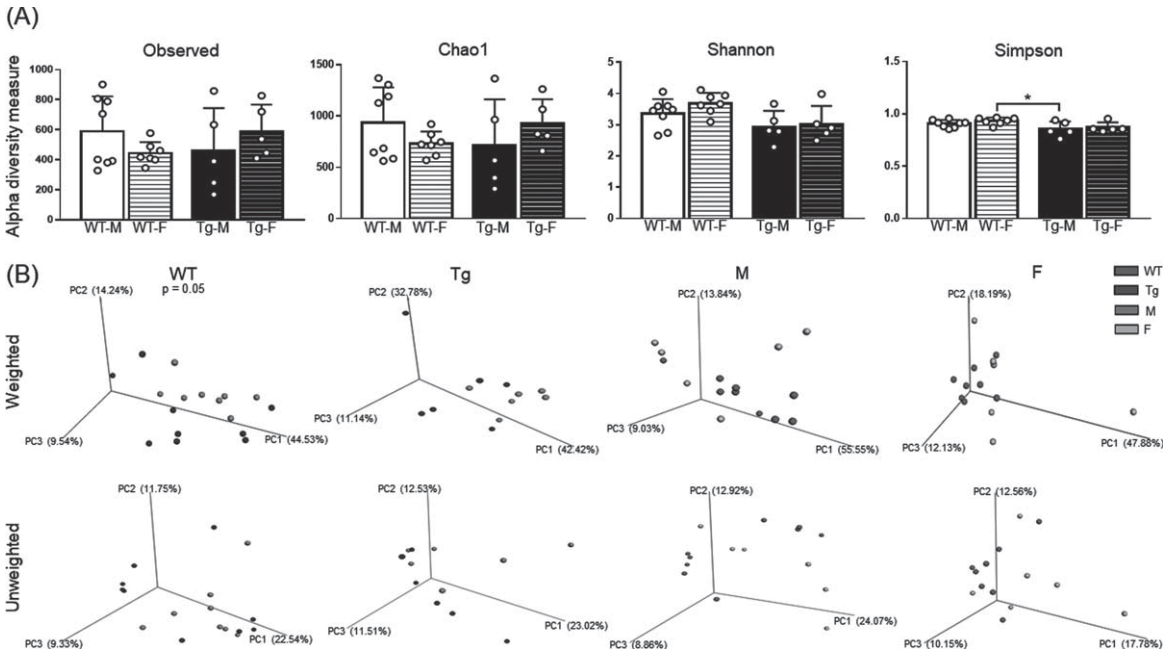


Fig. 4. Fecal microbiota bacterial diversity. Alfa diversity analyses for experimental groups are shown in (A) by observed number of *taxa* [F (1, 21) = 2.71, 0.01 and 0.01 for interaction, row and column factor respectively], Chao1 richness [F (1, 21) = 2.74, 0.0001 and 0.01 for interaction, row and column factor respectively], Shannon index [F (1, 21) = 0.38, 1.19, and 8.02 for interaction, row and column factor respectively] and Simpson index [F (1, 21) = 0.09, 1.15 and 8.65 for interaction, row and column factor respectively]. Beta diversity analyses for experimental groups plotted as principal coordinates analysis (PCoA) in (B) of weighted and unweighted variables. WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. For A, white color circles within bars indicate experimental subjects. For B, each subject is represented by a color sphere. Data are shown as mean ± SD bars. For A, data between square brackets indicate F (DFn, DFd) values of two-way ANOVA with Tukey *post-hoc* correction and statistical significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. For B, p values for PCoA schemes with significative clustering are showed according to the following results: ADONIS: $R = 0.07$, $p = 0.33$ and ANOSIM: $R = 0.02$, $p = 0.34$ for weighted and ADONIS: $R = 0.10$, $p = 0.05$ and ANOSIM: $R = 0.16$, $p = 0.06$ for unweighted variables comparing WT mice; ADONIS: $R = 0.18$, $p = 0.08$ and ANOSIM: $R = 0.11$, $p = 0.14$ for weighted and ADONIS: $R = 0.09$, $p = 0.47$ and ANOSIM: $R = –0.01$, $p = 0.46$ for unweighted variables comparing Tg mice; ADONIS: $R = 0.10$, $p = 0.18$ and ANOSIM: $R = 0.06$, $p = 0.24$ for weighted and ADONIS: $R = 0.09$, $p = 0.20$ and ANOSIM: $R = 0.11$, $p = 0.14$ for unweighted variables comparing M mice; and ADONIS: $R = 0.10$, $p = 0.23$ and ANOSIM: $R = 0.15$, $p = 0.07$ for weighted and ADONIS: $R = 0.09$, $p = 0.08$ and ANOSIM: $R = 0.26$, $p = 0.01$ for unweighted variables comparing F mice. F, female; M, male; Tg, transgenic; WT, wild type.

Table 2
Alpha diversity indexes for experimental groups

Experimental groups	Statistical parameter	Observed taxa	Chao1 richness	Shannon index	Simpson index
WT (M)	mean ± SD	588.63 ± 234.04	933.99 ± 345.31	3.36 ± 0.46	0.91 ± 0.03
WT (F)		443.00 ± 75.09	730.47 ± 118.92	3.68 ± 0.34	0.93 ± 0.03
Tg (M)		459.80 ± 284.79	717.88 ± 443.44	2.94 ± 0.50	0.86 ± 0.07
Tg (F)		588.20 ± 179.51	924.42 ± 238.42	3.02 ± 0.57	0.87 ± 0.05
WT-M versus WT-F	p	0.3301	0.3744	0.3461	0.497
Tg-M versus Tg-F		0.5512	0.5016	0.9442	0.8584
WT-M versus Tg-M		0.4810	0.3998	0.2393	0.1395
WT-F versus Tg-F		0.4175	0.4921	0.0500	0.0677

Alpha diversity indexes and statistical values for comparisons between WT-M ($n = 9$), WT-F ($n = 8$), Tg-M ($n = 6$), and Tg-F ($n = 6$) mice in Figure 5. Statistical significances (p values) were calculated by two-way ANOVA with Sidak *post-hoc* correction. F, female; M, male; N.A., not applicable; SD, standard deviation; Tg, transgenic mice; WT, wild type mice.

recognition memory was determined by the NOR test. Impaired recognition memory was observed in both Tg-M and Tg-F mice, as they presented lower DIs compared to their WT-counterparts (Fig. 1D). Spatial-learning and spatial-memory were assessed by the WM test. Learning curves are shown in Fig. 1E. Average time to find the scape platform during all learning trials indicate that Tg-M mice had higher

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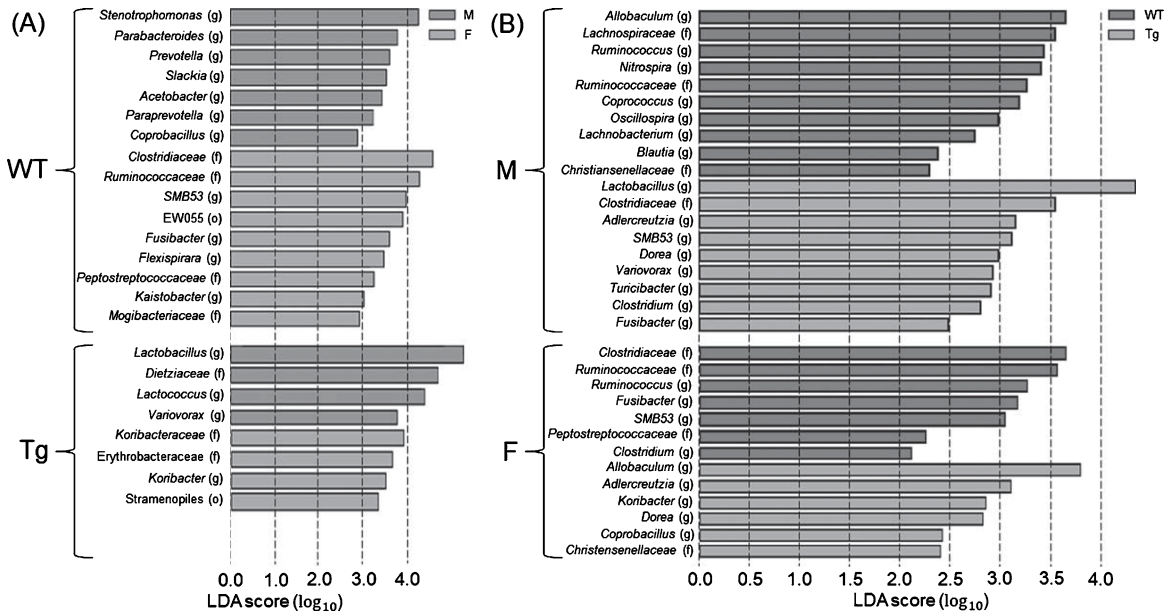


Fig. 5. Fecal enriched bacterial *taxa* associated to gender and genotype. The figure shows the results of the linear discriminant analysis (LDA) effect size (LEfSe) by gender (A) and genotype (B) comparison of differentially abundant bacterial *taxa* (orders, families and genera) significantly associated with WT-M ($n = 9$), WT-F ($n = 8$), Tg-M ($n = 6$), and Tg-F ($n = 6$). For A and B, the color tags on the top right side indicate experimental groups. LDA score (shown at logarithmic scale underneath the bars and indicate with vertical dotted lines) cutoff of 2 was used to discriminate bacterial *taxa*. Specific LDA scores and statistical significances are shown in Supplementary Table 4. F, female; f, family; g, genus; M, male; o, order; Tg, transgenic; WT, wild type.

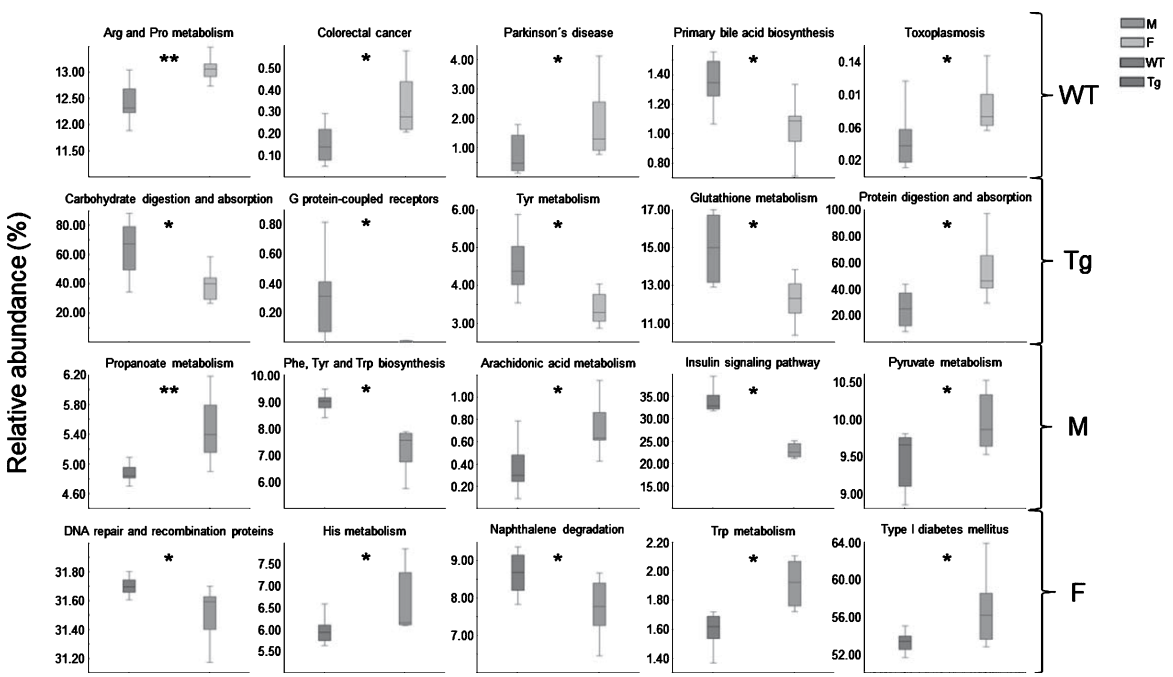


Fig. 6. Prediction of the functional metagenome of the fecal bacterial microbiota. The figure shows graphic representations of significant predicted metabolic pathways determined by PICRUSt v1.1.1 regarding the corresponding bacterial OTU table. Y-axis shows relative abundance to corresponding pathway according to bacterial *taxa* composition for each experimental group (WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$). Data are shown as mean ± SD bars. Statistical significances calculated by *t*-test are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. F, female; M, male; Tg, transgenic; WT, wild type.

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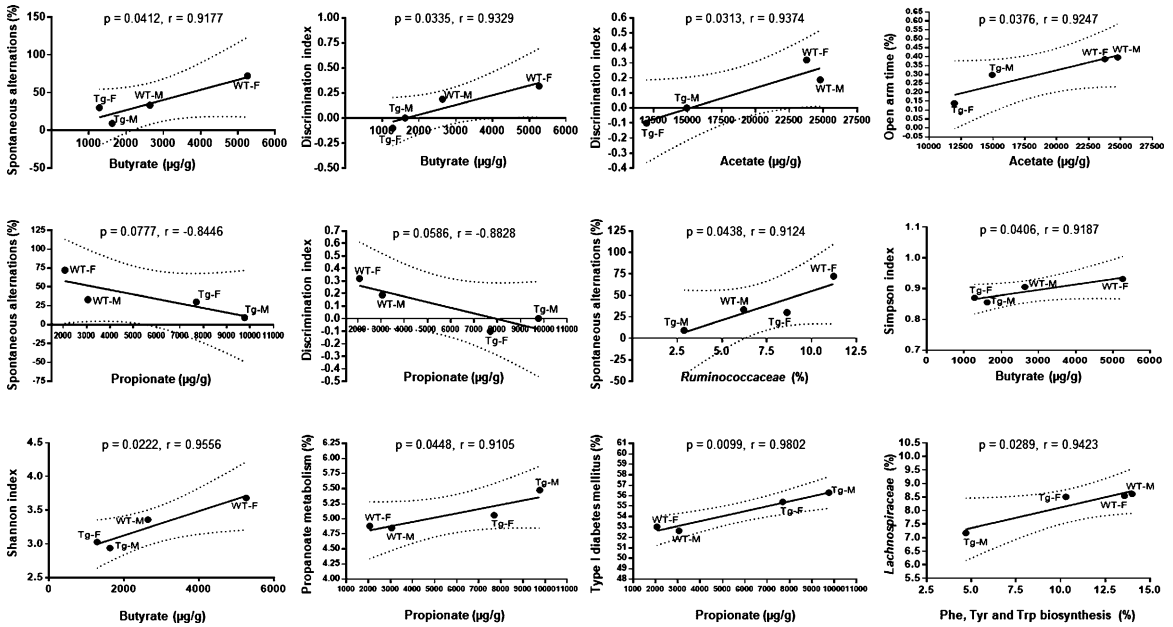


Fig. 7. Correlations between determined parameters. Pearson's correlation between butyrate concentration and spontaneous alternation proportion, butyrate concentration and discrimination index, acetate concentration and discrimination index, acetate concentration and open arm time proportion, propionate concentration and spontaneous alternation proportion, propionate concentration and discrimination index, *Ruminococcaceae* relative abundance and spontaneous alternation proportion, butyrate concentration and Simpson index, butyrate concentration and Shannon index, propionate concentration and propanoate metabolism predicted pathway, propionate concentration and type I diabetes mellitus predicted pathway, and Phe, Tyr and Trp biosynthesis predicted pathway and *Lachnospiraceae* relative abundance are shown. WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. Data are shown as mean. One-tailed p values (p), Pearson's correlation coefficients (r), trend (solid line) and 95% confidence interval (dotted line) are shown for each correlation. F, female; M, male; Tg, transgenic; WT, wild type.

latencies compared to their WT-counterpart (Fig. 1E, F). To determine effective learning, we compared the latencies to find the scape platform in trial 1 (E1) compared to trial 12 (E12). We observed that only WT mice (both, males and females) were able to show an effective learning as E12 <E1, but Tg mice (both, males and females) did not showed significant differences between E12 and E1 (Fig. 1G). Spatial memory test was carried out after learning trials. WT-F showed better spatial memory than WT-M, Tg-M and Tg-F (Fig. 1H). Representative trajectories during learning and memory phases for M and F subjects are shown in Fig. 1I and 1J, respectively.

Short chain fatty acids concentration

SCFAs were quantified in fecal samples. The concentration of acetate was decreased in Tg mice (male: 14939.03 ± 5496.59 µg/g, female: 11978.96 ± 5480.01 µg/g) compared to their WT counterparts (male: 24776.47 ± 6380.95 µg/g, female: 23768.91 ± 3387.26 µg/g). In contrast, propionate concentration increased in Tg mice (male: 9765.39 ± 3128.70 µg/g, female: 7696.35 ± 5746.66 µg/g) compared to their WT counterparts (male: 3056.42 ± 2163.77 µg/g, female: 2071.83 ± 742.01 µg/g). Butyrate concentration was higher in WT-F mice (5263.79 ± 1455.44 µg/g) compared to WT-M (2639.47 ± 1039.08 µg/g), Tg-F (1284.66 ± 432.52 µg/g), and Tg-M mice (1623.90 ± 558.06 µg/g).

Fecal microbiota analysis

To determine whether 6-month-old mice presented sex- and/or genotype-dependent GM dysbiosis, we analyzed the fecal microbiota *taxa* relative abundance in all experimental groups. At *phylum* level, we found a higher abundance of Bacteroidetes in Tg-F compared Tg-M (Figs. 2A and 3, and Table 1). At class level, a higher relative abundance of Bacilli was observed in Tg-M compared to Tg-F (Fig. 3, Supplementary Figure 1, and Supplementary Table 3). At order level, higher abundances of Lactobacillales and Turicibacterales were observed in Tg-M compared to Tg-F mice and compared to WT-F only for Turicibacterales (Fig. 3, Table 1, Supplementary

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Figure 1, and Supplementary Table 3). At family level, *Clostridiaceae* abundance was higher in WT-F compared to WT-M mice. *Lactobacillaceae* relative abundance was higher in Tg-M compared to the rest of the groups. *S24-7* showed an increase in Tg-F compared to Tg-M, and *Turicibacteraceae* was higher in Tg-M compared to Tg-F and WT-F (Fig. 3, Table 1, Supplementary Figure 1, and Supplementary Table 3). At genus level, abundance of *Klebsiella* was higher in Tg-F compared to WT-F and WT-M, while *Lactobacillus* was higher in Tg-M compared to Tg-F. *Lactococcus* showed higher proportions in Tg-M compared to the other three groups, and *SMB53* showed a higher abundance in WT-F compared to WT-M and Tg-F, increasing its abundance in Tg-M in comparison to WT-M mice (Figs. 2B and 3 and Table 1).

Alpha diversity was estimated by the Observed number of *taxa*, Chao1 richness and Shannon and Simpson indexes (rarefaction curves are shown in Supplementary Figure 2). A decreased bacterial diversity was observed in Tg-M compared to WT-F according to Simpson index (Fig. 4A). No significant differences were found in observed number of *taxa*, Chao1 richness and Shannon index (Table 2). The clustering the bacterial communities using PCoA of weighted and unweighted variables revealed a sex-dependent segregation between WT mice comparing weighted variables, without statistical clustering for the rest of comparisons (Fig. 4B).

Next, we used a linear discriminant analysis effect size (LEfSe) to determine enriched *taxa* per condition. Seven *taxa* were enriched in WT-M versus WT-F (*Stenotrophomonas, Parabacteroides, Prevotella, Slackia, Acetobacter, Paraprevotella* and *Coprobacillus*), whereas nine *taxa* were enriched in WT-F versus WT-M (*Clostridiaceae, Ruminococcaceae, SMB53*, *EW055*, *Fusibacter, Flexispira, Peptostreptococcaceae, Kaistobacter and Mogibacteriaceae*). Additionally, four *taxa* were enriched in Tg-M versus to Tg-F (*Lactobacillus*, Dietziaceae, *Lactococcus* and *Variovorax*) and four in Tg-F versus Tg-M (*Koribacteraceae, Erythrobacteraceae, Koribacter and* Stramenopiles) (Fig. 5A and Supplementary Table 4). Also, ten *taxa* were enriched for WT-M versus Tg-M (*Allobaculum*, Lachnospiraceae, *Ruminococcus*, *Nitrospira*, *Ruminococcaceae*, *Coprococcus*, *Oscillospira*, *Lachnobacterium*, *Blautia* and *Christiansenellaceae*), whereas nine *taxa* were enriched in Tg-M versus WT-M (*Lactobacillus*, Clostridiaceae, *Adlercreutzia*, *SMB53*, *Dorea*, *Variovorax*, *Turicibacter*, *Clostridium* and *Fusibacter*). Regarding females, seven *taxa* were enriched in WT-F versus Tg-F (Clostridiaceae, Ruminococcaceae, *Ruminococcus, Fusibacter*, *SMB53*, *Peptostreptococcaceae*, and *Clostridium*), while six enriched *taxa* were observed in Tg-F versus WT-F (*Allobaculum*, *Adlercreutzia*, *Koribacter*, *Dorea*, *Coprobacillus* and *Christiansenellaceae*; Fig. 5B and Supplementary Table 4).

Metagenome prediction analysis

Functional metabolic pathways related to fecal microbiota abundances was determined by PICRUSt analysis using the OTU table. The main 20 pathways showing statistically significant difference between experimental groups were mostly linked with metabolism, genetic information processing, human diseases, organismal systems, and environmental information processing (Fig. 6 and Supplementary Table 5). Among these, arginine (Arg) and proline (Pro) metabolism, colorectal cancer, Parkinson's disease, and toxoplasmosis were increased in WT-F with respect to WT-M, while primary bile acid biosynthesis was decreased in WT-F regarding WT-M. On the other hand, carbohydrate digestion and absorption, G protein-coupled receptors, tyrosine (Tyr), and glutathione metabolism were decreased in Tg-F in comparison to Tg-M mice. Protein digestion and absorption was decreased in Tg-M compared to Tg-F. Tg-M presented increases in propanoate, arachidonic acid and pyruvate metabolic related-pathways, and decreases in insulin signaling pathway and phenylalanine (Phe), Tyr and tryptophan (Trp) biosynthesis compared to WT-M. On the other hand, Tg-F presented increases in type I diabetes mellitus and histidine (His) and Trp metabolism, and decreases in naphthalene degradation, DNA repair, and recombination proteins compared to WT-F (Fig. 6).

Correlations analysis

The determine the possible association between behavioral/cognitive performance in the experimental groups versus SCFAs concentration, *taxa* relative abundance, alpha diversity metrics, and predicted metabolic pathways we used the Pearson's correlation coefficient test (Fig. 7). Butyrate concentrations correlated positively with working- and object recognition memory with the highest values in WT-F mice, and the lowest in Tg-F mice. Acetate concentration correlated positively with a better object recognition memory and with lower anxiety index,

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as WT mice showed the higher values and Tg-F mice the lower values. Propionate levels in fecal samples showed a negative correlation with working memory and object-recognition memory, as Tg-M mice showed the highest values. Among all detected *taxa*, only *Ruminococcaceae* and *Lachnospiraceae* families were positively correlated with working memory and Phe, Tyr, and Trp biosynthesis predicted pathway, respectively. Simpson and Shannon indexes were positively correlated with butyrate concentrations, with highest values found in WT-F mice. Finally, propionate concentrations in feces were positively correlated with propanoate metabolism, and Type I diabetes mellitus predicted pathways, with highest values in Tg-M mice (Fig. 7).

DISCUSSION

The higher risk for women to develop AD has been associated with a decline in sex hormones during the transition to the menopause [5, 6]. However, brain alterations can be observed even before menopause [69, 70], despite ovarian estrogen production and an active reproductive function. Therefore, it remains to be explained why women are at higher risk to develop AD than men. Here, we aimed to describe whether GM alterations can be associated with the cognitive decline in 6-month-old APP/PS1 mice (Tg) based on a sex/genotype segregation design. We observed sex-dependent differences in cognitive skills in male versus female WT mice, favoring females. However, in Tg-F mice the cognitive advantage over males was lost. GM composition showed few differences between female and male WT mice, with only a marked abundance of *Clostridiaceae* in WT-F compared to their opposite sex. Tg mice showed several sex-dependent GM alterations, being more pronounced in male mice. In addition, Tg mice showed decreased concentrations of butyrate and acetate compared to sex-matched WT mice. However, propionate was highly abundant in Tg mice (both sexes) compared to their sex-matched controls. Butyrate and acetate levels were directly associated with better working- and object recognition memory. Contrary, propionate was inversely associated with those types of memories.

For this work, we used APP/PS1 mice as they develop spatial, working, and recognition memory impairments starting at 3-4 months of age [71, 72]. In addition, APP/PS1 mice also presented detectable GM alterations since three months of age [73], being more pronounced after six months of age compared to WT animals [74–78]. Thus, APP/PS1 mice are an ideal AD model to evaluate dysbiosis-related to memory alterations.

The first objective of our study was to describe sex-dependent cognitive alterations in WT and Tg mice. In AD mice models, it has been shown that male and female 3xTgAD mice presented equally impaired cognition at 4 months of age and a faster rate of cognitive decline in females compared to male mice at 9 months of age, but this sex-differences were lost at 12 months of age, as both sexes performed worse than their WT counterparts [34]. We observed a better working memory in WT-F compared to WT-M mice in the T-maze test. However, this sexual dimorphism disappeared in Tg mice, as both male and females Tg mice showed similar percentage of spontaneous alternations and an impaired spatial learning compared to WT mice. APP/PS1 mice model develops neuropathological and cognitive alterations early than the previously used 3xTgAD mice. Therefore, our data supports early studies in humans and Tg models where sexual dimorphism in healthy subjects (WT) favors females, while AD pathology abates the sexual dimorphism in cognition.

The pathological hallmarks of AD also showed a sexual dimorphism that is present in young and old Tg mice as females exhibit an increased plaque burden and higher $A\beta_{40}$ levels than age-matched males along aging [31–33]. Notwithstanding, female Tg mice do not show worst cognitive impairment as amyloid pathology progresses, compared to males. Previous studies addressed the concept of compensatory mechanisms against brain dysfunction during the perimenopausal period in females. Perimenopause is characterized by brain alterations such as a decline in glucose metabolism and neuronal bioenergetics [79, 80], allowing a better utilization of ketone bodies as an alternative brain fuel [70, 81, 82]. Those compensatory mechanism are observed around 6–9 months of age in healthy mice, but as early as 3 months in Tg mice [80]. Notably, peripheral metabolic alterations are also observed in perimenopause rodents [79, 80, 83]. GM composition has important role in host-metabolism, and dysbiosis has been associated with obesity and diabetes onset [84–86]. In this study, we did not measure brain or peripheral bioenergetics markers. However, our predicted metabolic pathway showed that Tg-F mice has an enhanced metabolic pathway linked to diabetes type-1 than WT-F, that may reflect an impaired peripheral glucose metabolism.

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Recent focus has been paid to GM alterations and their possible impact in brain function. AD patients show an altered GM composition compared to healthy age-matched subjects [41–46]. However, those previous reports did not discriminate between genders. Cox et al. (2019) showed more age-related microbiome changes in female Tg mice (Tg2576) compared to female WT mice [87]. Importantly, GM alterations in Tg mice can be already detected since the first and third month of age compared to their WT littermates, preceding brain amyloid-β aggregation [73]. The impact of GM on the neuropathological hallmarks of AD was demonstrated in germ-free Tg mice and antibiotic-treated Tg mice, as under both experimental conditions Tg mice showed a reduction in brain amyloid-β aggregation in male mice [75, 76]. However, similar antibiotic treatment did not affect amyloid-β aggregation in female Tg mice [47]. This females' antibiotic resistant effect abating amyloid aggregation may have important implications in the etiology of AD. Our data showed a lower GM diversity trend in Shannon and Simpson indexes for Tg-F compared to WT-F group. Similarly, AD patients show lower microbial diversity compared to healthy controls [45]. We did not observe significant differences at *phylum* level between WT and Tg mice. However, at a more specific level of *taxa*, several differences were detected between Tg-F and Tg-M mice, while a more homogeneous ecology was observed between WT-M and WT-F mice. In the one side, WT-F show an increased abundance of *Clostridiaceace* compared to WT-M. On the other side, Tg-M mice presented an increased abundance of Bacilli (class), Lactobacillales, Turicibacterales (orders), *Lactobacillaceae, Turicibacteraceae* (families), *Lactobacillus*, and *Lactococcus* (genera) compared to Tg-F mice. Meanwhile, Tg-F showed an enhanced abundance of Bacteroidetes (*phylum*), *S24-7* (family), and *Klebsiella* (genus) compared to Tg-M mice. *Turibacteraceae* is a bacteria linked to inflammatory conditions [88], and *Lactococcus*, an acid lactic producing bacteria [89], has been associated with cognitive dysfunction and neurological impairment [90]. Sex-dependent expression of neuroinflammation markers have been reported in humans [91] and mice [92] with aging, with a more exacerbated response in females. It remains to be explored whether GM dysbiosis in Tg-F mice may initiate a stronger inflammatory reaction compared to their males counterparts at six months of age.

When comparing GM composition between WT-F and Tg-F, we observed an increased abundance of *Ruminococcaceae* and *Clostridiaceae* WT-F mice. *Ruminococcaceae* and *Clostridiales* have been associated with higher circulating estrogens levels in postmenopausal women [93]. Correlation analysis raveled a positive association between *Ruminococcaceae* and working memory, with highest values in WT-F. WT-F mice also presented an enrichment of *Clostridiaceae* and *Clostridium* compared to Tg-F mice. The presence of those gut bacteria may have important consequences in estrogen bioavailability. Regretfully, we did not measure serum estrogen levels in these animals.

The effect size analysis (LEfSe) by sex and genotype showed substantial differences between groups. *Allobaculum, Adlercreuzia, Koribacter*, and *Dorea* were enriched in Tg-F mice compared to WT-F. Increased abundances of *Allobaculum* is considered a risk factor of female hepatocellular carcinoma [94]. In addition, mucin degradation and gut-barrier dysfunction have been associated with higher *Dorea* abundance [95] in alcohol-dependent subjects [96], and certain species of *Dorea* promote IFNγ production and chronic inflammation in multiple-sclerosis patients [97]. Tg-M showed significant enrichment of *Lactobacillus*, *Clostridiaceae*, *Adlercreutzia*, *SMB53*, *Dorea*, *Variovorax*, *Turicibacter, Clostridium*, and *Fusibacter*, compared to WT-M. *Clostridium butyricum* has showed neuroprotective effects [98, 99]. Contrastingly, *Clostridium* type-structures have been detected by immunohistochemistry in AD brain tissue [100]. Thus, our data showed that Tg-M suffer a more severe GM dysbiosis compared to Tg-F mice.

One relevant data collected in this study was the genotype-dependent alterations in bacterial fermented products such as SCFAs. We observed an enhanced propionate, but a reduced butyrate concentration in Tg mice (male and female) compared to their WT-counterparts. Increased propionate and acetate levels have been found in saliva of AD patients [101, 102], indicating that microbiota dysbiosis in AD is not restricted to the gut. We previously reported a prominent abundance of *Prevotella copri*, a propionate producing bacteria, in fecal samples of another Tg mice model (3xTgAD), that was associated with an enhanced propionate concentration in Tg brain tissue compared to the control mice [56]. Here, we also observed a significant increase in propionate producing bacteria in Tg-M mice, such as *Lactobacillus* (propionate producing species: *L. buchneri*, *L. diolivorans*, *L. acidophilus*, *L. helviticus, L. rhamnosus*, and *L.* gasseri) [103–105]. Previous studies also

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reported a *Lactobacillus* enrichment in fecal samples of male Tg mice [106, 107] and AD patients [108]. *Lactobacilli* are often considered to be commensal or beneficial participants to the host microbial ecology; however, they have been identified as potential pathogens in elderly and immunocompromised patients [109, 110]. In addition, patients with myalgic encephalomyelitis/chronic fatigue syndrome present a direct correlation between *Lactobacillus* distribution and symptom, neurocognitive, and neurosensory factors associated with neurological impairment [111]. Thus, caution may be taken for future probiotic design and supplementation protocols for AD patients. In addition, Tg mice (both, males and females) also presented a significant reduction in butyrate-producing bacteria (e.g., Lachnospiraceae, Ruminococcaceae, Peptostreptococcaceae, and *Oscillospira*) and acetate-producing bacteria (*Blautia*, Christiansenellaceaea, and *Ruminococcus* [43, 112–117]. Previous investigations have reported a general reduction in SCFAs concentration in brain and fecal samples of APP/PS1 mice [77]. However, significant biological differences among the principal bacterial fermented products have been described. Intracerebroventricular infusion of propionate in rats results in neuroinflammation [118, 119], progressive development of limbic kindled seizures, oxidative stress (e.g., lipid peroxidation), microglial activation-associated with decreased glutathione activity [120], and impairments in social behavior and working memory [121]. In the present study, WT-F mice presented an increase in butyrate-producing *taxa* (i.e., *Clostridiaceae*) compared to Tg-F, and highest butyrate concentration compared to the other groups. Correlation analysis further indicates that butyrate concentration correlates positively with working- and object-recognition memory, while propionate concentrations had a negative correlation with those memory capabilities. Sodium butyrate treatment protect against cerebral Ischemia [99], improving associative memory in APP/PS1 mice even at an advance stage of the disease [122]. Moreover, butyrate suppresses microglia activation [123] and decreases astrocyte activation [124] in cell cultures. Therefore, our results may indicate that Tg mice lack several butyrate-producing bacteria, with important neuroprotective effects, but they carry specific gut bacteria capable of releasing neurotoxic substances (i.e., propionate).

Predicted metabolic pathways of the fecal bacterial microbiota by functional metagenome indicate that GM modulation by genotype and gender is related to different bacterial products that interact with the host-physiology. We observed an enhanced propanoate metabolism pathway in Tg-M that could also be associated with *Lactobacillus* abundance

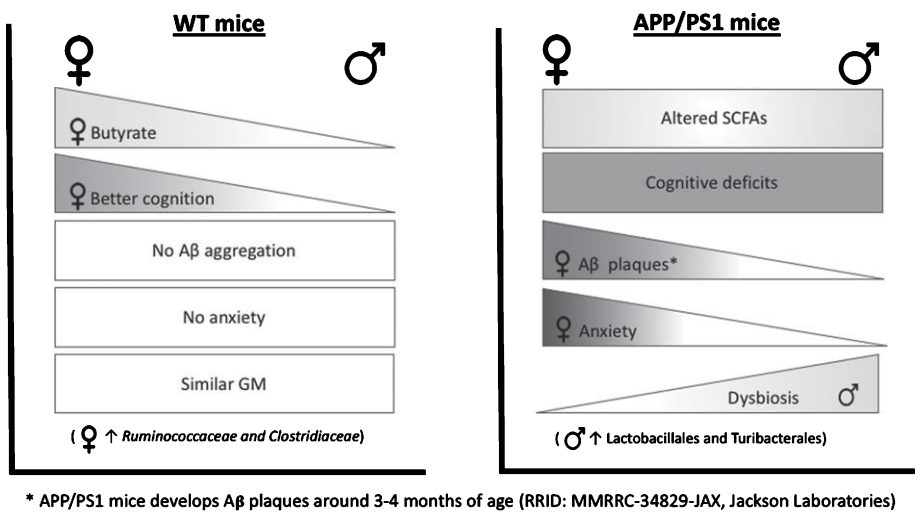


Fig. 8. Graphical abstract: WT-F mice showed higher butyrate concentration in fecal samples, which was associated with better cognitive performance compared WT-M and Tg-F mice. WT mice (without Aβ aggregates) did not showed anxiety. WT-F and WT-M mice present similar GM composition Notably, WT-F mice presented an enrichment of *Ruminococcaceae* and *Clostridiales* compared to Tg-F mice. APP/PS1 (Tg) mice (that develop Aβ plaques), showed altered short-chain-fatty acids (SCFAs) concentration (lower acetate/butyrate, but higher propionate) compared to their WT counterparts, cognitive deficits, and enhanced anxiety compared to WT mice. GM dysbiosis was stronger in Tg-M than Tg-F. Tg-M showed enrichment of *Lactobacillaceae* and *Turicibacteraceae* compared to WT-M and Tg-F mice.

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and fecal propionate levels in this group. Protein digestion and absorption were increased in Tg-F compared to Tg-M mice. A recent study showed a reduced protein digestion and absorption pathway in APPPS1-21 mice treated with antibiotics (both, female and male animals) [47]. Our study, based in a sex-discrimination analysis, showed that this metabolic pathway was reduced in Tg-M compared to Tg-F mice. The PICRUSt analysis also revealed a reduced Phe, Tyr, and Trp pathway in Tg-M mice compared to their WT counterparts. Low concentrations of Phe, Tyr, and Trp have been found in AD patients compared to control individuals [125], and lower Trp and kynurenic acid plasma concentrations were correlated with dementia severity in AD patients [126]. Thus, the collected data may indicate that several metabolic and cellular alterations observed in AD patients and Tg mice can be associated with GM dysbiosis.

In conclusion, this study shows a direct association among GM, cognitive decline, and sexual dimorphism in an AD mice model (Fig. 8). Sex differences in GM composition were observed mainly in Tg mice, which implies that sex itself exerted specific influences on the composition of the microbiota in AD pathology. The changes in composition of the GM between male and female Tg mice, and its physiological implications may be related with the dimorphism observed in the incidence of AD, affecting more women than men. Further studies are needed to understand the impact of female Tg dysbiosis in AD pathology, and to explore whether specific taxa may confer protection against brain dysfunction.

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SUPPLEMENTARY MATERIAL

Gut Microbiota Alterations and Cognitive Impairment Are Sexually Dissociated in a Transgenic Mice Model of Alzheimer's Disease

Supplementary Table 1
V3 region of the 16S rDNA gene amplification conditions

Step		Temperature	Time (s)
Initial denaturation		95°C	300
30 thermocycles	Denaturing	94°C	15
	Annealing	62°C	15
	Extension	72°C	15
Final extension		72°C	600
		4°C	Hold

V3 hypervariable region of the 16S rDNA gene amplification end-point PCR conditions are shown.

Supplementary Table 2
Sequencing summary

Parameter	M		F		Total $n = 29$
	WT ($n = 9$)	Tg ($n = 6$)	WT ($n = 8$)	Tg ($n = 6$)	
Reads	918474.0	610078.0	234680.0	226339.0	1989571.0
Length mean (bases) ± SD	186.9 ± 4.6	189.1 ± 5.8	182.7 ± 2.0	184.8 ± 8.1	185.9 ± 2.8
Reads mean (bases)	102053.0	101680.0	29335.0	37723.0	67698.0
Reads min-max (bases)	9612.0-288017.0	8722.0-227456.0	10921.0-92902.0	27054.0-61535.0	8722.0-288017.0
OTUs count	5638.0	3738.0	3994.0	3152.0	16522.0
Identified taxa	215.0	208.0	161.0	187.0	771.0
Samples with<10000 reads	1.0	1.0	0.0	0.0	2.0

Sequencing data summary for the bacterial 16S rDNA gene V3 hypervariable region amplicon for WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. OUT, operational taxonomic unit; SD, standard deviation; Tg-C, transgenic mice fed with control diet; Tg-F, transgenic mice fed with agave fructans supplemented diet; Tg-F-Abx, transgenic mice fed with agave fructans supplemented diet and treated with antibiotics as described in materials and methods; WT-C, wild type mice fed with control diet.

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Supplementary Table 3
Fecal microbiota relative abundance (%) of bacterial taxa in Fig. 3 and Supplementary Figure 1

Taxa	WT-M	WT-F	Tg-M	Tg-F
Actinobacteria (c) in A	4.7292	2.4386	2.8151	2.0476
Bacilli (c) in A	38.87	25.0478	53.3505	17.3650°°
Bacteroidia (c) in A	7.5073	8.3392	3.5882	21.2915°°
Clostridia (c) in A	23.53	52.2668	27.0055	42.1818
Coriobacteriia (c) in A	4.1747	5.81394	3.155	2.3215
δ-bacteria (c) in A	0.4336	0.36092	1.2959	0.1874
ε-bacteria (c) in A	0.2188	0.83691	0.1552	0.0373
Erysipelotrichi (c) in A	12.0828	2.14508	5.9533	11.122
γ-bacteria (c) in A	0.2143	0.10733	0.4964	0.0373
TM7-3 (c) in A	0.7324	0.8888	0.4314	1.1676
Verrucomicrobiae (c) in A	0.8034	1.36117	1.4812	1.0249
Others (c) in A	0.2542	0.3935	0.2722	0.9416
Bacteroidales (o) in B	7.5073	8.3392	3.5882	21.2916
Bifidobacteriales (o) in B	4.5078	2.25	2.408	1.8489
Clostridiales (o) in B	36.5121	52.2636	27.0047	42.1763
Clostridiales (o) in C	11.3837	15.5697	4.1053	17.0178
Coriobacteriales (o) in B	4.1747	5.8139	3.155	2.3214
CW040 (o) in B	0.7324	0.8848	0.4306	1.1449
Desulfovibrionales (o) in B	0.4328	0.3609	1.2959	0.1874
Enterobacteriales (o) in B	0.0557	0.0188	0.0301	0.0128
Erysipelotrichales (o) in B	12.0828	2.1451	5.9533	11.1221
Lactobacillales (o) in B	31.6879	24.4017	51.4736	17.2794°
Pseudomonadales (o) in B	0.1508	0.0834	0.462	0.0198
Turicibacterales (o) in B	0.616	0.6327	1.8487♂#	0.0813°
Verrucomicrobiales (o) in B	0.8034	1.3612	1.4812	1.0249
Others (o) in B	0.7364	1.4446	0.8688	1.4893
Anaeroplasmataceae (f) in C	0.0541	0.0005	0.0367	0.0486
Bacteroidaceae (f) in C	0.5871	0.4721	0.1611	0.5256
Bifidobacteriaceae (f) in C	4.5078	2.2501	2.408	1.9339
Clostridiaceae (f) in C	2.331	12.809	13.5516	9.0401
Coriobacteriaceae (f) in C	4.1747	5.8139	3.155	2.2576
Desulfovibrionaceae (f) in C	0.4328	0.3609	1.2959	0.1553
Enterobacteriaceae (f) in C	0.0557	0.0188	0.0301	0.0145
Erysipelotrichaceae (f) in C	12.0828	2.1451	5.9533	8.0608
F16 (f) in C	0.7324	0.8849	0.4306	1.1406
Helicobacteraceae (f) in C	0.2176	0.8369	0.1552	0.3111
Lachnospiraceae (f) in C	16.3396	12.1593	6.1687	12.4207
Lactobacillaceae (f) in C	26.1609	22.2087	44.5275♂♂##	18.0844°°
Pseudomonadaceae (f) in C	0.1456	0.0734	0.4531	0.0121
Ruminococcaceae (f) in C	6.2104	11.2312	2.8872#	8.6251
S24-7 (f) in C	6.7278	7.6272	3.28	14.4931
Streptococcaceae (f) in C	5.5198	2.1881	6.932	2.3743
Turicibacteraceae (f) in C	0.616	0.6327	1.8487#	0.0884°
Verrucomicrobiaceae (f) in C	0.8034	1.3612	1.4812	1.5413
Others (f) in C	0.9169	1.3559	1.1388	1.8546

Fecal microbiota proportions are shown for bacterial *phyla*, orders, families and genera in Fig. 3 and Supplementary Figure 1. WT-M $n = 9$, WT-F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. Data are shown as mean. Statistical significances (p values) were calculated by two-way ANOVA with Tukey *post-hoc* correction. Statistical significance between WT-M versus WT-F is shown by □, Tg-M versus Tg-F by ○, WT-M versus Tg-F by ◊, WT-F versus Tg-M by #, WT-M versus Tg-M by ♂ and WT-F versus Tg-F by ♀; one symbol represents $p < 0.05$, two symbols $p < 0.01$, three symbols $p < 0.001$, and four symbols $p < 0.0001$. C, class; f, family; o, order; Tg-C, transgenic mice fed with control diet; Tg-F, transgenic mice fed with agave fructans supplemented diet; Tg- F-Abx, transgenic mice fed with agave fructans supplemented diet and treated with antibiotics as described in materials and methods; WT-C, wild type mice fed with control diet. A, B, and C refers to sections in Supplementary Figure 1.

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Supplementary Table 4
Values for LDA scores, p and q for taxa between experimental groups

Section	Group	Phylum	Class	Order	Family	Genus	LDA	p	q
		Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Slackia*	3.60	0.04	1.00
					Paraprevotellaceae	*Paraprevotella*	3.30	0.04	1.00
		Bacteroidetes	Bacteroidia	Bacteroidales	*Porphyromonadaceae*	*Parabacteroides*	3.90	0.04	1.00
	WT-M				*Prevotellaceae*	*Prevotella*	3.70	0.04	1.00
		Firmicutes	Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*	*Coprobacillus*	2.90	0.02	1.00
		Proteobacteria	α-bacteria	Rhodospirillales	*Acetobacteraceae*	*Acetobacter*	3.50	0.04	1.00
			γ-bacteria	Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas*	4.40	0.04	1.00
					Acidaminobacteraceae	*Fusibacter*	3.60	0.04	1.00
		Firmicutes	Clostridia	Clostridiales	Clostridiaceae	—	4.70	0.01	1.00
						SMB53	4.00	0.01	0.73
	WT-F				Mogibacteriaceae	—	2.90	0.04	1.00
A					*Peptostreptococcaceae*	—	3.30	0.01	0.40
					Ruminococcaceae	—	4.40	0.03	1.00
		Proteobacteria	α-bacteria	Sphingomonadales	*Sphingomonadaceae*	*Kaistobacter*	3.10	0.03	1.00
			ε-bacteria	Campylobacterales	*Helicobacteraceae*	*Flexispira*	3.50	0.02	1.00
		TM7	TM7-3	EW055	—	—	3.90	0.02	1.00
		Acidobacteria	Acidobacteria	Actinomycetales	*Dietziaceae*	—	4.70	0.02	1.00
	Tg-M	Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	5.20	0.04	1.00
					Lactococcus	*Lactococcus*	4.40	0.04	1.00
		Proteobacteria	β -bacteria	Burkholderiales	*Comamonadaceae*	*Variovorax*	3.80	0.02	1.00
	Tg-F	Acidobacteria	Acidobacteriia	Acidobacteriales	*Korobacteraceae*	—	3.90	0.02	1.00
						Koribacter	3.50	0.02	1.00
		Cyanobacteria	Chloroplast	Stramenopiles	—	—	3.30	0.02	1.00
		Proteobacteria	α-bacteria	Sphingomonadales	*Erythrobacteraceae*	—	3.60	0.02	1.00
					Christensenellaceae	—	2.30	0.01	0.11
						—	3.60	0.01	0.06
						Blautia	2.40	0.01	0.06
	WT-M	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Coprococcus*	3.20	0.01	0.05
						Lachnobacterium	2.80	0.02	0.18
						Ruminococcus	3.40	0.01	0.03
						—	3.30	0.01	0.06
					Ruminococcaceae	*Oscillospira*	3.00	0.01	0.14
			Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*	Allobaculum	3.70	0.03	0.31
		Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	3.40	0.03	0.30
			Bacilli	Lactobacillales	*Peptostreptococcaceae*	—	2.30	0.01	0.26
					Acidaminobacteraceae	*Fusibacter*	3.20	0.03	0.44
						—	3.70	0.01	0.26
	WT-F	Firmicutes			Clostridiaceae	*Clostridium*	2.10	0.01	0.26
			Clostridia	Clostridiales		*SMB53*	3.10	0.01	0.26
B					*Lachnospiraceae*	Ruminococcus	3.30	0.01	0.26
					Ruminococcaceae	—	3.60	0.02	0.43
		Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Adlercreutzia*	3.20	0.01	0.03
			Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	4.40	0.01	0.07
				Turicibacterales	*Turicibacteraceae*	*Turicibacter*	2.90	0.01	0.09
					Acidaminobacteraceae	*Fusibacter*	2.50	0.01	0.08
	Tg-M					—	3.60	0.01	0.08
		Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*SMB53*	3.10	0.01	0.13
					Lachnospiraceae	*Dorea*	3.00	0.01	0.07
					Peptostreptococcaceae	*Clostridium*	2.80	0.01	0.06
		Proteobacteria	β-bacteria	Burkholderiales	*Comamonadaceae*	*Variovorax*	2.90	0.01	0.17
	Tg-F	Acidobacteria	Acidobacteriia	Acidobacteriales	*Koribacteraceae*	*Koribacter*	2.90	0.01	0.26
		Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Adlercreutzia*	3.10	0.01	0.26
					Christensenellaceae	—	2.40	0.01	0.26
		Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Dorea*	2.80	0.01	0.26
						Allobaculum	3.80	0.03	0.46
			Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*	*Coprobacillus*	2.40	0.02	0.26

LDA score, p and q values for enriched orders, families and genera in fecal samples of WT-M ($n = 9$), WT-F ($n = 8$), Tg-M ($n = 6$), and Tg-F ($n = 6$) mice are shown. The threshold for LDA score was set to 2.0. Statistical significances (p values) were calculated and corrected (q values) by two- stage linear step-up procedure of Benjamini-Hochberg in order to evidence an FDR per p value. F, female; FDR, false discovery rate; LDA, linear discriminant analysis; M, male; Tg, transgenic mice; WT, wild type mice; —, unidentified taxa. A and B refers to sections in Figure 5.

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Supplementary Table 5
Statistical values of predicted functional metagenome significant pathways

Compared groups	Category	Pathway	Difference between means	p p
	Cellular processes	P53 signaling pathway	0.0264	0.0392
		Colorectal cancer	0.1625	0.0256
		Influenza A	0.0372	0.0461
	Human diseases	Parkinson´s disease	1.017	0.0429
		Small cell lung cancer	0.1625	0.0256
		Toxoplasmosis	0.0374	0.0414
WT-M versus		Arg and Pro metabolism	0.7308	0.0024
WT-F		Atrazine degradation	0.6311	0.0254
		Butirosin and neomycin biosynthesis	1.0441	0.0461
	Metabolism	Glycosphingolipid biosynthesis	0.0001	0.0374
		Primary bile acid biosynthesis	0.2680	0.0246
		Secondary bile acid biosynthesis	0.2725	0.0209
		Selenocompound metabolism	1.1791	0.0232
	Organismal systems	Bile secretion	0.4773	0.0456
	Environmental information processing	G protein-coupled receptors	0.2972	0.0381
	Humandiseases	Epithelial cell signaling in *Helicobacter pylori* infection	8.4921	0.0236
		β-alanine metabolism	3.6584	0.0292
		Biosynthesis of siderophore group nonribosomal peptides	0.4919	0.0018
		Glutathione metabolism	2.7030	0.0179
		Glycolysis / Gluconeogenesis	1.3690	0.0495
		Glycosphingolipid biosynthesis	2.2649	0.0389
		Glycosyltransferases	4.8385	0.0371
	Metabolism	Other glycan metabolism	5.7855	0.0268
		Pantothenate and CoA biosynthesis	0.6835	0.0395
Tg-M versus Tg-F		Phe, Tyr and Trp biosynthesis	1.3314	0.0106
		Polyketide sugar unit biosynthesis	3.4145	0.0364
		Sphingolipid metabolism	3.4284	0.0368
		Tyr metabolism	1.1549	0.0163
		Vitamin B6 metabolism	0.8051	0.0448
		Antigen processing and presentation	3.5655	0.0328
		Carbohydrate digestion and absorption	28.5524	0.0329
		Hematopoietic cell lineage	0.0358	0.0418
	Organismal systems	NOD-like receptor signaling pathway	3.5644	0.0328
		Protein digestion and absorption	29.705	0.0306
		RIG-I-like receptor signaling pathway	7.0787	0.0334
	Cellular processes	Bacterial chemotaxis	1.537	0.0057
	Environmental Information Processing	Secretion system	0.7532	0.0162
	Genetic Information Processing	mRNA surveillance pathway	0.0002	0.0424
		Type I diabetes mellitus	3.5500	0.0079
	Human Diseases	Type II diabetes mellitus	3.5499	0.0079
		alpha-Linolenic acid metabolism	1.5874	0.0002
		Arachidonic acid metabolism	0.3285	0.2555
		Biosynthesis of ansamycins	0.3836	0.0029
		Biosynthesis of siderophore group nonribosomal peptides	0.8258	0.0089
		Butanoate metabolism	0.4034	0.0095
		Carbon fixation pathways in prokaryotes	1.1095	0.0097
		Chloroalkane and chloroalkene degradation	0.8905	0.0105
WT-M versus Tg-M				
		Citrate cycle (TCA cycle)	0.3433	0.0115
		Drug metabolism - other enzymes	0.3437	0.0119
	Metabolism	Galactose metabolism	1.7642	0.0167
		Pantothenate and CoA biosynthesis	0.9654	0.0171
		Phe, Tyr and Trp biosynthesis	1.4423	0.0364

(*Continued*)

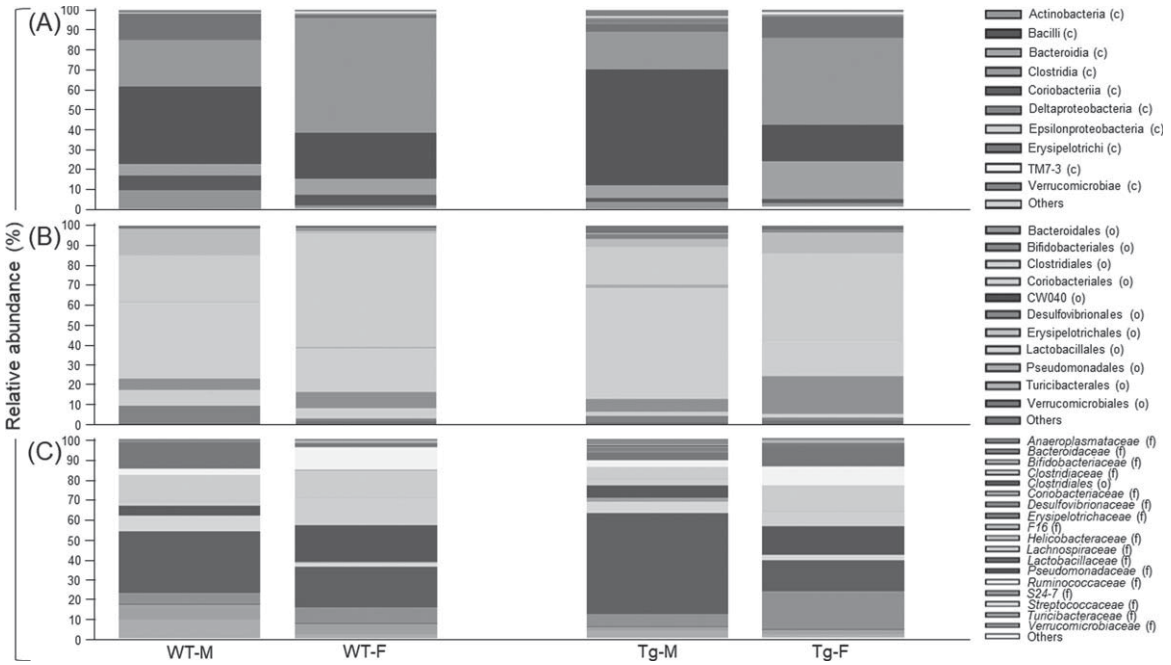
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Supplementary Table 5
(*Continued*)

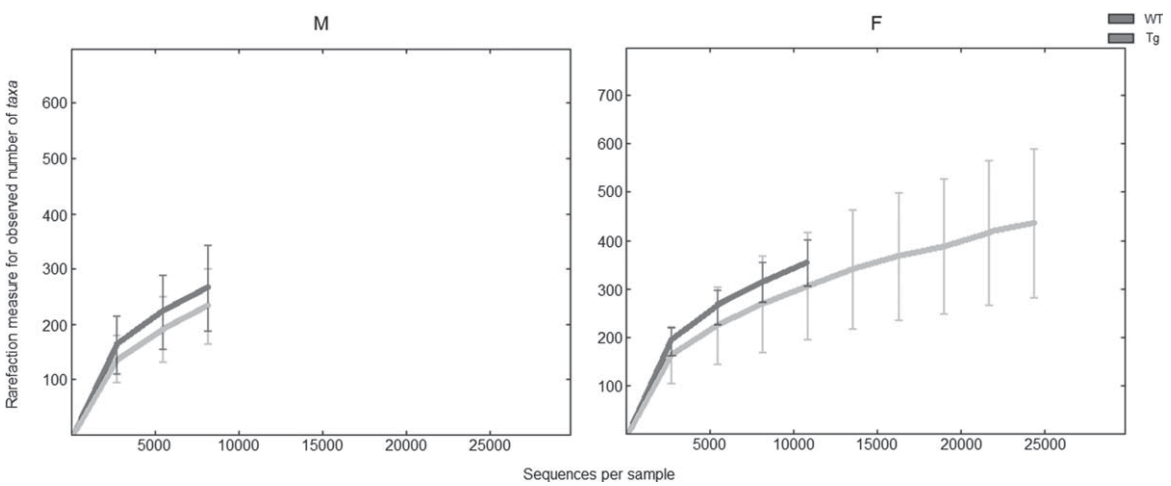
Compared groups	Category	Pathway	Difference between means	p p
		Polycyclic aromatic hydrocarbon degradation	0.8245	0.0176
		Primary bile acid biosynthesis	1.6893	0.0191
		Propanoate metabolism	0.6298	0.0031
		Pyruvate metabolism	0.5266	0.025
		Secondary bile acid biosynthesis	0.5601	0.02
		Sphingolipid metabolism	1.7063	0.0236
		Starch and sucrose metabolism	1.2796	0.031
		Taurine and hypotaurine metabolism	0.1341	0.0324
		Toluene degradation	1.3369	0.033
		Tryptophan metabolism	0.3001	0.0381
		Ubiquinone and other terpenoid- quinone biosynthesis	0.6978	0.0389
		Valine, leucine and isoleucine degradation	2.0219	0.0424
	Organismalsystems	Hematopoietic cell lineage	0.0321	0.0241
		Insulin signaling pathway	8.8075	0.0356
	Genetic information processing	DNA repair and recombination proteins	0.2177	0.0238
		Type I diabetes mellitus	3.5343	0.0405
	Humandiseases	Type II diabetes mellitus	3.534	0.041
WT-F versus Tg-F		His metabolism	0.7613	0.0456
	Metabolism	Naphthalene degradation	0.9286	0.0296
		Trp metabolism	0.2828	0.0121

Statistical values of predicted functional metagenome significant pathways determined using PICRUSt v1.1.1 to analyze the corresponding bacterial OTU table are shown. WT-M $n = 9$, WT- F $n = 8$, Tg-M $n = 6$, and Tg-F $n = 6$. Data are shown as mean $\pm$ SD. Statistical significances (p values) were calculated by t-test. F, female; FDR, false discovery rate; M, male; Tg, transgenic mice; WT, wild type mice fed with control diet.

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Supplementary Figure 1. Relative abundance of bacterial classes, orders, and families in fecal samples of the different mice groups. A) Graph bars show relative abundance of bacterial classes. Y-axis shows the percentage of abundance. The color of each tag on the right side of the bars indicates class *taxon*. Other groups classes < 1% of relative abundance. B) Graph bars show relative abundance of bacterial classes and orders. Y-axis shows the percentage of abundance. The color of each tag on the right side of the bars indicates class and order taxa. Other groups *taxa* < 1.5% of relative abundance. C) Graph bars show relative abundance of bacterial orders and families. Y-axis shows the percentage of abundance. The color of each tag on the right side of the bars indicates order and family *taxa*. Other groups *taxa* < 1.9% of relative abundance. WT-M n = 9, WT-F n = 8, Tg-M n = 6, and Tg-F n = 6. Data are shown as mean. Statistical analyzes and significances are shown in Supplementary Table 3. c, class; f, family; o, order.



Supplementary Figure 2. Rarefaction curves per experimental group for observed number of *taxa*. Rarefaction curves shows the bacterial richness based on observed OTUs at 97% similarity. WT-M n = 9, WT-F n = 8, Tg-M n = 6, and Tg-F n = 6. Each curve shows the minimum value of sequences per sample, and none of them approached a plateau, suggesting that additional sequencing would result in more OTUs for each group.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Gut Microbiota and Alzheimer's Disease: Pathophysiology and Therapeutic Perspectives

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Abstract. Alzheimer's disease (AD) is the most common cause of dementia in the elderly and is characterized by a progressive decline in cognitive function. Amyloid-β protein accumulation is believed to be the key pathological hallmark of AD. Increasing evidence has shown that the gut microbiota has a role in brain function and host behaviors. The gut microbiota regulates the bidirectional interactions between the gut and brain through neural, endocrine, and immune pathways. With increasing age, the gut microbiota diversity decreases, and the dominant bacteria change, which is closely related to systemic inflammation and health status. Dysbiosis of the gut microbiota is related to cognitive impairment and neurodegenerative diseases. The purpose of this review is to discuss the impacts of the gut microbiota on brain function and the development of AD. It is a feasible target for therapeutic invention. Modulating the composition of the gut microbiota through diet, physical activity or probiotic/prebiotic supplements can provide new prevention and treatment options for AD.

Keywords: Alzheimer's disease, dysbiosis, gut, microbiota, microbiota-gut-brain axis, pathophysiology, therapeutics

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, accounting for up to 60% of all dementia cases [1], and its incidence increases with increasing age. AD typically manifests through a progressive loss of learning and recall of recently learned information, and in the later stages, a deficiency of language, visuospatial abilities, and cognitive function. Such changes are often accompanied by behavioral disorders such as apathy, aggressiveness, and depression [2]. The occurrence of this disease has a huge impact on the life of patients' families, in addition to imposing a high financial cost on society.

Amyloid-β protein accumulation is believed to be the key pathological hallmark of AD, which can trigger tau protein hyperphosphorylation, neuroinflammation, oxidative stress, and neurite degeneration and ultimately lead to dementia [3]. However, increasing evidence has shown that the amyloid hypothesis alone cannot explain the pathogenesis of AD, which indicates that the pathogenesis of AD involves many different mechanisms [4]. Therefore, the exploration of the mechanism of AD and finding effective prevention and treatment approaches for AD is urgent.

A growing body of experimental and clinical data has confirmed a key role of gut dysbiosis and gut microbiota-host interactions in neurodegeneration

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[5–9]. Alterations in the gut microbiota composition induce increased permeability of the gut barrier and immune activation, leading to systemic inflammation, which in turn may impair the blood-brain barrier and promote neuroinflammation, neural injury, and ultimately neurodegeneration [10, 11]. More importantly, the pathological permeability of the intestinal mucosa and blood-brain barrier begin to increase over time and form a vicious cycle, leading to an irreversible destruction of neurons. The combination of gut inflammation, aging, and an unhealthy diet in the elderly may be one of the pathogenesis of AD. Adjusting the composition of the gut microbiota through diet, physical exercise, or probiotic/prebiotic supplements can provide new prevention and treatment options for AD. In this review, we discuss the role of gut microbiota in the pathogenesis of AD and then discuss the possibility of its prevention based on modulation of the gut microbiota.

GUT MICROBIOTA AND BRAIN HEALTH

Gut microbiota

The human intestine harbors up to 100 trillion microorganisms, ten times greater than the total number of human cells in the body. It is a complex ecosystem comprised of bacteria, fungi, and viruses, which is called the gut microbiota and is known as the "second genome" of humans [12]. Microbial densities in the proximal and middle small intestine are relatively low but increase dramatically in the distal small intestine (approximately 10^8 bacteria/ml of luminal contents) and colon ($10^{11} \sim 10^{12}$/g) [13]. The gut microbiota of healthy adults has two dominant bacterial phyla: *Firmicutes* and *Bacteroidetes*. The two bacterial phyla commonly dominate this ecosystem [14]. The remnants belong to *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, and *Verrucomicrobia* [15]. *Proteobacteria* are common but usually not dominant [16].

The microbiota is established shortly after birth and stabilizes during the first 2 to 3 years of life [17]. For decades, the microbiota has coevolved with the host to live together and establish a symbiotic relationship. The human gut microbiota plays a significant role on nutrient metabolism, maintenance of structural integrity of the gut mucosal barrier and immunomodulation [18]. For example, *Bacteroides thetaiotaomicron* is a prominent mutualist in the distal intestinal habitat of adult humans [19]. It allows us to extract calories from otherwise indigestible dietary polysaccharides, including plant-derived pectin, cellulose, hemicellulose, and resistant starches [20]. It occurs because the microbiota may adaptively deploy a large array of glycoside hydrolases and polysaccharide lyases that we humans do not encode in our genome [19, 21]. There is also evidence that gut microbiota maintain the structure and function of gut mucosal barrier. It is reported that *Bacteroides thetaiotaomicron* induce expression of the small proline-rich protein 2A (sprr2A), which is required for maintenance of desmosomes at the epithelial villus [22]. And the tight junction is maintained by Toll-like receptor 2 (TLR2) mediated signaling that is stimulated by the microbial cell wall peptidoglycan [23, 24]. In addition, *Bacteroides* are shown to activate intestinal dendritic cells (DCs), which induces plasma cells in the intestinal mucosa to express secretory IgA (sIgA). It can restrict the translocation of the microbiota from the intestinal lumen to the circulation, thereby preventing a systemic immune response [25]. The gut microbiota plays a crucial role in the development of innate and adaptive immune responses, modulation of gut motility and intestinal barrier homeostasis, absorption of nutrients, and distribution of fat [26, 27].

Microbiota-gut-brain axis

The gut-brain axis describes the complex and bidirectional communication system between the brain and gut [28, 29]. Increasing evidence also suggests that the gut microbiota greatly impacts gut-brain communication and plays a very important role in communication between the brain and gut [30]. The gut microbiota regulates the bidirectional interactions between the gut and brain through neural, endocrine, and immune pathways, which has been called the microbiota-gut-brain axis (Fig. 1) [31–35].

The vagus nerve has been shown to have a crucial role in modulating gut-brain axis function [36, 37]. As a primary nerve, the vagus nerve connects the enteric nervous system (ENS) with the central nervous system (CNS), which can transmit the peripheral state to the CNS [38]. The gut microbiome generates nerve signaling molecules such as catecholamine, γ-GABA, 5-HT, melatonin, and acetylcholine and influences the central nervous system through the vagus nerve. The vagus nerve has the ability to regulate the expression of γ-GABA, c-fos protein, 5-HT, and other neurotransmitters and can transmit the signaling molecules released by the gut microbiota into the brain to regulate brain function and

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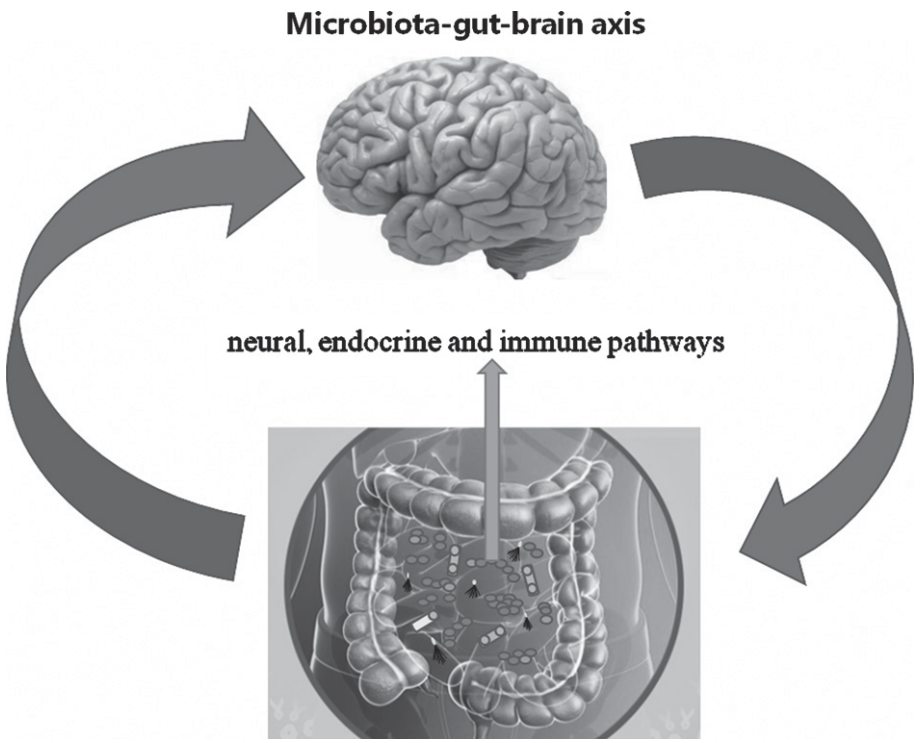


Fig. 1. Microbiota-gut-brain axis. The gut microbiota regulates the bidirectional interactions between the gut and brain through neural, endocrine and immune pathways.

host behavior. *L. rhamnosus* (JB-1) has been proven to regulate central GABA receptor expression and reduce stress-induced corticosterone and anxiety- and depression-related behavior. However, these neurochemical and behavioral effects will disappear in vagotomized mice, identifying the vagus as a major modulatory constitutive communication pathway between the gut and the brain [36]. Infection with the noninvasive parasite *Trichuris muris* can cause mild to moderate colonic inflammation and anxiety-like behavior in mice. Anxiety-like behavior was still present in infected mice after vagotomy. It has been suggested that there are other avenues of communication besides the vagal pathway [39].

The gut microbiota can also modulate mucosal immune responses, and the effect of the microbiota on the central nervous system may be transmitted through the immune system [40]. The immune system plays an important role in maintaining gastrointestinal homeostasis and health. The immune function of elderly individuals declines, so communication between the microbiota and brain may be reduced. Behavioral changes observed in patients with systemic or intestinal infections also support this immune pathway. Some molecular structures present on microbiota, such as lipopolysaccharide (LPS), bacterial lipoproteins, and flagellin, can activate various immune cells, such as macrophages, neutrophils, and dendritic cells. Once activated, immune cells produce a large number of proinflammatory cytokines, such as IL-1α, IL-1β, TNF-α, and IL-6. Locally produced cytokines can either activate neurons that project to specific brain areas or diffuse by volume transmission into the brain parenchyma to reach their targets [41].

The gut microbiota can regulate hormone secretion by endocrine cells and produce hormones such as brain-gut peptide, leptin, corticotropin-releasing hormone, and adrenocorticotropic hormone, which act directly on the brain [42]. The microbiota itself is capable of producing neuroendocrine hormones, which are known as microbial endocrinology-based mechanisms and are involved in gut-to-brain interactions [43]. Tryptophan, a precursor of 5-HT, is synthesized by the gut microbiota and can pass through the blood-brain barrier to generate 5-HT in the central nervous system [44]. Approximately 90% of the human body's total 5-HT is located in enterochromaffin cells in the gut. Enterochromaffin cells are hormone-secreting enteroendocrine cells scattered in the intestinal epithelium, accounting for only 1% of the intestinal epithelium. They express

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specific chemosensory receptors and can sense various stimuli in the intestine. Recent studies have found that bacterial metabolites may act on enterochromaffin cells and transmit information directly from the gut to the nervous system through the secretion of 5-HT [45]. Serum 5-HT is decreased in germ-free (GF) mice, and the levels of 5-HT precursors and metabolites are decreased accordingly in the intestine and urine [46]. The neurotransmitters regulated by the gut microbiota also include γ-GABA and dopamine [47].

Impacts of gut microbiota dysbiosis on brain health

The gut microbiota shows host specificity and may change in response to environmental variations inside and outside of their hosts, such as life stresses, current health status, diet, and age. An imbalance in gut microbiota is called dysbiosis and is characterized by decreased diversity of the gut microbiota, a reduced number of beneficial bacteria and an increased number of pathobionts or bacterial species associated with disease [48].

The gut microbiota is essential for the neurodevelopment and maturation of the nervous system. Microbiota dysbiosis may affect neurodevelopment. GF mice show significant abnormalities in the myenteric plexi of the jejunum and ileum, characterized by a decrease in nerve density, a decrease in the number of neurons per ganglion, and an increase in the proportion of myenteric nitrergic neurons [49]. The expression of postsynaptic density protein 95 (PSD-95) and synaptophysin in the striatum is increased, so GF mice display increased motor activity and reduced anxiety compared with specific pathogen-free (SPF) mice with a normal gut microbiota [50]. Administration of oral antimicrobials to SPF mice transiently alters the composition of the microbiota and increases exploratory behavior and hippocampal expression of brain-derived neurotrophic factor (BDNF). BDNF can stimulate neurogenesis and synapse formation and regulate synaptic plasticity. The gut microbiota influences host behavior through the hypothalamus pituitary adrenal axis (HPA axis), which may suggest that the hippocampal expression of BDNF is related to the host behavior [51].

Accumulating evidence has supported that the gut microbiota plays an important role in regulating cognitive functions, such as learning and memory, anxiety, development of schizophrenia, and autism-like behaviors. GF mice exhibit deficits in nonspatial memory in the novel object test and deficits in working memory in the T-maze test compared with SPF mice colonized with intact gut microbiota [52]. SPF mice infected with *C. rodentium* exhibit stress-induced memory dysfunction at 10 and 30 days after infection. Probiotics administered before and during infection prevent memory dysfunction [52]. Savignac et al. suggested that an improvement in learning and memory was found on the mice fed *Bifidobacterium longum* 1714, which was demonstrated by increased performance in an object recognition test and a Barnes maze test [53]. Gut microbiota dysbiosis induced by a combination of antibiotics (ampicillin, bacitracin, meropenem, neomycin, and vancomycin) impaired novel object recognition memory in mice but not spatial memory [54]. *Lactobacillus fermentum* strain NS9 alleviated the ampicillin-induced impairment in memory retention [55]. In addition, Yuan et al. reported that a mechanistic link between cognition and gut microbiota might be in a nematode model of AD, the gut microbiota-derived metabolite 'urolithins' can prevent beta-amyloid fibrillation [56]. Also, Duerkop et al. suggested that gut microbiota can cause cognition deficits through inflammation [57]. These results highlight the role of the gut microbiota in mediating cognition.

Dysbiosis of the gut microbiota and aging

Aging is one of the greatest risk factors for many chronic diseases, including AD. Increasing evidence has shown that the gut microbiota changes continuously with age. With increasing age, gut microbiota diversity decreases, the dominant bacteria change, and probiotics and short-chain fatty acids decrease [58]. 16S rRNA gene sequencing of stool samples, which were collected from 161 elderly people and 9 non-elderly adults who lived in the same area, showed that the gut microbiota of the elderly individuals was significantly different from that of non-elderly adults. Compared with that of the non-elderly adults, the proportion of *Firmicutes* in the elderly decreased, and the ratio of *Bacteroidetes/Firmicutes* increased [59]. However, the findings from different studies were quite variable. For example, some studies found an increase in *Bacteroides* with age, while others found a decrease [60, 61]. These differences may be due to the different populations or different research methods [62].

A recent study analyzed 3,663 gut microbiota samples from 1,165 healthy people around the world and developed a method to predict the physiological age

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of the host based on the gut microbiota using a deep learning method. This prediction model is called the human microbiome aging clock. Further study found that 39 bacteria were significantly correlated with age prediction. As people get older, some bacteria increase, such as *Eubacterium hallii*, while others decrease, such as *Bacteroides vulgatus*. This microbiome clock can be used as a baseline to predict an individual's age and aging rate [63]. The composition of the gut microbiota in the elderly is closely related to systemic inflammation and health status [64]. Transplanting the microbiota from aged rats into young rats led to increased systemic inflammation and neuroinflammation in the young recipient rats, which caused cognitive decline [65]. Ragonnaud et al. have shown that gut dysbiosis is increased in aging, and the dysbiosis triggers a chain of pathological and inflammatory events, and result in consequent aging-associated pathology [66]. Coman et al. indicated that aging-related gut dysbiosis may contribute to the initiation and/or progress of other metabolic diseases, and consequently, to a decrease in healthy longevity [67]. In addition, a previous study discussed the possible mechanisms between gut microbiota and aging, and the results shown that prebiotics, probiotics, and synbiotics might contribute to longevity through gut microbiota modulating [68]. Thus, gut microbiota might emerge as a therapeutic target in the elderly.

DYSBIOSIS OF THE GUT MICROBIOTA AND AD

Evidence has indicated a link between the gut microbiota and cognition. As AD is characterized by memory loss and multiple cognitive impairments, we have reason to speculate that gut microbiota plays a significant role in the occurrence and development of AD. Some clinical studies have shown that the gut microbiota of AD patients differs from that of healthy individuals. Microbial diversity is decreased in AD patients compared with healthy controls [6]. In addition, decreased *Firmicutes*, increased *Bacteroidetes*, and decreased *Bifidobacterium* were observed in AD participants [6]. Our previous study of 43 AD patients from China and 43 healthy people also found microbiological differences between groups in *Bacteroidetes* and *Actinobacteria* [69]. In another study, *Escherichia* was observed to be increased at the genus level in patients with AD and mild cognitive impairment (MCI) [70]. Moreover, compared with the normal controls, the abundance of 7 genera in the fecal microbiota was higher (*Lactobacillus*, *Blautia*, *Streptococcus*, etc.), and the abundance of 11 genera was lower in the patients with AD (*Prevotella*, *Sutterella*, *Haemophilus*, etc.) [70]. A previous study showed that the abundance of the genus *Bacteroides* was decreased in patients with MCI and AD [71].

Similar results have been observed in AD model animals. It was found that amyloid precursor protein (APP) transgenic mice have a remarkable shift in the gut microbiota compared to nontransgenic wild-type (WT) mice, including reduced *Firmicutes*, *Verrucomicrobia*, *Proteobacteria*, and *Actinobacteria* and a concurrent increase in *Bacteroidetes* and *Tenericutes*. Subsequently, they generated germ-free APP transgenic mice and found a drastic reduction in cerebral Aβ pathology as compared to control mice with gut microbiota. Importantly, colonization of germ-free APP transgenic mice with microbiota from conventionally raised APP transgenic mice increased cerebral Aβ pathology, while colonization with microbiota from wild-type mice was less effective in increasing cerebral Aβ levels [72]. In addition to fecal microbiota transplantation (FMT), other interventions, such as antimicrobial-based strategies, were used to assess the role of the microbiota in cognition or AD-related changes. It was reported that prolonged shifts in gut microbial composition and diversity induced by long-term broad-spectrum antibiotic treatment decreased Aβ plaque deposition in the APPswe/PS1ΔE9 mouse model of AD [73]. These results indicated microbial involvement in the development of Aβ pathology and suggested that the microbiota may contribute to the development of AD.

The precise mechanisms of how the gut microbiota contributes to AD pathogenesis are far from completely understood. However, there is a clear indication that the gut microbiota influences AD pathogenesis through multiple pathways, including metabolites, neurotransmitters, and chronic neuroinflammation (Fig. 2).

Metabolites

Some metabolic products of the gut microbiota, such as endotoxin LPS, trimethylamine N-oxide (TMAO), and β-methylamino-l-alanine (BMAA), have been reported to influence the central nervous system. It has previously been demonstrated that bacterial LPS is significantly increased in brain tissue from AD patients. In AD, mean LPS levels had twofold increases in the neocortex and threefold increases in the hippocampus when compared

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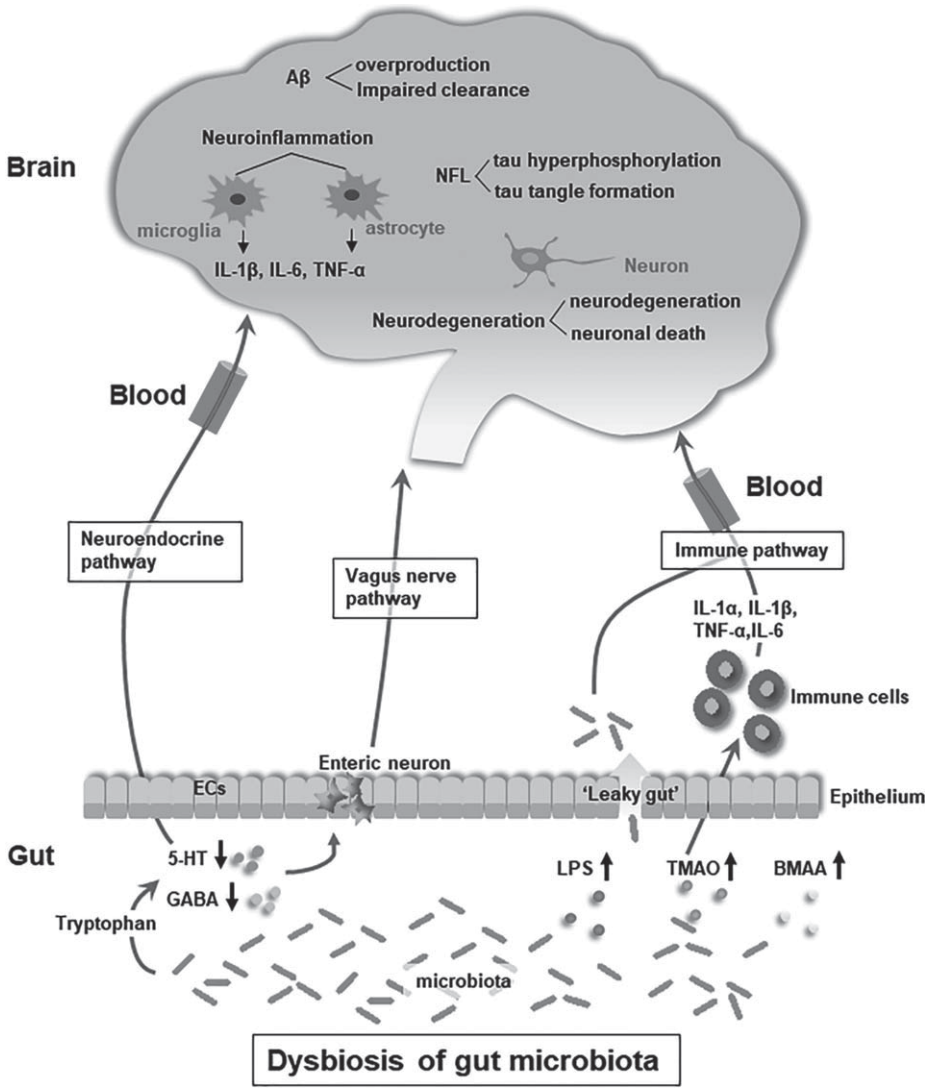


Fig. 2. Mechanism underlying the impact of the gut microbiota on AD. The gut microbiota regulates the interactions between the gut and brain through vagus nerve, immune, and neuroendocrine pathways. Dysbiosis of the gut microbiota leads to altered levels of neurotransmitters, such as 5-HT and GABA, which may contribute to abnormal signaling through vagus nerve. Gut bacteria and bacterial endotoxins may disrupt the intestinal barrier and result in the "leaky gut". Metabolites and bacteria from gut microbiota activate immune cells and cause a chronic inflammatory. These signal pathways influence AD pathogenesis, including Aβ deposition, neurofibrillary tangles (NFL), neuroinflammation and neurodegeneration in the brain. 5-HT, 5-hydroxytryptamine; GABA, gamma-aminobutyric acid; TMAO, Trimethylamine N-oxide; LPS, lipopolysaccharide; BMAA, β-N-methylamino-L-alanine; NFL, neurofibrillary tangles; ECs, endocrine cells.

to age-matched non-AD brains [74]. Compared to control brains, *Escherichia coli* K99 and LPS levels were higher in the brain parenchyma and vessels of AD brains. LPS colocalizes with $A\beta_{1-40/42}$ in amyloid plaques [75]. In vitro studies found that *Escherichia coli* LPS can promote the fibrillogenesis of Aβ peptides [76]. Gut microbiota disturbance augments LPS, which enters the brain by impairing the blood-brain barrier, thereby exacerbating the progression of Aβ pathology. Trimethylamine N-oxide (TMAO) is a small molecule produced via gut microbial metabolism. Elevated levels of biomarkers (NFT and $A\beta_{42}$) and TMAO have been reported in AD and MCI patients and are accompanied by neuronal degeneration [77]. β-N-methylamino-L-alanine (BMAA) is a neurotoxic nonproteinogenic amino acid from *cyanobacteria* that possibly causes protein misfolding and aggregation. This may be a possible mechanism of Aβ aggregation and senile plaque formation in AD

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patients [78]. In addition, studies have shown that some bacterial strains, such as *Escherichia coli, Salmonella enterica* serovar Typhimurium, *Bacillus subtilis, Staphylococcus aureus,* and *Mycobacterium tuberculosis,* produce extracellular amyloid fibers [79]. Bacterial amyloids can aggregate into toxic oligomers, which cause damage to lipid membranes and promote amyloid fibril formation by acting as nucleators for aggregation [80]. The aggregation of proteins into amyloid fibers is a common characteristic of many neurodegenerative disorders, including AD and Parkinson's disease.

Neurotransmitters

Some neurotransmitters, such as GABA and 5-HT, are involved in the pathology of AD. In severe cases of AD, GABA levels in the brain have been shown to be significantly reduced, which could underlie the cognitive and neuropsychiatric symptoms of AD [81]. Compared with WT mice, the 5-HT content in 18-month-old APPswe/PS1ΔE9 (APP/PS1) transgenic mice was reduced in the neocortex [82]. The gut microbiota is capable of producing most neurotransmitters found in the human brain. *Lactobacillus* and *Bifidobacterium* spp. can produce gamma-aminobutyric acid (GABA); *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* spp. can produce 5-HT; *Bifidobacterium infantis* has been shown to elevate plasma tryptophan levels and thus influence central 5-HT transmission [83]. These neurotransmitters produced by the gut microbiota may be closely related to clinical syndromes of AD. Ingestion of *L. rhamnosus* regulated GABA (Aα2) mRNA expression in the brain and reduced stress-induced anxiety- and depression-related behavior [36]. Despite this, these microbially synthesized neurotransmitters are incapable of crossing the blood-brain barrier, so their impact on brain function is likely to be indirect.

Chronic neuroinflammation

It has been reported that gut microbe-triggered inflammatory responses are involved in the pathogenesis of AD. Early epidemiological studies have shown that long-term use of nonsteroidal anti-inflammatory drugs was associated with a reduction in the risk of AD [84]. In clinically diagnosed AD patients, the abundance of proinflammatory gut bacterial taxa, including *Escherichia* and *Shigella*, increased, while anti-inflammatory *E. rectale* decreased [71]. In addition, increasing support for the role of the gut microbiota in AD is provided from AD mouse models. In AD mice, alteration of the gut microbiota composition leads to the peripheral accumulation of phenylalanine and isoleucine, which stimulates the differentiation and proliferation of proinflammatory T helper 1 (Th1) cells. Brain-infiltrated peripheral Th1 immune cells are associated with M1 microglial activation, contributing to AD-associated neuroinflammation [10]. Transplantation of the fecal microbiota from WT mice into amyloid and neurofibrillary tangles ($ADLP^{APT}$) transgenic mice ameliorated the formation of Aβ plaques and neurofibrillary tangles, glial reactivity, and cognitive impairment [85]. Long-term broad-spectrum antibiotic treatment decreased glial reactivity and Aβ plaque deposition in the APPswe/PS1ΔE9 mouse model of AD [73]. The results indicate that microbiota-mediated intestinal and systemic immune aberrations contribute to the pathogenesis of AD.

AD PREVENTION FROM THE MICROBIOTA PERSPECTIVE

Despite all of the research attempting to understand and treat AD, there has been a high rate of failure in AD drug development programs [86]. The World Alzheimer Report of 2014 and the Lancet Commission on Dementia Intervention, Prevention, and Care of 2017 emphasized modifiable risk factors, which have been shown to potentially delay the onset and/or progression of cognitive impairment [87, 88]. Here, we review some interventions, including physical activity, different types of diets, probiotics or prebiotics, and FMT.

Physical activity

Physical inactivity is an important risk factor for cognitive decline in aging and for AD [89]. In a systematic review, most experiments showed that physical activity was negatively correlated with the risk of AD [90]. Maintaining high leisure-time physical activity (LTPA) or increasing LTPA after midlife was associated with a lower dementia risk [91]. Regular physical activity increases the endurance of cells and tissues to oxidative stress, vascularization, energy metabolism, and neurotrophin synthesis, all of which are important in neurogenesis, memory improvement, and brain plasticity [92]. There is some evidence that physical activity levels may modify disease pathology in preclinical AD. NSE/APPsw Tg

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mice were subjected to exercise on a treadmill for 16 weeks, and their $A\beta_{42}$ peptides were significantly decreased [93]. In humans, habitual physical activity levels, as measured by self-report questionnaires, are associated with a lower brain amyloid load and lower insulin, triglyceride, and $A\beta_{1-42/1-40}$ levels [94].

Aside from these direct interactions, physical activity also has the potential to influence other modifiable risk factors for AD, such as insulin resistance [95]. Insulin resistance in middle-aged to older adults is associated with reduced glucose metabolism in the medial temporal lobe, and this association is even stronger than that with *APOE4* status [96], which is a risk factor for AD and is associated with reduced brain glucose metabolism [97]. Exercise can improve insulin sensitivity [98] and could thereby potentially contribute indirectly to improving memory function.

In recent years, several studies have demonstrated that physical activity increases the microbiota diversity and modulates its distribution [99, 100]. Bacterial diversity is decreased in sedentary elderly individuals compared with elderly individuals who take physical exercise. The gut microbiota of professional rugby players was more diverse than that of nonathlete healthy subjects [99]. Santacruz et al. compared changes in the gut microbial distribution of obese adults who underwent moderate to strenuous aerobic exercise for 10 weeks and found increased *Bacteroidetes* and decreased *Firmicutes* [101]. Evans et al. showed that the ratio of *Bacteroidetes* to *Firmicutes* correlated inversely with the amount of exercise performed [102]. A previous study showed that during diet induced obesity, the ratio of *Bacteroidetes* to *Firmicutes* of fecal microbiota were increased by high-intensity interval training [103]. Besides, it was suggested that physical exercise could decrease the proportions of *Bacteroides*/*Prevotella spp*. and *Methanobrevibavter spp.* [104]. These studies suggest that exercise can alter the microbiota diversity and distribution. It has been suggested that changes in the human gut microbiota mediated by exercise could influence the development and progression of AD, and a new term, the "muscle-gut-brain axis", has been introduced [105]. Physical activity in humans seems to correlate with the gut microbiota and can prevent the incidence and development of AD. However, further research is needed.

Diet

It is well known that diet can influence the microbiota composition in the gut. A Mediterranean diet (MeDi) has been recognized as a healthy dietary pattern. The MeDi is characterized by frequent consumption of bread, legumes, vegetables, fruits, and fats rich in unsaturated fatty acids, moderate intake of fish and poultry, low intake of dairy and meat, and regular but moderate consumption of wine with meals [106]. The diet has previously been related to the composition of the gut microbiota and related metabolome. Subjects who consume a MeDi have higher levels of fecal short-chain fatty acids, while low adherence to the MeDi corresponds to an increase in urinary trimethylamine oxide levels, a potential risk factor for cardiovascular disease [107].

Epidemiologic investigations showed that higher adherence to MeDi was associated with a lower risk of incident cognitive impairment [108]. Adherence to MeDi has been associated with a reduced risk of MCI and AD [109]. Numerous studies have explored the potential mechanisms by which MeDi may protect against AD. MeDi may have a protective role due to its high content of antioxidants and anti-inflammatory compounds [110].

Recently, some studies have explored whether MeDi has an effect on the gut microbiota to protect against AD. Eleven subjects with MCI underwent a modified Mediterranean-ketogenic diet (MMKD) for 6 weeks, and their abundances of *Enterobacteriaceae*, *Akkermansia*, *Slackia*, *Christensenellaceae*, and *Erysipelotriaceae* increased, while those of *Bifidobacterium* and *Lachnobacterium* decreased in the gut. MMKD slightly reduces fecal lactate and acetate while increasing propionate and butyrate. The data suggest that MMKD can modulate the gut microbiome and metabolites in association with improved AD biomarkers in the cerebrospinal fluid [111]. MeDi contains an unusually large quantity of *lactobacilli*, which reduces blood ammonia levels, is very effective in preventing AD [112]. After intervention with coconut oil-enriched MeDi, improvements in episodic, temporal orientation, and semantic memory in women with mild-moderate states were observed [113]. To date, there is sufficient evidence to recommend the MeDi to delay the onset of AD. Future research should focus on larger, controlled trials in diverse populations [114].

Probiotics and prebiotics

Probiotics are bacteria that have a beneficial effect on the health of the recipient, while prebiotics are mainly fiber substances that serve as food for these bacteria. AD mice with exercise training and

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probiotic treatments significantly outperformed controls in the Morris maze test, and the number of Aβ plaques decreased in the hippocampus [115]. Treatment with the SLAB51 probiotic formulation in AD mice caused changes in the composition of the gut microbiota and its metabolites, such as short chain fatty acids, that improve cognitive functions [116]. Oral administration of *Bifidobacterium* breve strain A1 ameliorated the cognitive decline observed in AD mice [117]. Pistollato et al. have found that healthy diet patterns characterized by a large intake of probiotics and prebiotics are related to other nutrients, which can delay the decline of neurocognitive ability and reduce the AD risk [118]. Tillisch et al. suggested that supplementation of probiotic diet has an effect on normal brain activity [119], and Akbari et al. based on 60 AD patients to evaluate the probiotics effects, and the results also shown that compared to controls, the probiotic could significantly improve the cognitive function in AD patients [120]. These data revealed that probiotic treatment can decrease the progression of AD and that the beneficial effects could be partly mediated by alteration of the microbiota.

More importantly, the latest randomized, double-blind, and controlled clinical trial demonstrated that a mixture of probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum*) consumed for 12 weeks had a positive effect on cognitive function (Mini-Mental State Examination score) and some metabolic statuses in AD patients [120]. To summarize, certain probiotic strains show efficacy in modulating cognitive behavior and AD-related pathogenesis. Consequently, there is a need for future studies focusing on the best combinations of probiotic strains, the timing of administration, and the identification of the mechanism by which each strain exerts its effects.

Fecal microbiota transplantation

FMT is a type of bacteriotherapy that introduces healthy donor stool into the bowel of the recipient to re-establish a protective gut flora. FMT can change the gut microecology more robustly than food or probiotics. FMT has become an important therapeutic option for *Clostridium difficile* infection [121]. Whether FMT is feasible in treating various nervous system diseases remains to be investigated.

Recent studies have shown that transplantation of the fecal microbiota from WT mice into ADLP^{APT} transgenic mice ameliorated the formation of Aβ plaques and neurofibrillary tangles, glial reactivity, and cognitive impairment [85]. Jiang et al. indicated that although FMT seems to be a drastic treatment, the change in the composition of the gut microbiota may positively affect the cognition or pathology of AD [122]. Hazan et al. found that in AD patients, FMT may prevent the translocation of neuroactive compounds and metabolites within the CNS that regulate mood and cognition and contribute to inflammation [123]. In addition, Sun et al. found that FMT alleviated AD-like pathogenesis in APP/PS1 transgenic mice [124]. Thus, FMT may have beneficial effects on AD treatment. However, further human clinical research is needed to evaluate its effectiveness in AD patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

It is widely recognized that the human gut microbiota is critical to health and disease. Dysbiosis of the gut microbiota is relevant to a variety of diseases. In AD, at least a proportion of individuals show dysbiosis of the gut microbiota. However, it remains unclear whether dysbiosis of the gut microbiota is caused by disorders of the nervous system modulating gut function or is a major factor affecting brain function. Hence, there is a need for further investigations of the interactions between the gut microbiota and AD. It is expected that in the near future, a better understanding of the pathological contribution of gut microbiota dysbiosis in AD development and the molecular mechanisms of the bacterial species significantly regulated by drug administration to retard AD will provide useful directions for the development of gut microbiota-based therapeutic interventions for this disorder [125].

A large number of studies have confirmed the interaction between the gut microbiota and the CNS and preliminarily described the interaction through neural, endocrine and immune pathways, the so-called microbiota-gut-brain axis [31]. However, the underlying mechanisms of the gut microbiota and its metabolites in AD remain unclear. Metagenomics and metabolomics as the tools to reveal the relationship between disease and gut microbiota, which could help to identify more metabolic pathways and provide a strong rationale for approaches to the treatment of many CNS disorders.

It is a feasible strategy to regulate the gut microbiota through long-term individualized diet adjustment, physical activity, probiotic interventions or

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FMT, which can be used for innovative treatment of neurodegenerative diseases, including AD. The gut microecology environment contains the largest number and types of microorganisms in the human body. It is of great significance to study gut microecology for the prevention and treatment of various human diseases. Thus, intervention research in microecology will possibly bring hope to understanding AD and finding new methods to prevent and treat AD.

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Relationship Between Plasma Neurofilament Light Chain, Gut Microbiota, and Dementia: A Cross-Sectional Study

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Abstract.
Background: Previous studies have demonstrated associations between gut microbiota, microbial metabolites, and cognitive decline. However, relationships between these factors and neurofilament light chain (NfL; a disease-nonspecific biomarker of neural damage) remain controversial.
Objective: To evaluate the associations between plasma NfL, gut microbiota, and cognitive function.
Methods: We performed a cross-sectional sub-analysis of data from our prospective cohort study that was designed to investigate the relationship between gut microbiota and cognitive function. Patients who visited our memory clinic were enrolled and demographics, dementia-related risk factors, cognitive function, brain imaging, gut microbiomes, and microbial metabolites were assessed. We evaluated the relationships between the gut microbiome, microbial metabolites, and plasma NfL. Moreover, the relationships between plasma NfL and cognitive function were assessed using multivariable logistic regression analyses.
Results: We analyzed 128 participants (women: 59%, mean age: 74 years). Participants with high (above the median) plasma NfL concentrations tended to be older, women, and hypertensive and have a history of stroke, chronic kidney disease, and dementia. Plasma NfL was also associated with cerebral small vessel disease. However, plasma NfL levels were not significantly correlated with gut microbial metabolites. Multivariable analyses revealed that a higher plasma NfL concentration was independently associated with the presence of dementia (odds ratio: 9.94, 95% confidence interval: 2.75–48.2, $p < 0.001$).

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Conclusion: High plasma NfL concentration was independently associated with the presence of dementia as previously reported. However, plasma NfL levels were not significantly correlated with gut microbial metabolites in this preliminary study.

Keywords: Biomarkers, cerebral small vessel disease, cognitive decline, dementia, gut microbiota

INTRODUCTION

Dementia is a significant global healthcare problem; in 2015, 47 million people worldwide were living with dementia [1]. The number of people with dementia is expected to increase to 66 million by 2030 and 131 million by 2050 [2]. Additionally, the global cost of dementia was estimated at almost US$820 billion in 2015, and this figure has continued to increase [1, 2]. Japan is also facing financial pressure on its healthcare system because of the increasing proportion of people with dementia [3]. Thus, a comprehensive strategy for dementia research has been introduced in Japan to improve the healthcare system [3].

Recently, associations between gut microbiota and cognitive decline have gained increasing attention [4–6] because these associations may reveal the mechanisms underlying the onset of dementia. Previous studies have suggested that disruption of the neuroinflammatory system [7], vascular inflammation [8], and remote relationships driven by various metabolites [9] are mechanisms underlying cognitive decline caused by the gut microbiome. Furthermore, Cryan et al. summarized multiple direct (e.g., vagus nerve) and indirect (e.g., short-chain fatty acids, cytokines, and key dietary amino acids, such as tryptophan) gut–brain pathways and proposed that the gut microbiota modulates the gut–brain axis via these pathways [6]. Specifically, microbes locally synthesize neurotransmitters in the gut, which is an important mode of communication. Neuroactive bacterial metabolites and metabolites from the diet can modulate the brain and behavior, which include the influence of epithelial cells on gut-barrier function, enteroendocrine cells on hormone release, and dendritic cells on immune and microglial function, all of which play fundamental roles in aging and neurological disorders [6]. However, knowledge regarding the effects of gut microbiota on cognitive function remains limited. Examining blood biomarkers that indicate neural damage and analyzing the links between such biomarkers, gut microbiota, and cognitive function may fill this knowledge gap.

Neurofilament light chain (NfL) is an emerging, non-invasive, disease-non-specific marker of neurodegeneration and neuronal injury [10]. Neurofilaments are intermediate filament proteins of the cytoskeleton that are integral to the structure and function of axons. NfL is one of the subunits of neurofilaments. During neurodegeneration, neurons break down, and this neural breakdown releases NfL into the blood and cerebrospinal fluid (CSF) [11]. Previous studies have identified relationships between NfL and several neurological disorders, such as dementia [10, 11] and cerebral small vessel disease (SVD) [12]. NfL is associated with poorer cognitive performance across most neurological disorders and cognitive domains [11] and also reflects the severity and predicts the progression of cerebral SVD [12]. Additionally, Vogt et al. revealed that gut microbial-derived metabolite trimethylamine N-oxide in the CSF of people with Alzheimer's disease (AD) is positively correlated with CSF biomarkers of neuronal degeneration indicated by tau and NfL, which suggests the presence of the gut–brain axis [13]. However, associations between NfL in blood samples, gut microbiota, and cognitive function have not been comprehensively investigated. Such analysis will clarify the link between gut microbiota and damage to the brain parenchyma, such as that observed in patients with cerebral SVD or brain atrophy.

We have recently conducted an observational study that was originally designed to investigate the relationship between gut microbiota and cognitive function. In this study, gut microbial dysregulation was revealed to be associated with cognitive decline [14, 15], vascular risk factors [16], and cerebral SVD, such as white matter hyperintensity [17]. These findings suggest links among gut microbiota, vascular risk factors, cerebral SVD, and cognitive decline. Furthermore, gut microbial metabolites and dietary components play important roles in these associations [18]. More specifically, gut microbial metabolites are associated with cognitive decline, independent of the gut microbiome [18]. Furthermore, adherence to a Japanese-style diet is associated with gut microbial metabolites and is inversely associated with cognitive

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decline [19, 20]. Thus, these potential factors may also affect the diet–microbiome–dementia cascade.

The present study aimed to evaluate the relationships between NfL, gut microbiota, and cognitive function by performing a sub-analysis of the data from our ongoing clinical study. We hypothesized that plasma NfL would be associated with cognitive decline in our cohort data and that there would be an association between plasma NfL, the gut microbiome, and microbial metabolites.

MATERIALS AND METHODS

Study design

We performed a cross-sectional sub-analysis of data from a hospital-based prospective cohort study, the Gerontological Investigation of Microbiome: a Longitudinal Estimation Study (the Gimlet study), conducted at the National Center for Geriatrics and Gerontology (NCGG) in Japan [14]. Briefly, patients who visited the Memory Clinic at the NCGG and agreed to undergo both a medical assessment of their cognitive function and a fecal examination were enrolled. Activities of daily living and cognitive functions of participants were assessed annually after enrollment. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the NCGG (no. 1191-3). Written informed consent was obtained from all patients and their families before their participation in the study. The Gimlet study is ongoing and is registered on the UMIN Clinical Trials Registry (UMIN000031851). Detailed information regarding the Gimlet study and the present sub-analysis is provided in the Supplementary Material.

Participants

We enrolled consecutive patients who had subjective memory impairment and visited the Memory Clinic at the NCGG between March 2016 and March 2017. Participants in the Gimlet study were eligible for this sub-study if they met the following criteria: 1) able to undergo brain magnetic resonance imaging (MRI); 2) blood samples were successfully preserved; and 3) provided written informed consent. Patients who had potential confounding factors or effect modifiers for the variables of interest (e.g., recent use of antibiotics) were excluded at the time of enrollment in the Gimlet study.

Assessments

All participants underwent a comprehensive geriatric assessment based on the following features: 1) demographic characteristics; 2) risk factors, such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, and a history of stroke; 3) activities of daily living; 4) global cognitive function, which was assessed using the Mini-Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) scales; 5) neuropsychological testing using evaluations such as the Alzheimer's Disease Assessment Scale-Cognitive Subscale and Frontal Assessment Battery; 6) behavioral and psychological symptoms; 7) burden on caregivers; 8) depression status, which was assessed using the Geriatric Depression Scale; 9) laboratory parameters, such as apolipoprotein E $\varepsilon 4$ and C-reactive protein; 10) ankle brachial index and pulse wave velocity as indicators of arteriosclerosis [21] and the 'impact' of pulse [22], respectively; 11) results of brain imaging, such as MRI and single-photon emission-computed tomography; and 12) dietary assessments. Clinical samples and data were provided by the NCGG Biobank, which collects clinical data for research. In this sub-analysis, we used a single dataset that was obtained at enrollment in the Gimlet study.

Brain imaging

Participants underwent a 1.5-T brain MRI (Philips Ingenia, Eindhoven, Netherlands). The presence of cerebral SVD and its components, such as silent lacunar infarcts, white matter hyperintensity, cerebral microbleeds, and enlarged periventricular space, were assessed. The voxel-based specific regional analysis system for Alzheimer's Disease (VSRAD) software (Eisai Co., Ltd., Tokyo, Japan) was used to quantify cortical and hippocampal atrophy. A high VSRAD score indicated the presence of AD [23]. Participants also underwent N-isopropyl-p-[^{123}I]-iodoamphetamine single-photon emission-computed tomography, in which low blood flow in the area of the posterior cingulate gyrus and/or precuneus was regarded as a surrogate marker of AD [24].

Dietary assessments

We evaluated the Japanese Diet Index (JDI_{12}), which is a questionnaire on dietary composition that consists of 12 items: 10 beneficial components (rice,

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miso, fish and shellfish, green and yellow vegetables, seaweed, pickles, green tea, soybeans and soybean-derived foods, fruit, and mushrooms) and two less beneficial components (beef and pork, and coffee) [19, 20]. Participants were assigned one point if their daily beneficial intake was equal to or greater than the sex-specific median intake and their daily less beneficial intake was below the sex-specific median intake [19, 20].

Classification of cognitive function

Dementia was defined as an MMSE score < 20 and/or a CDR score ≥ 1 [14]. Participants who did not have dementia were further categorized as having either mild cognitive impairment (MCI) or normal cognition (NC). MCI was defined as an MMSE score ≥ 20 and a CDR score $= 0.5$; this implies possible, very mild dementia, and suggests a higher risk of dementia [15]. NC was defined as an MMSE score ≥ 20 and a CDR score $= 0$.

Plasma neurofilament light chain

Blood samples were collected, processed onsite to isolate plasma, aliquoted, and frozen at –81°C in the NCGG Biobank. Plasma NfL concentrations were measured according to manufacturer instructions using the NF-Light Advantage Kit on a highly sensitive single-molecule array assay Simoa HD-1 platform (Quanterix, Lexington, MA, USA) [25, 26]. Plasma samples were measured at a 1 : 4 dilution and were run in duplicate by a trained technician. All samples were measured blinded. All NfL values were within the linear ranges of the assays.

Gut microbiome

Fecal samples were collected, frozen, and stored at –81°C in the NCGG Biobank. The gut microbiome of each participant was analyzed by Techno Suruga Laboratory (Shizuoka, Japan) using terminal restriction fragment length polymorphism (T-RFLP) analysis [27]. The T-RFLP analysis was used to classify gut microbes. By referencing the Human Fecal Microbiome T-RFLP profile, each gut microbiome was categorized as representing one of three enterotypes: enterotype I, which included *Bacteroides* at $> 30\%$; enterotype II, which included *Prevotella* at $> 15\%$; and enterotype III, which comprised other combinations of microorganisms. The Firmicutes/Bacteroidetes (F/B) ratio was also calculated because a high ratio is indicative of dysbiosis [28].

Analysis of microbial metabolites in feces

The fecal concentrations of organic acids, short-chain fatty acids, ammonium ions, indoles, phenol, skatole, and p-cresol were measured, as previously described [18]. The concentrations of organic acids and short-chain fatty acids (acetic, propionic, butyric, iso-butyric, succinic, lactic, formic, valeric, and iso-valeric acids) were measured using high-performance liquid chromatography. Ammonium ion concentrations were quantified using ion chromatography, and fecal concentrations of indoles, phenol, skatole, and p-cresol were quantified using gas chromatography/mass spectrometry.

Statistical analysis

Continuous, ordinal, and categorical variables are expressed as means $\pm$ standard deviations, medians and interquartile ranges, or frequencies and proportions (percentage), respectively. Data were compared using Student's unpaired *t*-test, Wilcoxon rank-sum test, and χ^2 test, respectively. Natural logarithm-transformed plasma NfL (Ln-NfL) was occasionally used to reduce the right skewness of plasma NfL concentrations for subsequent analysis. First, we compared clinical characteristics between participants with high concentrations of plasma NfL and those with low concentrations, who were divided according to the median value of plasma NfL. Second, relationships between the gut microbiome, microbial metabolites, and plasma NfL concentrations were evaluated. Furthermore, we compared the differences in dietary composition between participants with high concentrations of plasma NfL and those with low concentrations. Third, participants were allocated to groups according to the presence or absence of dementia, the presence or absence of enterotype I, and MCI or NC status (among those who did not have dementia), to compare plasma NfL concentrations. Additionally, participants were allocated to four subgroups according to high or low plasma NfL concentrations divided by the median value and the presence or absence of dementia-related items. Dementia-related items consisted of items, such as age, sex, years of education, and risk factors. The presence of dementia-related items was categorized using the median value of the continuous variables and was based on our previous findings (i.e., being

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a woman, higher age, higher VSRAD score, higher pulse wave velocity, higher F/B ratio, fewer years of education, lower Mini-Nutritional Assessment-Short Form score, lower ankle brachial index, lower JDI_{12} score, and having categorical items). Finally, multivariable logistic regression model was used to identify independent associations between the presence of dementia and plasma NfL concentrations. Backward stepwise multivariable logistic regression analyses were performed adjusting for age, sex, years of education, risk factors, enterotype I, F/B ratio, and brain imaging. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All comparisons were two-tailed, and $p < 0.05$ represented statistical significance. Data were analyzed using the JMP 14.3 software package (SAS Institute Inc., Cary, NC, USA) and R software (http://www.r-project.org).

RESULTS

Participant characteristics

A total of 128 participants were enrolled in this study, as previously reported (women: 59%, mean age: 74.2 ± 8.7 years). The levels of cognitive function and enterotypes were as follows: 34 participants (26.6%) were classified as having dementia, 71 (55.5%) as having MCI, and 23 (18.0%) as having NC; 47 (36.7%) participants were classified as having enterotype I, 5 (4%) as having enterotype II, and 76 (59.4%) as having enterotype III.

Clinical data and plasma NfL

Compared with participants with low concentrations of plasma NfL, those with high concentrations tended to be older (high versus low: 77 versus 73 years, $p < 0.001$), women (69.8% versus 46.9%, $p = 0.012$), and hypertensive (74.6% versus 50.0%, $p = 0.006$) and have a history of stroke (14.3% versus 3.1%, $p = 0.030$), chronic kidney disease (44.4% versus 20.3%, $p = 0.005$), and dementia (47.6% versus 4.7%, $p < 0.001$). They also tended to have cerebral SVD, higher scores in the VSRAD (median score: 1.6 versus 0.8, $p < 0.001$), and reduced cognitive function (Tables 1 and 2 and Supplementary Table 1).

Plasma NfL concentrations were significantly higher in participants with cognitive decline and those with cerebral SVD (Fig. 1). Higher levels of Ln-NfL were significantly associated with greater cognitive decline, as indicated by MMSE, CDR-Sum of Boxes (CDR-SB), Alzheimer's Disease Assessment Scale-Cognitive Subscale, and Frontal Assessment Battery scores (Fig. 2). Higher levels of Ln-NfL were also associated with increased VSRAD and decreased JDI_{12} scores (Fig. 3). Cognitive function, VSRAD scores, and JDI_{12} scores were significantly associated with Ln-NfL levels in both the unadjusted analyses and analyses adjusted for age (Supplementary Table 2). However, there were no significant correlations between levels of Ln-NfL and gut microbial metabolites.

Cutoff values of plasma NfL

The plasma NfL cutoff value for the detection of dementia was 24.2 pg/mL, with 91% sensitivity and 65% specificity (area under the receiver operating characteristic curve [AUC] = 0.80, $p < 0.0001$). In participants without dementia, the plasma NfL cutoff value for the detection of MCI was 21.0 pg/mL, with 64% sensitivity and 83% specificity (AUC = 0.74, $p < 0.0001$).

Dietary assessments and NfL

Compared with participants with high plasma NfL concentrations, those with low concentrations tended to consume more fish and shellfish (71.7% versus 42.1%, $p = 0.008$), mushrooms (65.2% versus 39.5%, $p = 0.028$), fruit (89.1% versus 73.7%, $p = 0.088$), soybeans and soybean-derived foods (93.5% versus 71.1%, $p = 0.008$), and coffee (80.4% versus 60.5%, $p = 0.055$), although some of these differences were not significant (Supplementary Table 3). Participants with low plasma NfL concentrations also tended to have higher JDI_{12} scores than participants with high plasma NfL concentrations (median score: 8 versus 7, $p = 0.036$).

Gut microbiota, metabolites, and NfL

There were no significant correlations between levels of Ln-NfL and concentrations of gut microbiome metabolites (Supplementary Figure 1). Regarding the enterotypes, there were no significant differences between participants with high and low plasma NfL concentrations (Supplementary Table 4).

Plasma NfL was significantly different between participants with and without dementia, and between participants with MCI and those with NC among participants without dementia (Supplementary Table 5). F/B ratios were significantly different between participants with and without dementia, and between

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Table 1
Comparisons of participant background information between participants with high neurofilament light chain (NfL) concentrations and those with low NfL concentrations

	High NfL ($n = 63$)	Low NfL ($n = 64$)	p
Demographic factor			
Age, y	77, 75–82	73, 66.3–78	< 0.0001
Women, *n* (%)	44 (69.8)	30 (46.9)	0.012
Education, y	12, 9–12	12, 12–14	0.0005
Risk factor			
Hypertension, *n* (%)	47 (74.6)	32 (50.0)	0.006
Diabetes mellitus, *n* (%)	14 (22.2)	6 (9.4)	0.054
Dyslipidemia, *n* (%)	30 (47.6)	29 (45.3)	0.860
IHD, *n* (%)	7 (11.1)	5 (7.8)	0.560
Stroke, *n* (%)	9 (14.3)	2 (3.1)	0.030
CKD, *n* (%)	28 (44.4)	13 (20.3)	0.005
Smoking habit, *n* (%)	9 (14.3)	23 (35.9)	0.007
Alcohol consumption, *n* (%)	18 (28.6)	31 (48.4)	0.029
Apolipoprotein E ε4 carrier, *n* (%)	22 (34.9)	16 (25.0)	0.249
Dementia, *n* (%)	30 (47.6)	3 (4.7)	< 0.0001
Comprehensive geriatric assessment			
Barthel Index	100, 95–100	100, 100–100	0.009
IADL impairment, *n* (%)	41 (65.1)	17 (26.6)	< 0.0001
DBDS	12, 5–18.3	6, 3–11.8	0.0002
GDS	3, 1–5	2, 1–5	0.578
ZBI	15, 6–24	7, 3–19	0.007
MNA-SF	12, 11–13	13, 12–14	0.015
Arterial stiffness			
Ankle brachial index	1.11, 1.04–1.15	1.08, 1.06–1.15	0.842
Pulse wave velocity, m/s	18.0, 16.1–20.5	19.3, 15.8–23.3	0.275
Laboratory finding			
eGFR, mL/min/1.73 m^2	60.9, 50.2–72.3	72.4, 61.3–84.8	< 0.0001

Data are presented as medians, interquartile ranges, or number of patients (%). Wilcoxon rank-sum test and χ^2 test were used. CKD, chronic kidney disease; DBDS, Dementia Behavior Disturbance Scale; eGFR, estimated glomerular filtration rate; GDS, Geriatric Depression Scale; IADL, instrumental activities of daily living; IHD, ischemic heart disease; MNA-SF, Mini-Nutritional Assessment-Short Form; ZBI, Zarit Caregiver Burden Interview.

participants with enterotype I and those with other enterotypes (Supplementary Table 5). However, serum C-reactive protein was not significantly different between any of the groups.

Subgroup analyses stratified by plasma NfL and dementia-related items

There were significant correlations among the four groups stratified by plasma NfL and dementia-related items (Supplementary Table 6). Those with both high plasma NfL and dementia-related items, such as fewer years of education and higher VSRAD score, tended to have a higher prevalence of dementia compared with those who had no dementia-related items. Regarding the gut microbiome, having both high plasma NfL and high F/B ratio also tended to be associated with the presence of dementia (Supplementary Table 6). Additionally, those who had high plasma NfL who presented with enterotype III tended to be more associated with higher prevalence of dementia compared with that of those who presented with enterotype I (prevalence of dementia: 63.2% versus 18.2%; Supplementary Table 6).

Compared with the AUC of NfL alone for the detection of dementia (AUC [95% CI]: 0.81 [0.73–0.88]), those of NfL+F/B ratio, NfL+enterotype I, and NfL+enterotype III were equal or slightly higher (0.81 [0.74–0.89], 0.83 [0.74–0.91], and 0.84 [0.75–0.92], respectively); however, there were no significant differences (Supplementary Figure 2).

Multivariable analyses

Multivariable analyses revealed that plasma NfL was independently associated with the presence of dementia. Specifically, compared with participants with low plasma NfL concentrations, those with high concentrations had a higher OR (OR: 9.94,

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Table 2
Comparisons of participant characteristics between participants with high neurofilament light chain (NfL) concentrations and those with low NfL concentrations

	High NfL ($n=63$)	Low NfL ($n=64$)	p
Cognitive function			
MMSE	22, 18–24	27.5, 24–29	<0.0001
CDR-GB			<0.0001
0, *n* (%)	2 (3.2)	21 (32.8)	
0.5, *n* (%)	43 (68.3)	42 (65.6)	
≥1, *n* (%)	18 (28.6)	1 (1.6)	
CDR-SB	3.5, 2–4.5	0.5, 0.5–1.9	<0.0001
ADAS-cog	13.2, 8–17.3	6.3, 4.7–10	<0.0001
RCPM	24.5, 20.8–28	31, 28–33.5	<0.0001
FAB	10, 8–11	13, 10–14	<0.0001
LM-WMSR I	4, 2–9	12, 7–21	<0.0001
LM-WMSR II	0, 0–3	7, 1–14	<0.0001
Brain MRI finding			
SLI, *n* (%)	11 (17.5)	2 (3.1)	0.009
WMH, *n* (%)	22 (34.9)	12 (18.8)	0.047
CMB, *n* (%)	20 (31.8)	8 (12.5)	0.011
EPVS, *n* (%)	43 (68.3)	53 (82.8)	0.065
VSRAD	1.6, 0.9–2.3	0.8, 0.5–1.2	<0.0001
SPECT			
Low blood flow*	44 (73.3)	41 (68.3)	0.688

Data are presented as medians, interquartile ranges, or number of patients (%). Wilcoxon rank-sum test and χ^2 test were used. *Blood flow reduction in the posterior cingulate gyrus and/or precuneus, as seen in SPECT images. ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; BP, blood pressure; CDR-GB, Clinical Dementia Rating Global Score; CDR-SB, Clinical Dementia Rating-Sum of Boxes; CMB, cerebral microbleed; EPVS, enlarged periventricular space; FAB, Frontal Assessment Battery; LM-WMSR, Logical Memory subtests I and II of the Wechsler Memory Scale-Revised; MMSE, Mini-Mental State Examination; MRI, magnetic resonance imaging; RCPM, Raven's Coloured Progressive Matrices; SLI, silent lacunar infarct; SPECT, single-photon emission-computed tomography; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease; WMH, white matter hyperintensity.

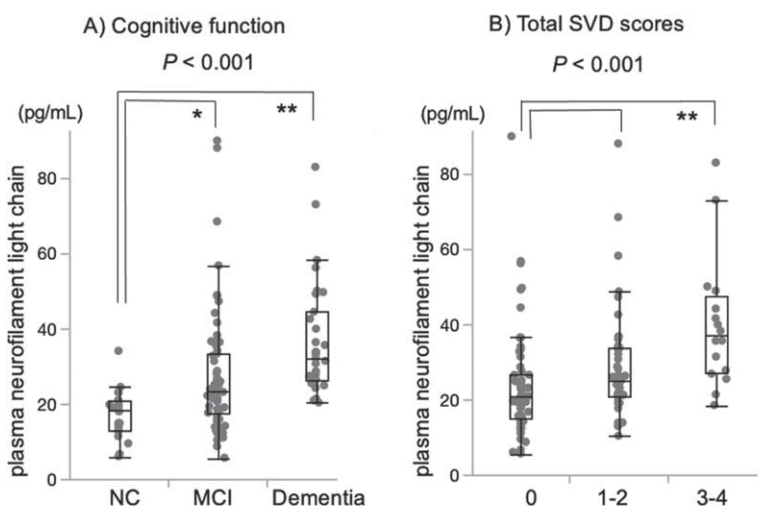


Fig. 1. Comparisons of plasma neurofilament light chain (NfL) concentrations and (A) cognitive function or (B) total cerebral small vessel disease (SVD) scores. The *x*-axes show: (A) cognitive function stratified into normal cognition (NC), mild cognitive impairment (MCI), and dementia groups; (B) total cerebral SVD scores stratified as scores of 0, 1–2, or 3–4. The *y*-axes show plasma NfL concentrations. The plasma NfL concentrations increased with increasing degrees of cognitive decline and total cerebral SVD scores (Kruskal-Wallis test). *Post hoc* analysis performed using Dunnett's test. *$p<0.05$; **$p<0.0001$.

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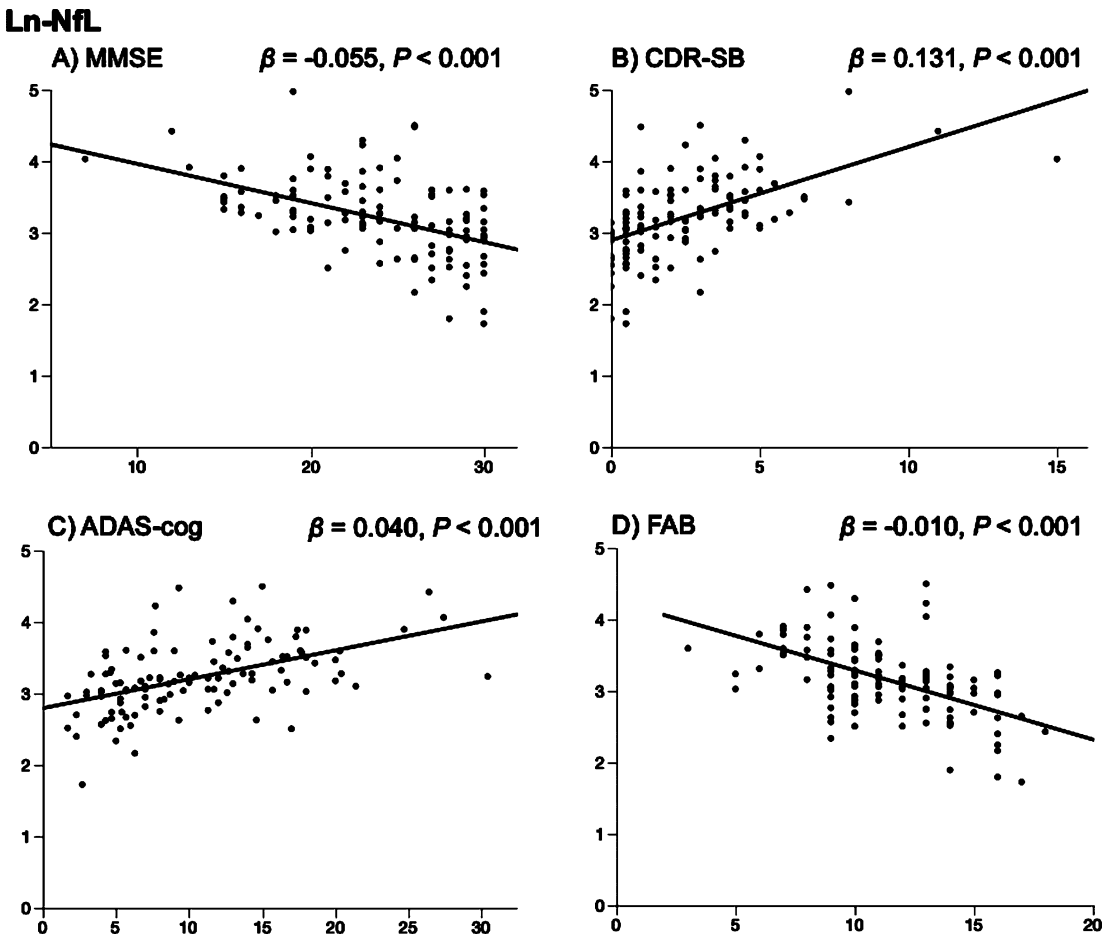


Fig. 2. Comparisons of plasma neurofilament light chain (NfL) concentrations and cognitive function. The *x*-axes show: (A) Mini-Mental State Examination (MMSE) scores; (B) Clinical Dementia Rating-Sum of Boxes (CDR-SB) scores; (C) Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-cog) scores; (D) Frontal Assessment Battery (FAB) scores. The *y*-axes show the levels of the natural logarithm-transformed plasma NfL (Ln-NfL). The levels of Ln-NfL increased with increasing degrees of cognitive decline (single regression analysis).

95% CI: 2.75–48.2, $p < 0.001$) calculated in the stepwise multivariable logistic regression analyses after adjusting for other confounding covariables (Table 3 and Supplementary Table 7). Similarly, the stepwise multivariable logistic regression analysis revealed that higher plasma NfL (categorized as a continuous variable) was more likely than lower plasma NfL to be associated with the presence of dementia; however, this result was not significant (per 10 pg/mL, OR: 1.26, 95% CI: 0.94–1.79; $p = 0.139$; Supplementary Table 8).

DISCUSSION

The main finding of the present study was that plasma NfL was independently and significantly associated with the presence of dementia, as previously reported. Moreover, plasma NfL was associated with dementia-related items, such as cognitive function, total SVD score, and JDI_{12} score. Furthermore, those with high plasma NfL and dementia-related items tended to have a higher prevalence of dementia compared with those with no dementia-related items. Regarding the gut microbiome, high plasma NfL presenting with either enterotype III or high F/B ratio also tended to be associated with the presence of dementia. However, there were no significant correlations between the levels of NfL and gut microbial metabolites.

NfL is an intermediate filament in the axonal cytoskeleton, and NfL changes in biological fluids, such as blood and CSF, have been proposed as a biomarker for brain damage and atrophy in multiple

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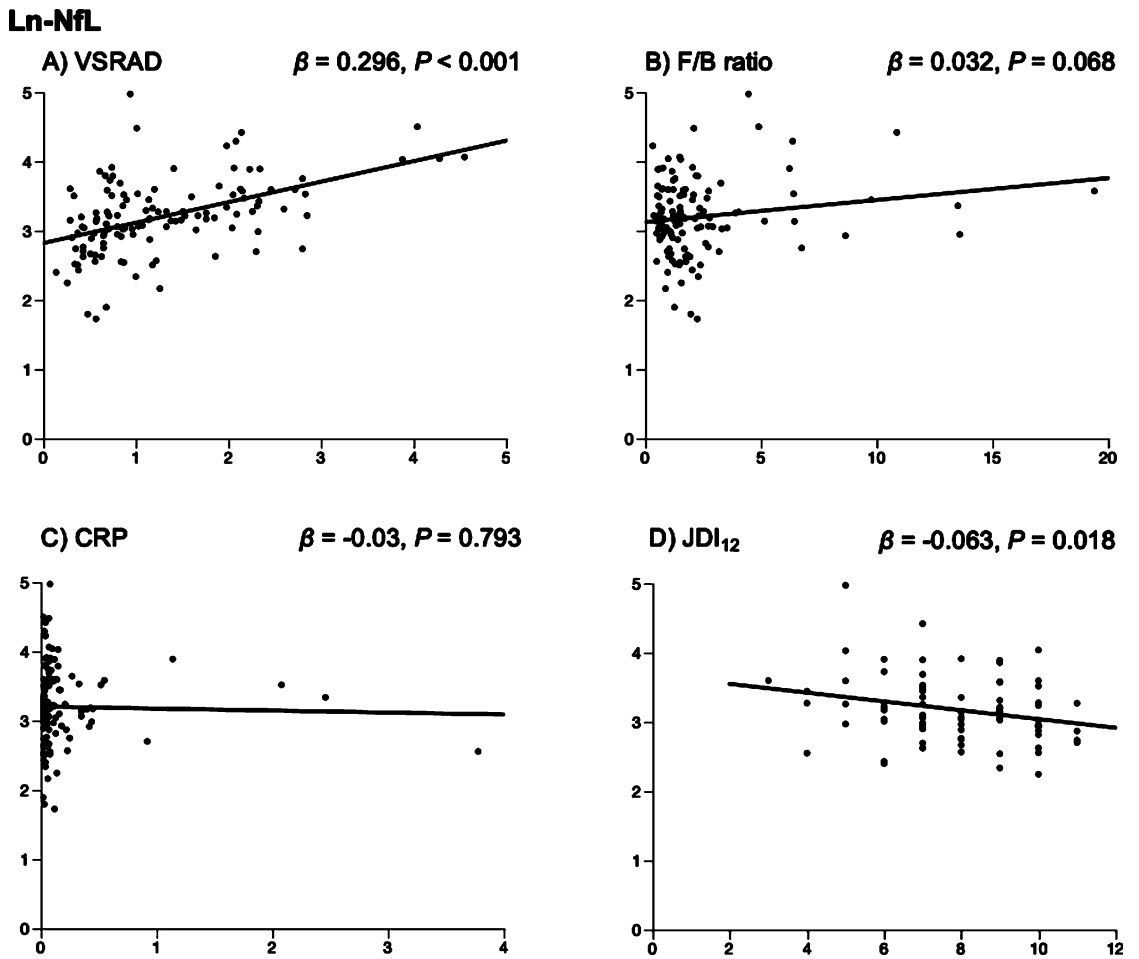


Fig. 3. Comparisons of plasma neurofilament light chain (NfL) concentrations and clinical biomarkers. The *x*-axes show: (A) voxel-based specific regional analysis system for Alzheimer's disease (VSRAD); (B) Firmicutes/Bacteroidetes ratio (F/B ratio); (C) C-reactive protein (CRP); (D) Japanese Diet Index (JDI_{12}). The *y*-axes show the levels of the natural logarithm-transformed plasma NfL (Ln-NfL). The levels of Ln-NfL increased with increasing VSRAD scores, which suggested more cortical and hippocampal atrophy. They also decreased with increasing adherence to a traditional Japanese diet (single regression analysis).

neurological disorders, including neurodegenerative diseases [25, 26]. Both amyloid-β and tau are considered robust biomarkers of dementia, such as AD [29]. In addition, recent studies have proposed the A/T/N system, which comprises three biomarkers (Amyloid-β, Tau, and Neurodegeneration or neural injury) to describe and categorize multidomain biomarkers findings [29]. Both amyloid-β and tau are more specific indicators of AD than is NfL an indicator of neurodegeneration. Therefore, we speculated that, of these three biomarkers, NfL is useful for estimating disease-non-specific neural injury or neurodegeneration caused by the gut microbiome and/or microbial metabolites. In our dataset, plasma NfL was related to cognitive impairment and was associated with several clinical biomarkers, such as VSRAD and cerebral SVD scores. The cutoff value of plasma NfL to detect the presence of dementia in the present study (24.2 pg/mL) was similar to that of a previous study to detect the presence of AD (25.7 pg/mL) [30]. Thus, these associations are reasonable and consistent with the results of previous studies [10–12].

In our previous study, the F/B ratio was associated with the presence of dementia. Specifically, the F/B ratio was higher in participants with dementia compared with those without dementia, which indicated that advanced dysbiosis may be related to the onset of dementia [14]. However, both the F/B ratio and gut microbiome, which we categorized according to enterotypes, did not significantly differ

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Table 3
Multivariable logistic regression analyses for the presence of dementia

	OR	95% CI	*p*
High concentration of plasma NfL*	9.94	2.75–48.2	<0.001
Enterotype I	0.11	0.03–0.39	<0.001
Alcohol consumption	0.47	0.13–1.51	0.206
CMBs	3.22	0.98–11.0	0.054
VSRAD	2.48	1.26–5.58	0.007

The dependent variable was the presence of dementia. *Participants were allocated to two groups according to their plasma NfL concentrations: high NfL group if the NfL value was above the median; low NfL group if the NfL value was below the median. Model: backward stepwise multivariable logistic regression analyses adjusted for age, sex, years of education, and risk factors, such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking, alcohol consumption, apolipoprotein E, enterotype I, F/B ratio, silent lacunar infarct, white matter hyperintensity, cerebral microbleeds, enlarged perivascular space, VSRAD score, single-photon emission-computed tomography findings (presence or absence of low blood flow in the posterior cingulate gyrus and/or precuneus). CI, confidence interval; CMBs, cerebral microbleeds; NfL: neurofilament light chain; OR, odds ratio; VSRAD; voxel-based specific regional analysis system for Alzheimer's disease.

between different plasma NfL concentrations. This negative finding may be a result of our methodological limitations regarding the T-RFLP analysis and the F/B ratio. The use of next-generation sequencing can reveal more detailed microbial data than can the T-RFLP method. Furthermore, several biomarkers, other than the F/B ratio, such as lipopolysaccharides [31] and trimethylamine N-oxide [13], are attributed to the gut microbiome and may affect the brain. Lipopolysaccharides are the major outer membrane components of gram-negative bacteria and are capable of triggering systemic inflammation and the release of pro-inflammatory cytokines [31]. Trimethylamine N-oxide is a small molecule produced by the meta-organismal metabolism of dietary choline and has been implicated in human disease pathogenesis, including as a risk factor for AD [13]. Analyzing these biomarkers in addition to NfL may reveal more precise associations between the gut microbiome, microbial metabolites, blood biomarkers, and cognitive function. Furthermore, these associations may also clarify the presence of the gut–microbiome–brain axis.

We found that plasma NfL concentrations were not significantly correlated with gut microbial metabolite concentrations. Previously, we reported that several gut microbial metabolites are associated with dementia [18], where several metabolites were positively associated with dementia, whereas others were inversely associated with dementia, which suggested a multiplex linkage among the gut microbiome, microbial metabolites, and cognitive function. Alkasir et al. reported the presence of a brain–gut link and proposed a novel strategy for the management of dementia by modulating microbiota [32]. Furthermore, Rosa et al. found a significant correlation between gastrointestinal disease and high CDR score, which indicated the presence of a brain–gut link [33]. A recent study reported that older individuals with subjective cognitive decline had a significant reduction in the genus *Faecalibacterium*, which suggested an alteration of anti-inflammatory gut bacteria [34]. Another study also demonstrated that some microbial metabolites, such as indole and propionic acid, promote brain health [35]; moreover, microbiota-derived short-chain fatty acids are critical mediators along the gut–brain axis and promote amyloid-β deposition [36]. These findings support the presence of a gut–microbiome–brain axis. However, our sub-analysis revealed no significant correlations between microbial metabolites and plasma NfL. Nevertheless, associations between metabolites and blood biomarkers, other than NfL, may exist, and further comprehensive analyses that include these factors will likely reveal the nature of the gut–microbiome–brain axis.

Another notable result of the present study was that fish and shellfish, mushrooms, fruits, soybeans and soybean-derived foods, and coffee may have protective effects on cognitive decline. We found that greater consumption of these foods was associated with lower concentrations of plasma NfL, which indicated that these foods may alter gut microbiota and suppress cognitive decline. A previous large-scale study also reported an association between gut microbiota and healthy dietary habits, which was related to favorable cardiometabolic markers [37]. In addition, we revealed that a healthier diet pattern indicated by JDI_{12} was associated with lower concentrations of plasma NfL. This will be investigated in more detail in the future.

The present study has several strengths. First, we revealed novel relationships among gut microbiota, microbial metabolites, plasma NfL, and cognitive decline. Specifically, plasma NfL was strongly associated with cognitive decline. Second, we also revealed that NfL levels were not correlated with gut microbial metabolites. Although we did not observe a positive association, this is the first study to analyze

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such associations and thus, offers new research directions in this emerging field. Third, we confirmed the utility of plasma NfL for detecting the presence of cognitive decline as well as several biomarkers for cognitive function, such as VSRAD and cerebral SVD scores, which are consistent with previous studies. Finally, we systematically evaluated cognitive function using a comprehensive geriatric assessment and a range of neuropsychological tests. Our findings encourage further focus on the relationships between blood biomarkers and cognitive function through examinations of the gut microbiome.

The present study also has several limitations. We did not assess amyloid-β or tau in the present study because the Gimlet study did not perform CSF testing or positron emission tomography to identify these biomarkers. The small number of participants and large number of potential variables may have also statistically underpowered our study. However, because this was a sub-analysis, sample size was not calculated. Furthermore, selection bias may also exist because our sample was a single hospital-based cohort. Another limitation of our study is that the distribution of enterotypes was unbalanced; however, enterotypes were likely unbalanced according to the characteristics of the cohort. Moreover, the specific effects of each microbial metabolite on blood biomarkers have not yet been determined. Thus, a comprehensive assessment that includes other biomarkers, such as amyloid-β precursor protein and non-neurodegenerative inflammatory biomarkers, may be useful given that they are associated with the risk of cognitive impairment [38].

Although this sub-study was a preliminary analysis that included a small number of patients, we provided evidence for relationships between plasma NfL, gut microbiota, and cognitive function. Detailed assessments of these relationships should be conducted in future studies to determine the underlying mechanisms.

Conclusions

High plasma NfL concentration was independently and significantly associated with the presence of dementia and was also associated with cerebral SVD, as previously reported. However, plasma NfL levels were not significantly correlated with gut microbial metabolites in this preliminary study. Further studies are warranted to examine these associations in relation to the microbiome–gut–brain axis.

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SUPPLEMENTARY MATERIAL

Relationship Between Plasma Neurofilament Light Chain, Gut Microbiota, and Dementia: A Cross-Sectional Study

SUPPLEMENTARY METHODS

Outline of the Gimlet Study

Subjects

Between March 2016 and March 2017, we enrolled consecutive patients visiting the memory clinic at the National Center for Geriatrics and Gerontology (NCGG) in Japan who agreed to undergo a medical assessment of their cognitive function and a fecal examination.

Patients were eligible for the Gimlet study if they met the following criteria: 1) were able to undergo a brain MRI; 2) provided informed consent in writing; 3) provided informed consent for the NCGG Biobank to store their clinical data, blood, and fecal samples; and 4) were accompanied by a study partner who could assess the patient's condition. Patients were excluded if they met the following criteria: 1) were unable to undergo an MRI examination or the MRI was unable to be evaluated because of movement; 2) had local lesions, such as cerebral infarctions, that were detected by MRI before enrolment and might significantly affect cognitive function; 3) had a history of a major psychological disorder or current, serious, or unstable alcohol or drug abuse; 4) had $\leq$6 years of education; 5) had a history of cancer of the digestive tract; or 6) were judged by an investigator to be ineligible to participate as a study subject (e.g., recent use of antibiotics, digestive disorder such as liver cirrhosis or inflammatory bowel disease, neurological disorder such as brain tumor, encephalitis/meningitis, normal pressure hydrocephalus, or Huntington's disease).

Baseline assessment

All participants underwent a comprehensive geriatric assessment [1] using the following: 1) demographic characteristics; 2) risk factors, such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking habits, or a history of stroke and alcohol consumption; 3) basic and instrumental activities of daily living scales, assessed using the Barthel Index [2] and Lawton and Brody scale [3]; 4) global cognitive function, assessed using the Mini-Mental State Examination [4] and Clinical Dementia Rating [5]; 5) neuropsychological testing, using the Alzheimer's Disease Assessment Scale-Cognitive Subscale [6], Raven's Coloured Progressive Matrices [7], Frontal Assessment Battery [8], and Logical Memory subtests of the Wechsler Memory Scale-Revised [9]; 6) behavioral and psychological symptoms, assessed using the Dementia Behaviour Disturbance Scale [10]; 7) burden of caregivers, assessed by the Zarit Caregiver Burden Interview [11]; 8) depressive status, assessed by the Geriatric Depression Scale [12]; 9) laboratory variables, including *APOE* ε4 as a risk factor for AD; 10) ankle brachial index and pulse wave velocity, as indicators of arteriosclerosis [13] and the 'impact' of pulse [14]; 11) brain imaging, such as MRI and SPECT; 12) an assessment of other factors, such as the presence of frailty [15] and subjective hearing loss; and 13) an assessment of social and lifestyle factors, such as the Mini-Nutritional Assessment-Short Form to assess nutritional status [16]. All clinical samples and data were provided by the NCGG Biobank, which collects clinical data for research.

Risk factors

Hypertension was defined by a systolic blood pressure of $\geq$140 mmHg or a diastolic blood pressure of $\geq$90 mmHg, and/or the use of antihypertensive drugs. Dyslipidemia was defined by a serum low-density lipoprotein cholesterol concentration of $\geq$140 mg/dL, a serum high-density lipoprotein cholesterol concentration of <40 mg/dL, a serum triacylglycerol concentration of $\geq$150 mg/dL, and/or the use of statins. Diabetes mellitus was defined by a hemoglobin A1c concentration of $\geq$6.5%, and/or the use of oral hypoglycemic drugs or insulin, and/or a fasting serum glucose concentration of $\geq$69.9 mol/L (126 mg/dL). Ischemic heart disease was defined by a history of physician-diagnosed angina pectoris and/or evidence of a prior myocardial infarction or coronary revascularization procedure (percutaneous coronary intervention or coronary artery bypass surgery). Serum creatinine was measured and the estimated glomerular filtration rate (eGFR) was determined using the equation proposed by the Japanese Society of Nephrology, as follows: eGFR (mL/min/1.73 m^2) = $194 \times (\text{serum creatinine [mg/dL]})^{-1.094} \times (\text{age [years]})^{-0.287}$ ($\times$0.739 if female). Chronic kidney disease (CKD) was defined by an eGFR of <60 mL/min/1.73 m^2. We performed blood count and biochemical examination.

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MRI imaging and SPECT imaging

The participants underwent a 1.5-T MRI of their brains (Philips Ingenia, Eindhoven, the Netherlands). MRI scans were obtained, including diffusion-weighted images, fluid-attenuated inversion recovery images, T2-weighted images, T2*-weighted gradient-echo images, three-dimensional T1-weighted sagittal and axial coronal views, and 3D time-of-flight magnetic resonance angiography scans. The presence and components of cerebral small vessel disease, such as silent lacunar infarct, white matter hyperintensity, cerebral microbleeds, and enlarged periventricular space, were categorized using previously published standards for reporting vascular changes on neuroimaging [17]. The voxel-based specific regional analysis system for Alzheimer's Disease (VSRAD) software (Eisai Co., Ltd., Tokyo, Japan) was used to quantify cortical and hippocampal atrophy, using standardized z-scores [18]. A high VSRAD score suggests the presence of Alzheimer's disease (AD) because this score reflects hippocampal atrophy, which is one of the characteristics of the brain of a patient with AD. The participants also underwent N-isopropyl-p-[^{123}I]-iodoamphetamine-SPECT, in which the presence of low blood flow in the area of the posterior cingulate gyrus and/or the precuneus was regarded as a surrogate marker of AD [19].

Dietary assessments

The questionnaire regarding dietary composition consisted of 12 items. All the components of a typical Japanese diet were grouped on the basis of the definitions used in the Japanese National Health and Nutrition Survey and the previous studies [20]. We evaluated the Japanese dietary index (JDI) based on the items shown in the above survey. In detail, JDI_{12} was defined recently [21] and comprises 10 beneficial components (rice, miso, fish and shellfish, green and yellow vegetables, seaweed, pickles, green tea, soybeans and soybean-derived foods, fruit, and mushrooms) and two less beneficial components (beef and pork, and coffee). The participants were assigned one point if their daily beneficial intake was equal to or greater, and their daily less beneficial intake was below the sex-specific median intake.

Sample collection

Patients or their family members used a fecal sampler to collect a fecal sample as soon as possible after the patient's bowel movement. The sample was placed in a specimen container. Patients collected fecal samples on the day of their hospital consultation, and the samples were presented to the clinical laboratory center of the NCGG (preferably within 4 h of the bowel movement). Because one of the inclusion criteria for our study was that patients presented with study partners (family members), all of the demented patients were able to be supported if they needed support about excretion. Furthermore, when we received the fecal samples, we only accepted appropriate samples as per the analyzing company's criteria, while inadequate samples were disposed and resubmission was requested. The samples were frozen and stored at –81°C in the NCGG Biobank. After all samples had been collected, they were transported (frozen) to TechnoSuruga Laboratory (Shizuoka, Japan).

Gut microbiome

Fecal samples were collected at home, just after a bowel evacuation, by patients or their family members. The samples were collected using scoop collection tubes, and patients consumed their usual diets before and after sample collection. Samples were frozen and preserved at –81°C at the NCGG Biobank. After all samples had been collected, the gut microbiomes were analyzed by TechnoSuruga Laboratory (Shizuoka, Japan) using T-RFLP analysis [22]. T-RFLP analysis is one of the most well-established and reliable 16S ribosomal RNA-based methods, especially when considering its high throughput and reproducibility. First, T-RFLP was used to classify gut microbes into the following 10 groups: Prevotella, Bacteroides, Lactobacillales, Bifidobacterium, Clostridium cluster IV, Clostridium subcluster XIVa, Clostridium cluster IX, Clostridium cluster XI, Clostridium cluster XVIII, and 'others'. Second, by referencing the Human Fecal Microbiome T-RFLP profile [23, 24], the gut microbiome was stratified into three enterotypes: enterotype I included Bacteroides at > 30%, enterotype II included *Prevotella* at > 15%, and enterotype III included the remaining bacteria. Third, the F/B ratio was examined, because an increase in F/B ratio indicates dysbiosis [25]. The phylum Firmicutes included the Lactobacillales and *Clostridium* clusters, and the phylum Bacteroidetes included *Bacteroides* and *Prevotella*.

Terminal restriction fragment length polymorphisms

Fecal samples (approximately 10 mg each) were suspended in 900 μL of a solution containing 100 mM tris-HCl (pH 9.0), 40 mM ethylenedi-

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aminetetraacetic acid, 4 M guanidine thiocyanate and 0.001% bromothymol blue. Fecal solids in the suspension were broken down using a FastPrep FP100A Instrument (MP Biomedicals, CA, USA) and zirconia beads at 5 m/s for 2 min. DNA was then extracted from 200 μL of suspension using an automatic nucleic acid extractor and MagDEA® DNA 200 (Precision System Science, Chiba, Japan). PCR was performed using a Takara Thermal Cycler Dice TP650 (Takara Bio, Shiga, Japan) in a reaction mixture (20 μl) containing 1 × PCR buffer, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.2 μM, 10 ng of fecal DNA and 0.2 U of HotStar Taq DNA polymerase (Qiagen, Hilden, Germany). The primers used were 5' fluorescein amidite-labeled 516f (5'-TGC- CAGCAGCCGCGGTA-3'; *Escherichia coli* positions 516–532) and 1510r (5'-GGTTACCTTGTTACGA- CTT-3'; *E. coli* positions 1510–1492). The amplification program used was as follows: preheating at 95°C for 15 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30s, and extension at 72°C for 90s; and finally, a terminal extension at 72°C for 10 min. The size of the amplicon was verified by electrophoresis and it was purified using a MultiScreen PCR96 Filter Plate (Millipore, Billerica, MA, USA). The purified 16S rDNA amplicons were treated with 10 U of FastDigest *BseLI* (Thermo Fisher Scientific, MA, USA) for 10 min. An ABI PRISM 3130 × l genetic analyzer (Thermo Fisher Scientific) was used to analyze the resultant fluorescence-labeled terminal restriction fragments (T-RFs), and GeneMapper software (Thermo Fisher Scientific) was used to determine T-RF length and peak area for each sample. T-RFs were divided into 29 operational taxonomic units (OTUs). The OTUs were quantified as the percentage of each OTU of the total OTU area and expressed as the percentage of the area under the curve (% AUC). The reference database, Human Fecal Microbiota T-RFLP profiling (http://www.tecsrg-lab.jp/), was used to putatively identify the bacteria in each classification unit and the corresponding OTU.

Analysis of metabolites in feces

To determine metabolite levels, we measured fecal levels of organic acids, SCFAs, ammonium ions, indoles, phenol, skatole, and p-cresol. The organic acids and SCFAs, such as acetic acid, propionic acid, butyric acid, iso-butyric acid, succinic acid, lactic acid, formic acid, valeric acid, and iso-valeric acid, were measured using high-performance liquid chromatography (Prominence, Shimadzu, Kyoto, Japan) with a detector (CDD-10A, Shimadzu, Kyoto, Japan), two tandemly-arranged columns (Shim-pack SCR-102(H), 300 mm × 8 mm ID, Shimadzu), and a guard column (Shim-pack SCR-102(H), 50 mm × 6 mm ID, Shimadzu). We measured ammonium ion concentrations using an ion chromatography system (ICS-1000, DIONEX, Thermo Fisher Scientific K.K., Tokyo, Japan) with a column (IonPac CS12A, 4 mm × 250 mm, DIONEX) and a guard column (IonPac CG12A, 4 mm × 50 mm, DIONEX). Indoles, phenol, skatole, and p-cresol levels were measured using gas chromatography/mass spectrometry (QP-2010, Shimadzu) and a capillary column (Inert cap WAX, 30 m × 0.25 mm × 0.25 μm, GL Sciences, Tokyo, Japan).

Organic acids

To determine the presence of organic acids, 0.1 g of feces was placed into a 2.0-mL tube with zirconia beads and suspended in MilliQ water. Samples were heated at 85°C for 15 min, vortexed at 5 m/s for 45 s using FastPrep 24 (MP Biomedicals, CA, USA), and centrifuged at 15,350×*g* for 10 min. The supernatant was filtered using a 0.2-μm filter. Organic acids (acetic acid, propionic acid, butyric acid, iso-butyric acid, succinic acid, lactic acid, formic acid, valeric acid, and iso-valeric acid) in feces were measured using high-performance liquid chromatography (Prominence, Shimadzu) with a detector (CDD-10A, Shimadzu), two tandemly-arranged columns (Shim-pack SCR-102(H), 300 mm × 8 mm ID, Shimadzu), and a guard column (Shim-pack SCR-102(H), 50 mm × 6 mm ID, Shimadzu). A 5 mM p-toluenesulfonic acid solution was used in the mobile phase and a mixture (5 mM p-toluenesulfonic acid, 100 μM EDTA, and 20 mM Bis-Tris) was used as the reaction solution. The flow rate and oven temperature were 0.8 mL/min and 45°C, respectively. The detector cell temperature was maintained at 48°C. The measurements were performed with an absolute calibration curve (range 5–1,000 mg/L).

Ammonia

To measure ammonium ion concentrations, 0.1 g of feces was suspended in 0.9 mL MilliQ water, heated at 85°C for 15 min, and filtered through a 0.20-μm filter. Samples were diluted 200-fold in MilliQ water. Ammonium ion concentrations were

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then measured using an ion chromatography system (ICS-1000, DIONEX) with a column (IonPac CS12A, 4 mm × 250 mm, DIONEX) and a guard column (IonPac CG12A, 4 mm × 50 mm, DIONEX). In the mobile phase, a 10.7 mM sulfuric acid solution was used, and the flow rate was 1.0 mL/min. The detector temperature was maintained at 35°C. The measurements were performed with an absolute calibration curve (range 0.025–2.0 mg/L).

Indole, phenol, skatole, and p-cresol

A total of 0.1 g of feces was suspended in 2.5 mL phosphate buffer, including 0.4 mg/L 4-isopropylphenol as an internal standard. The sample was heated at 85°C for 15 min, mixed with 2.5 mL acetonitrile and 1 g NaCl, shaken for 30 min, and centrifuged at 1,300×*g* for 10 min. Next, 1 mL of the supernatant was dehydrated and purified using a sodium sulfate drying cartridge (Bond Elut LRC, Agilent Technologies, USA), C18 cartridge (Smart SPE C18-30, AiSTI SCIENCE, Japan), and PSA cartridge (Smart SPE PSA-30, AiSTI SCIENCE), and placed into a vial.

Indoles and phenols were measured using gas chromatography/mass spectrometry (QP-2010, Shimadzu) and a capillary column (Inert cap WAX, 30 m × 0.25 mm × 0.25 μm, GL Sciences). Helium was used as the carrier gas at 1.11 mL/min. The injector and interface temperatures were maintained at 240°C and 230°C, respectively. The oven temperature program was as follows: 70°C for 2 min; rose by 20°C/min to 200°C, held for 3 min; rose by 10°C/min to 240°C; and finally held for 16 min at 240°C. One microliter of the extract was injected in the splitless mode. The mass spectrometer was operated in electron impact ionization mode at 70 eV. The measurements were performed with an absolute calibration curve (range 0.02–5.0 mg/L). Data acquisition was performed in selected ion monitoring mode for quantification.

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Supplementary Table 1
Comparisons of participant characteristics between participants with high neurofilament light chain (NfL) concentrations and those with low NfL concentrations

	High NfL ($n=63$)	Low NfL ($n=64$)	p
Demographic factor			
Age, y	77, 75–82	73, 66.3–78	<0.0001
Female sex, *n* (%)	44 (69.8)	30 (46.9)	0.012
Education, y	12, 9–12	12, 12–14	0.0005
Body mass index, kg/m^2	22.4, 20–24.7	22.8, 21.3–24.5	0.120
Systolic BP, mmHg	139, 125–162	148, 127–158	0.845
Diastolic BP, mmHg	76, 68–86	82, 74–89	0.039
Risk factor			
Hypertension, *n* (%)	47 (74.6)	32 (50.0)	0.006
Diabetes mellitus, *n* (%)	14 (22.2)	6 (9.4)	0.054
Dyslipidemia, *n* (%)	30 (47.6)	29 (45.3)	0.860
IHD, *n* (%)	7 (11.1)	5 (7.8)	0.560
Stroke, *n* (%)	9 (14.3)	2 (3.1)	0.030
CKD, *n* (%)	28 (44.4)	13 (20.3)	0.005
Smoking habit, *n* (%)	9 (14.3)	23 (35.9)	0.007
Alcohol consumption, *n* (%)	18 (28.6)	31 (48.4)	0.029
Apolipoprotein E ε4 carrier, *n* (%)	22 (34.9)	16 (25.0)	0.249
Dementia, *n* (%)	30 (47.6)	3 (4.7)	<0.0001
Comprehensive geriatric assessment			
Barthel Index	100, 95–100	100, 100–100	0.009
IADL impairment, *n* (%)	41 (65.1)	17 (26.6)	<0.0001
DBDS	12, 5–18.3	6, 3–11.8	0.0002
GDS	3, 1–5	2, 1–5	0.578
ZBI	15, 6–24	7, 3–19	0.007
MNA-SF	12, 11–13	13, 12–14	0.015
Cognitive function			
MMSE	22, 18–24	27.5, 24–29	<0.0001
CDR-GB			<0.0001
0, *n* (%)	2 (3.2)	21 (32.8)	
0.5, *n* (%)	43 (68.3)	42 (65.6)	
≥1, *n* (%)	18 (28.6)	1 (1.6)	
CDR-SB	3.5, 2–4.5	0.5, 0.5–1.9	<0.0001
ADAS-cog	13.2, 8–17.3	6.3, 4.7–10	<0.0001
RCPM	24.5, 20.8–28	31, 28–33.5	<0.0001
FAB	10, 8–11	13, 10–14	<0.0001
LM-WMSR I	4, 2–9	12, 7–21	<0.0001
LM-WMSR II	0, 0–3	7, 1–14	<0.0001
Brain MRI finding			
SLI, *n* (%)	11 (17.5)	2 (3.1)	0.009
WMH, *n* (%)	22 (34.9)	12 (18.8)	0.047
CMB, *n* (%)	20 (31.8)	8 (12.5)	0.011
EPVS, *n* (%)	43 (68.3)	53 (82.8)	0.065
VSRAD	1.6, 0.9–2.3	0.8, 0.5–1.2	<0.0001
SPECT			
Low blood flow*	44 (73.3)	41 (68.3)	0.688
Arterial stiffness			
Ankle brachial index	1.11, 1.04–1.15	1.08, 1.06–1.15	0.842
Pulse wave velocity, m/s	18.0, 16.1–20.5	19.3, 15.8–23.3	0.275
Laboratory finding			
Total protein, g/dL	7.2, 6.8–7.5	7.4, 7.0–7.6	0.048
Albumin, g/dL	4.1, 3.9–4.3	4.3, 4.1–4.4	0.007
WBC	58.0, 47.0–73.0	56.5, 49.0–67.5	0.608
CRP, mg/dL	0.06, 0.03–0.12	0.05, 0.02–0.13	0.292
eGFR, mL/min/1.73 m^2	60.9, 50.2–72.3	72.4, 61.3–84.8	<0.0001
HbA1c, %	5.8, 5.6–6.2	5.7, 5.5–6.0	0.152

Data are represented as medians, interquartile ranges, or number of patients (%). Wilcoxon rank-sum test and χ^2 test were used. *Blood flow reduction in the posterior cingulate gyrus and/or precuneus, as seen in SPECT images. ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; BP, blood pressure; CDR-GB, Clinical Dementia Rating Global Score; CDR-SB, Clinical Dementia Rating-sum of boxes; CKD, chronic kidney disease; CMB, cerebral microbleed; CRP, C-reactive protein; DBDS, Dementia Behavior Disturbance Scale; eGFR, estimated glomerular filtration rate; EPVS, enlarged periventricular space; FAB, Frontal Assessment Battery; GDS, Geriatric Depression Scale; HbA1c, hemoglobin A1c; IADL, instrumental activities of daily living; IHD, ischemic heart disease; LM-WMSR, Logical Memory subtests I and II of the Wechsler Memory Scale-Revised; MMSE, Mini-Mental State Examination; MNA-SF, Mini-Nutritional Assessment-Short Form; MRI, magnetic resonance imaging; RCPM, Raven's Coloured Progressive Matrices; SLI, silent lacunar infarct; SPECT, single-photon emission-computed tomography; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease; WBC, white blood cells; WMH, white matter hyperintensity; ZBI, Zarit Caregiver Burden Interview.

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Supplementary Table 2
Correlations between the natural logarithm-transformed neurofilament light chain (Ln-NfL) and dementia-related items and gut microbiome metabolites, unadjusted and adjusted for age

Ln-NfL	Unadjusted β, 95% CI *p*	Adjusted for age β, 95% CI *p*
MMSE	–0.055, –0.072—0.038 <0.0001	–0.036, –0.051—0.020 <0.0001
CDR-SB	0.131, 0.096–0.165 <0.0001	0.030, 0.068–0.128 <0.0001
ADAS-cog	0.040, 0.027–0.054 <0.0001	0.030, 0.018–0.042 <0.0001
FAB	–0.010, –0.124—0.069 <0.0001	–0.055, –0.084—0.027 0.0002
VSRAD	0.296, 0.198–0.394 <0.0001	0.198, 0.113–0.284 <0.0001
F/B	0.032, –0.002–0.066 0.068	0.013, –0.015–0.041 0.364
CRP	–0.03, –0.240–0.183 0.793	–0.011, –0.180–0.158 0.895
JDI_{12}	–0.063, –0.116—0.011 0.018	–0.064, –0.110—0.018 0.007
Ammonia, mg/g	0.079, –0.114–0.272 0.418	0.013, –0.142–0.168 0.870
Succinic acid, mg/g	–0.006, –0.021–0.009 0.422	–0.011, –0.022—0.001 0.077
Lactic acid, mg/g	0.0003, –0.002–0.003 0.812	0.0013, –0.001–0.003 0.173
Formic acid, mg/g	–0.011, –0.126–0.105 0.858	–0.047, –0.139–0.045 0.316
Acetic acid, mg/g	0.002, –0.006–0.009 0.685	0.0018, –0.004–0.008 0.559
Propionic acid, mg/g	0.0014, –0.019–0.022 0.894	0.017, –7.463e–5–0.033 0.051
Iso-butyric acid, mg/g	0.0072, –0.116–0.130 0.908	–0.015, –0.113–0.084 0.768
n-Butyric acid, mg/g	0.019, –0.093–0.130 0.739	0.026, –0.063–0.115 0.557
Iso-valeric acid, mg/g	0.210, –0.129–0.550 0.222	0.016, –0.261–0.292 0.911
n-Valeric acid, mg/g	0.0032, –0.041–0.048 0.889	–0.0013, –0.037–0.034 0.944
Phenol, μg/g	–0.0049, –0.017–0.007 0.429	0.001, –0.009–0.010 0.895
P-cresol, μg/g	0.0006, –0.0003–0.002 0.218	–2.773e–5, –0.001–0.001 0.946
4-Ethylphenol, μg/g	0.0047, –0.034–0.043 0.809	0.012, –0.018–0.043 0.435
Indoles, μg/g	0.0027, –0.003–0.008 0.313	0.0023, –0.002–0.007 0.285
Skatole, μg/g	0.0034, –0.002–0.009 0.211	–0.001, –0.005–0.003 0.674

Data are represented as β and 95% confidence intervals. Linear regression analyses were employed. ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; CDR-SB, Clinical Dementia Rating-sum of boxes; CI, confidence interval; CRP, C-reactive protein; FAB, Frontal Assessment Battery; F/B, Firmicutes/Bacteroidetes ratio; JDI_{12}, Japanese Diet Index; MMSE, Mini-Mental State Examination; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease.

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Supplementary Table 3
Comparison of dietary compositions between participants with high plasma neurofilament light chain (NfL) concentrations and those with low concentrations

	High NfL ($n = 63$)	Low NfL ($n = 64$)	p
Food			
Rice, *n* (%)	38 (100)	44 (95.7)	0.499
Bread, *n* (%)	24 (63.2)	33 (71.7)	0.484
Noodles, *n* (%)	11 (29.0)	18 (39.1)	0.364
Miso, *n* (%)	22 (57.9)	33 (71.7)	0.250
Fish and shellfish, *n* (%)	16 (42.1)	33 (71.7)	0.008
Vegetables, *n* (%)	36 (94.7)	45 (97.8)	0.587
Seaweed, *n* (%)	17 (44.7)	25 (54.4)	0.511
Pickles, *n* (%)	22 (57.9)	30 (65.2)	0.508
Mushrooms, *n* (%)	15 (39.5)	30 (65.2)	0.028
Fruit, *n* (%)	28 (73.7)	41 (89.1)	0.088
Beef and pork, *n* (%)	25 (65.8)	33 (71.7)	0.638
Chicken, *n* (%)	19 (50.0)	30 (65.2)	0.187
Soybeans and soybean-derived foods, *n* (%)	27 (71.1)	43 (93.5)	0.008
Milk and dairy products, *n* (%)	28 (73.7)	40 (87.0)	0.165
Green tea, *n* (%)	26 (68.4)	31 (67.4)	1.000
Coffee, *n* (%)	23 (60.5)	37 (80.4)	0.055
Cooking method			
Boiling, *n* (%)	32 (84.2)	40 (87.0)	0.762
Steaming, *n* (%)	4 (10.5)	6 (13.0)	1.000
Raw, *n* (%)	27 (71.1)	44 (95.7)	0.002
Deep-frying, *n* (%)	12 (31.6)	18 (39.1)	0.502
Frying, *n* (%)	24 (63.2)	29 (63.0)	1.000
Use of soup stock, *n* (%)	35 (92.1)	44 (95.7)	0.654
Use of fermented seasoning, *n* (%)	36 (94.7)	46 (100.0)	0.202

Data are represented as the number of patients (%). Wilcoxon rank-sum test and χ^2 test were used.

Supplementary Table 4
Comparison of the gut microbiota and their metabolites between participants with high neurofilament light chain (NfL) concentrations and those with low concentrations

	High NfL ($n = 63$)	Low NfL ($n = 64$)	p
Gut microbiome			0.314
Enterotype I, *n* (%)	22 (34.9)	25 (39.0)	
Enterotype II, *n* (%)	1 (1.6)	4 (6.3)	
Enterotype III, *n* (%)	40 (63.5)	35 (54.7)	
F/B ratio	1.53, 0.99–2.67	1.44, 0.93–2.28	0.509
Metabolite			
Ammonia, mg/g	0.60, 0.27–0.97	0.64, 0.35–0.94	0.852
Succinic acid, mg/g	0.03, 0.03–0.39	0.03, 0.03–0.12	0.446
Lactic acid, mg/g	0.03, 0.03–0.15	0.03, 0.03–0.09	0.687
Formic acid, mg/g	0.05, 0.05–0.05	0.05, 0.05–0.05	0.224
Acetic acid, mg/g	3.52, 0.03–7.33	2.20, 0.03–6.39	0.430
Propionic acid, mg/g	0.56, 0.03–1.92	0.44, 0.03–1.47	0.749
Iso-butyric acid, mg/g	0.10, 0.03–0.21	0.06, 0.03–0.18	0.379
n-butyric acid, mg/g	0.15, 0.03–0.73	0.12, 0.03–0.69	0.728
Iso-valeric acid, mg/g	0.03, 0.03–0.32	0.03, 0.03–0.26	0.877
n-valeric acid, mg/g	0.22, 0.03–2.57	0.29, 0.03–1.99	0.860
Phenol, μg/g	1.08, 0.0003–2.15	0.79, 0.0003–1.79	0.396
P-cresol, μg/g	0.25, 0.0003–67.03	0.19, 0.0003–86.06	0.593
4-Ethylphenol, μg/g	0.0003, 0.0003–0.82	0.08, 0.0003–0.91	0.873
Indoles, μg/g	0.37, 0.0003–30.70	1.92, 0.03–23.88	0.961
Skatole, μg/g	0.0003, 0.0003–0.17	0.0003, 0.0003–3.73	0.259

Data are represented as the median, interquartile range or the number of patients (%). Wilcoxon rank-sum test and the χ^2 test were used.Enterotype I: *Bacteroides* > 30%, enterotype II: *Prevotella* > 15%, enterotype III: others. F/B ratio, Firmicutes/Bacteroidetes ratio.

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Supplementary Table 5
Comparison of clinical biomarkers between participants with dementia and those without; participants with enterotype I and those without; and participants with mild cognitive impairment (MCI) and normal cognition (NC) among participants without dementia

	Dementia (+) ($n=34$)	Dementia (–) ($n=94$)	p
Plasma NfL	33.2, 26.5–49.4	21.3, 15.5–27.2	<0.001
Serum CRP, mg/dL	0.06, 0.03–0.12	0.05, 0.02–0.13	0.791
F/B ratio	2.07, 1.26–2.95	1.38, 0.81–2.25	0.013

	Enterotype I (+) ($n=47$)	Enterotype I (–) ($n=81$)	p
Plasma NfL	23.4, 17.9–36.4	24.3, 19.0–34.0	0.621
Serum CRP, mg/dL	0.06, 0.02–0.10	0.05, 0.03–0.14	0.785
F/B ratio	0.77, 0.60–1.19	2.14,1.52–3.14	<0.001

	MCI ($n=71$)	NC ($n=23$)	p
Plasma NfL,	23.4, 17.6–33.3	18.2, 12.8–20.8	<0.001
Serum CRP, mg/dL	0.06, 0.02–0.14	0.03, 0.02–0.08	0.210
F/B ratio	1.25, 0.70–2.25	1.65, 1.11–2.26	0.082

Data are represented as the median, interquartile range. Wilcoxon rank sum test and χ^2 test were used. Enterotype I: *Bacteroides* > 30%. CRP, C-reactive protein; F/B ratio, Firmicutes/Bacteroidetes ratio; NC, normal cognition; NfL, neurofilament light chain; MCI, mild cognitive impairment.

Supplementary Table 6
Comparison of the prevalence of dementia stratified by plasma neurofilament light chain (NfL) concentrations and the presence or absence of dementia-related items

	NfL high		NfL low	
	risk (+)	risk (–)	risk (+)	risk (–)
Age (risk: higher age), *n* (%)	13 (33.3)	15 (68.2)	3 (12.0)	0 (0)
Sex (risk; women), *n* (%)	24 (55.8)	4 (22.2)	3 (10.0)	0 (0)
Education years (risk; fewer), *n* (%)	16 (53.3)	12 (38.7)	3 (6.0)	0 (0)
APOE ε4 carrier, *n* (%)	16 (76.2)	12 (30.0)	1 (6.3)	2 (4.2)
Smoking habit, *n* (%)	3 (33.3)	25 (48.1)	0 (0)	3 (7.3)
Alcohol consumption, *n* (%)	9 (50.0)	19 (44.2)	1 (3.2)	2 (6.1)
Hypertension, *n* (%)	19 (42.2)	9 (56.3)	3 (9.4)	0 (0)
Diabetes mellitus, *n* (%)	6 (50.0)	22 (44.9)	0 (0)	3 (5.2)
Dyslipidemia, *n* (%)	15 (51.7)	13 (40.6)	2 (6.9)	1 (2.9)
Ischemic heart disease, *n* (%)	2 (40.0)	26 (46.4)	0 (0)	3 (5.1)
Stroke, *n* (%)	2 (28.6)	26 (48.2)	0 (0)	3 (4.8)
Chronic kidney disease, *n* (%)	12 (46.2)	16 (45.7)	0 (0)	3 (5.9)
Silent lacunar infarct, *n* (%)	6 (66.7)	22 (42.3)	0 (0)	3 (4.8)
White matter hyperintensity, *n* (%)	7 (33.3)	21 (52.5)	1 (8.3)	2 (3.9)
Cerebral microbleed, *n* (%)	11 (57.9)	17 (40.5)	1 (12.5)	2 (3.6)
Enlarged periventricular space, *n* (%)	8 (40.0)	20 (48.8)	1 (9.1)	2 (3.8)
VSRAD, *n* (%)	22 (53.7)	5 (27.8)	3 (14.3)	0 (0)
SPECT*, *n* (%)	22 (51.2)	5 (33.3)	2 (4.9)	0 (0)
ABI (risk; lower), *n* (%)	13 (52.0)	9 (37.5)	1 (3.7)	2 (9.5)
PWV (risk; higher), *n* (%)	12 (48.0)	10 (41.7)	2 (7.4)	1 (4.8)
MNA-SF (risk; lower), *n* (%)	18 (51.4)	10 (38.5)	1 (3.7)	2 (5.4)
Enterotype I, *n* (%)	4 (18.2)	24 (61.5)	1 (4.0)	2 (5.1)
Enterotype III, *n* (%)	24 (63.2)	4 (17.4)	2 (5.7)	1 (3.5)
F/B ratio (risk; higher), *n* (%)	18 (58.1)	10 (33.3)	1 (3.5)	2 (5.7)
JDI_{12} (risk; lower), *n* (%)	6 (40.0)	11 (52.4)	0 (0)	3 (20.0)

Numbers and percentages indicate the number of the patients with dementia and the prevalence of dementia stratified by plasma NfL concentrations and the presence or absence of dementia-related items. The presence or absence of dementia-related items was categorized using the median value of the continuous variables and was based on our previous findings (i.e., being a woman, higher age, higher VSRAD score, higher pulse wave velocity, higher F/B ratio, fewer years of education, lower Mini-Nutritional Assessment-Short Form score, lower ankle brachial index, lower JDI_{12} score, and having categorical items). *Blood flow reduction in the posterior cingulate gyrus and/or precuneus, as seen in SPECT images. ABI, ankle brachial index; JDI, Japanese Diet Index; MNA-SF, Mini-Nutritional Assessment-Short Form score; PWV, pulse wave velocity; SPECT, single-photon emission-computed tomography; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease.

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Supplementary Table 7
Multivariable logistic regression analyses for the presence of dementia

	OR	95% CI	*p*
Plasma NfL, per 1 pg/mL			
Model 1	1.05	1.02–1.08	<0.001
Model 2	1.06	1.02–1.09	<0.001
Model 3	1.07	1.03–1.11	<0.001
Model 4	1.04	1.00–1.09	<0.001
High concentration of plasma NfL (versus low)*			
Model 1	17.9	5.80–78.6	<0.001
Model 2	17.8	4.99–90.6	<0.001
Model 3	18.5	5.13–94.5	<0.001
Model 4	9.94	2.75–48.2	<0.001

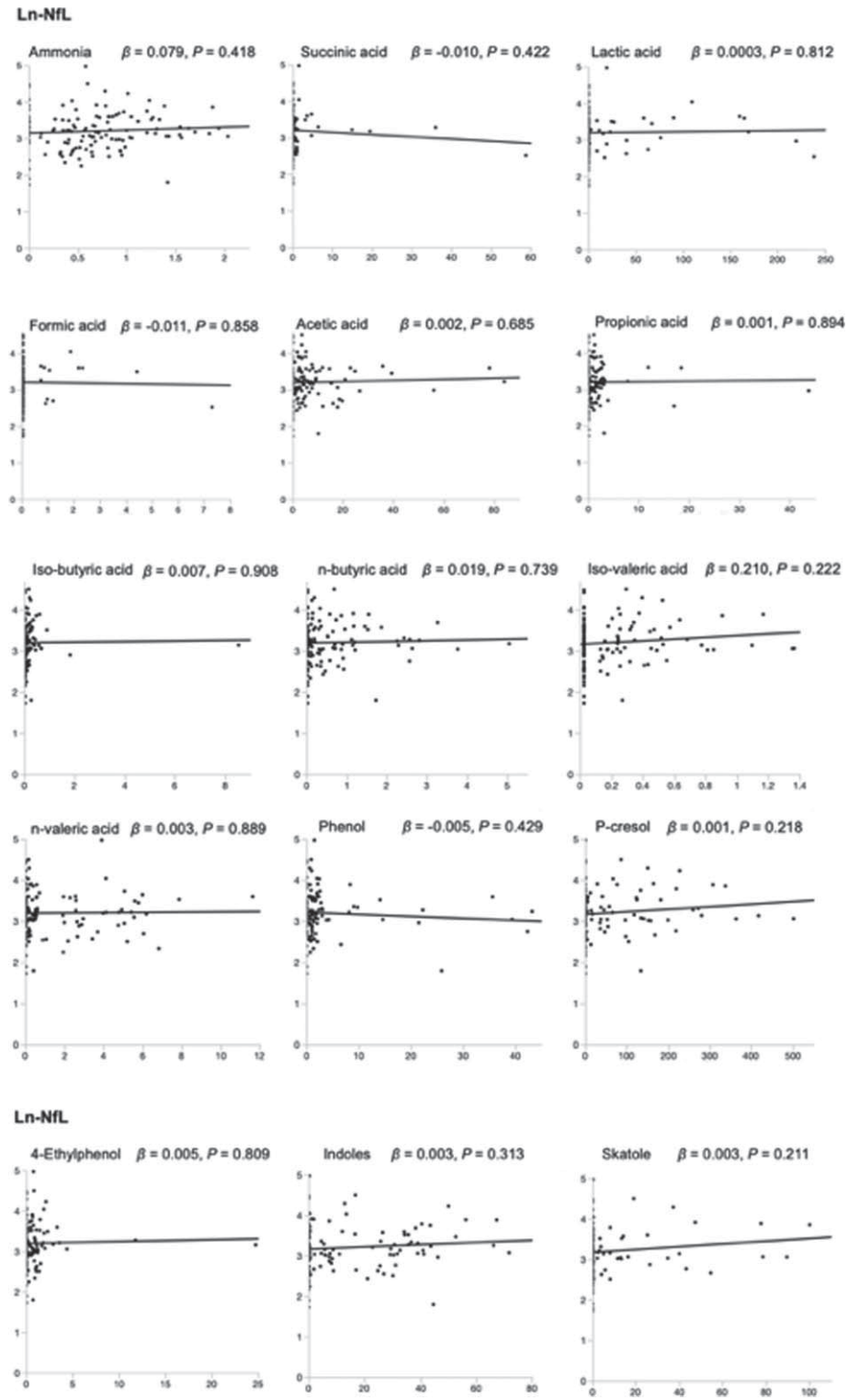
The dependent variable was the presence of dementia. *Participants were allocated to two groups according to plasma NfL concentrations: a high NfL group, if the NfL value was above the median; and a low NfL group, if the value was below the median. Model 1: univariate analyses. Model 2: adjusted for age and sex. Model 3: backward stepwise multivariable logistic regression analyses adjusted for model 2 factors, years of education, and risk factors such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking, alcohol consumption, and apolipoprotein E. Model 4: backward stepwise multivariable logistic regression analyses adjusted for model 3 factors, enterotype I (*Bacteroides* > 30%), Firmicutes/Bacteroidetes ratio, silent lacunar infarct, white matter hyperintensity, cerebral microbleeds, enlarged perivascular space, score of the voxel-based specific regional analysis system for Alzheimer's disease, single-photon emission-computed tomography findings (presence or absence of low blood flow in the area of the posterior cingulate gyrus and/or precuneus). CI, confidence interval; NfL: neurofilament light chain; OR, odds ratio.

Supplementary Table 8
Multivariable logistic regression analyses for the presence of dementia

	OR	95% CI	*p*
Plasma NfL*	1.26	0.94–1.79	0.139
Enterotype I	0.12	0.03–0.39	<0.001
Alcohol consumption	0.39	0.12–1.14	0.088
CMBs	3.43	1.11–10.8	0.033
VSRAD	3.42	1.69–7.66	<0.001

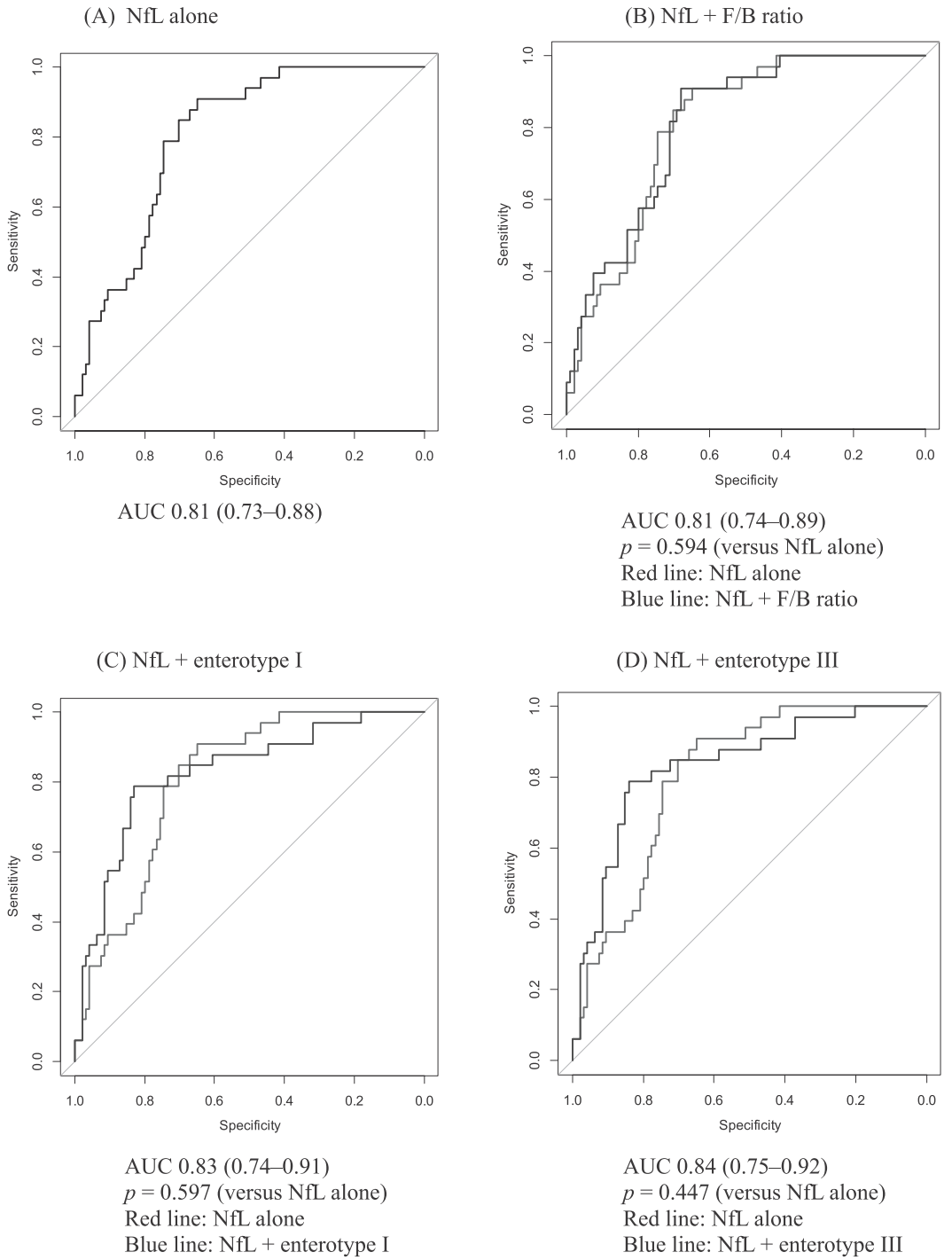
The dependent variable was the presence of dementia. *Per 10 pg/mL. Model: backward stepwise multivariable logistic regression analyses adjusted for age, sex, years of education, and risk factors such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking, alcohol consumption, apolipoprotein E, enterotype I (*Bacteroides* > 30%), Firmicutes/Bacteroidetes ratio, silent lacunar infarct, white matter hyperintensity, cerebral microbleeds, enlarged perivascular space, score of the voxel-based specific regional analysis system for Alzheimer's disease, single-photon emission-computed tomography findings (presence or absence of low blood flow in the area of the posterior cingulate gyrus and/or precuneus). CI, confidence interval; NfL: neurofilament light chain; OR, odds ratio.

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Supplementary Figure 1. Comparison of the concentrations of plasma neurofilament light chain (NfL) and gut microbiome metabolites. The x-axes show the microbiome metabolites. The *y*-axes show the concentrations of plasma NfL. Single regression analysis.

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Supplementary Figure 2. Comparison of the area under the receiver operating characteristic curve among (A) NfL alone, (B) NfL + F/B ratio, (C) NfL + enterotype I, and (D) NfL + enterotype III.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Influence of the Microbiota-Gut-Brain Axis on Cognition in Alzheimer's Disease

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Abstract. The gut microbiota is made up of trillions of microbial cells including bacteria, viruses, fungi, and other microbial bodies and is greatly involved in the maintenance of proper health of the host body. In particular, the gut microbiota has been shown to not only be involved in brain development but also in the modulation of behavior, neuropsychiatric disorders, and neurodegenerative diseases including Alzheimer's disease. The precise mechanism by which the gut microbiota can affect the development of Alzheimer's disease is unknown, but the gut microbiota is thought to communicate with the brain directly via the vagus nerve or indirectly through signaling molecules such as cytokines, neuroendocrine hormones, bacterial components, neuroactive molecules, or microbial metabolites such as short-chain fatty acids. In particular, interventions such as probiotic supplementation, fecal microbiota transfer, and supplementation with microbial metabolites have been used not only to study the effects that the gut microbiota has on behavior and cognitive function, but also as potential therapeutics for Alzheimer's disease. A few of these interventions, such as probiotics, are promising candidates for the improvement of cognition in Alzheimer 's disease and are the focus of this review.

Keywords: Alzheimer's disease, cognition, microbiota-gut-brain axis, mild cognitive impairment

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial, progressive, neurodegenerative disease and the most common form of dementia. Dementia is a general term for the mental decline that effects a person's ability to make decisions, think clearly, impairs memory, and causes changes in mood; AD is distinguished from other forms of dementia by the accumulation of extracellular amyloid-β (Aβ) fibers and intracellular neurofibrillary tangles (NFTs) which are thought to contribute to neuronal death and eventual symptom development. The strongest risk factor is advanced age as most cases are diagnosed in people over the age of 65; however, it is also known that genetic factors, i.e., *apolipoprotein* E (APOE), and environmental factors, i.e., air pollution exposure, contribute to disease [1–3]. Currently, the mechanism by which Aβ and NFT accumulation initially occurs is unknown. The current prevailing hypothesis is that lifelong exposures in a genetically susceptible person contribute to this phenomenon.

Recent evidence has shed light on a new potential contributor to AD development and progression: microbes (reviewed in [4, 5]). Spirochetes and the oral pathogen *Porphyromonas ginigvalis* have been found to be associated with Aβ plaques in postmortem

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brains of AD patients and AD animal models [6–10]. *In vitro* and *in vivo* evidence have both demonstrated that Aβ can act as an antimicrobial peptide, which has led to the hypothesis that Aβ accumulates in the brain in an effort to clear microbes that have penetrated the blood-brain barrier and entered the brain [11, 12]. The Gram negative bacterial membrane protein lipopolysaccharide (LPS) has also been found to be more prevalent in AD patient brains as compared to non-AD brains and can also trigger Aβ accumulation [13–15]. In examining other organ systems with bacterial alterations, AD patients have an altered gastrointestinal (GI) microbiota, termed dysbiosis, as compared to non-AD patients [16–18]. Transgenic animal models of AD also experience GI dysbiosis and AD mouse models that lack a gut microbiota have reduced amyloid plaques [19–21]. Furthermore, AD mouse models treated with antibiotics have reduced AD characteristics such as amyloid plaques and cognitive dysfunction related to memory [20, 22–24]. These studies strengthen the proposed relationship between the gut microbiota and the brain, specifically in regards to AD. Additionally, bacterial and amyloid proteins are thought to cross-seed resulting in heterogeneous amyloid formations. By acting as prions and self-seeding, infectious amyloids essentially spread their infectivity by eliciting additional amyloid conformations among other molecules. The eventual aggregation of these misfolded proteins results in neurodegeneration. Curli proteins from various bacterial species are an example of a microbial-derived protein with the ability to cross-seed amyloid formation [25]. Current understanding of prion cross-seeding, microbial amyloids, and their role in AD specifically is limited, but this is an area of growing interest due to the possibility of gut microbial molecules to perpetrate amyloid formation [26]. In this review, we will highlight the current state of the science regarding resident microbes and cognition in AD patients.

COGNITION IN AD

Cognitive health is a spectrum, and although some individuals maintain cognitive acuity with advanced age, cognitive decline is considered a common sign of aging [27]. Mild cognitive impairment (MCI) is a prodrome stage of AD, falling between normal cognitive function based on age and education-level and severe impairment due to AD dementia. Diagnostic criteria for MCI include subjective cognitive complaint, objective cognitive impairment, preserved independence in functional abilities, and no dementia. Although MCI does not always progress to AD, the cognitive difficulties commonly experienced by those suffering from MCI provide a preview of what is to come with an AD diagnosis [28]. The Mini-Mental State Examination (MMSE) is commonly implemented to assess cognitive function by testing orientation, attention, memory, language, and visual-spatial skills [29]. Typically, cognitive testing is used in conjunction with patient and family interviews and laboratory and brain-imaging tests to make an AD diagnosis [29]. Similar methods will be discussed throughout this review as varied degrees of cognitive function are explored.

Cerebral inflammation develops in AD patients years before disease onset is clinically apparent. During this asymptomatic time, Aβ plaques and activated microglial cells accumulate and contribute to the initial inflammation [30]. At first, neurological accumulation of Aβ proteins and NFTs are asymptomatic, but they later become critical hallmarks of AD brains. Aβ and NFTs in the frontal cortex and hippocampal regions of the brain decrease active neurons, resulting in a loss of function, leading to the cognitive impairment and memory deficits observed in AD patients. It is known that aberrant Aβ and NFTs are the underlying causes of AD, but why these proteins accumulate has yet to be established.

MICROBIOTA-GUT-BRAIN AXIS

There is a wealth of literature characterizing the bidirectional communication of the gut and the brain through various mechanisms [31]. The main communication pathways between the gut and the brain include: direct communication via the vagus nerve and indirect communication through the secretion of cytokines and neuroendocrine hormones to activate the immune and endocrine system [31]. Microbes that reside in the gut can also play a major role in the communication between the gut and brain, leading to the newly refined microbiota-gut-brain axis. These bacteria are able to produce substances that directly act on the enteric nervous system and vagus nerve, as well as substances that can contribute to indirect communication via activation of the immune system. Signaling molecules that are derived from or influenced by the gut microbiota include but are not limited to bacterial structural components, short-chain fatty acids (SCFAs), and

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neuroactive molecules. It is critical that we work to understand these signaling molecules and their relationship to cognition and AD in the presence of gut dysbiosis.

Gut dysbiosis refers to alterations of the resident bacterial populations of the gut, which can result from dietary changes, stress, medications, and alcohol or chemical consumption. Unbalanced bacterial populations may resolve themselves; however, persistent stressors can cause chronic dysbiosis, which is implicated in the development of inflammatory bowel disease, autism spectrum disorders, obesity, diabetes, cancer, and AD representing its vast pathophysiological outcomes [32–34]. The main consequences of gut dysbiosis are the production of noxious substances (e.g., lipopolysaccharide and trimethylamine) leading to an inflammatory response and an altered secretion of signaling molecules such as SCFAs [33, 35, 36]. Uncontrolled inflammation may induce aberrant immune activation, tissue destruction, and eventual loss of function. It can also cause malfunction of cell barriers. By increasing the permeability of the gastrointestinal epithelial layers, inflammation can cause dysregulation of local epithelial layers and allow microbial byproducts to escape the gut and enter the bloodstream. Furthermore, altered levels of signaling molecules such as SCFAs are problematic as they play important roles in the host [37]. SCFAs can induce gluconeogenesis, reduce inflammation, and directly interact with vagal afferents [38–40]. Additionally, circulating levels of SCFAs can cross the blood-brain barrier (BBB), with measurable amounts in the human brain [41, 42].

As gut dysbiosis can cause increased gut permeability and potentially increase the BBB permeability via altered SCFA secretion, systemic inflammation, and secretion of noxious compounds, this could lead to the development of cognitive impairment [31]. Systemic inflammation has been associated with reduced cognitive function specifically with short term memory and verbal learning and memory [43]. Researchers studying the relationship between brain amyloidosis and gut microbiota taxa found cognitively impaired patients with amyloidosis expressed greater levels of pro-inflammatory cytokines in blood compared to cognitively impaired patients without amyloidosis and healthy controls. Cognitively impaired patients with amyloidosis experienced varied taxa abundances compared to those without amyloidosis and controls; differences were determined via stool sampling and included lower abundance of *Eubacterium rectale* and higher abundances of *Escherichia* and *Shigella.* The enhanced pro-inflammatory and reduced anti-inflammatory taxon abundances associated with cognitively impaired patients suffering from amyloidosis supports the association between the gut-microbiota, inflammation, and brain amyloidosis [44]. Interestingly, a study of gut microbiota alterations experienced by AD versus MCI patients found no genera differences between AD and MCI patients [45]. However, 11 genera from feces and 11 from blood differed between AD patients and normal controls. These findings suggest that patients suffering from AD and MCI share similar gut microbiota profiles, indicating gut dysbiosis, may originate years before AD onset [45]. This research has the potential to have diagnostic implications, as differences in microbiota profiles could provide biomarker targets for earlier and noninvasive detection of AD [45]. Systemic inflammation can also lead to an activation of the brain-resident macrophages also known as microglia cells, which have been associated with the development of AD. Microglial cells in AD are thought to play a role in synapse loss, worsening tau pathology, and release of pro-inflammatory cytokines which can further damage neurons [46]. This can lead to reduced or altered cognitive function. Furthermore, as SCFAs have been associated with increased brain derived neurotrophic factor (BDNF) expression, a critical gene for neurogenesis and synaptic plasticity, and can also act to decrease inflammation, altered SCFA levels caused by gut dysbiosis could exacerbate the damage caused by systemic inflammation in the brain [38, 47, 48].

The various communication pathways between the gut microbiota and CNS demonstrate the complexities of their interrelationship, while also offering promising avenues for further investigation. Potential therapeutics to improve cognitive function in AD by targeting the gut microbiota have been studied using the following methods: germ-free mice, fecal microbiota transfer, humanized gnotobiotic mice, and probiotic supplementation.

GERM-FREE MICE

In order to understand the effects of the gut microbiota on the brain, several studies utilize mice free of bacteria, known as germ free (GF) mice [49]. GF mice and specific pathogen free mice (SPF), mice free of specific pathogens also sometimes referred to as conventional mice, allow researchers to determine the influence of the gut microbiota on behavior

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and the brain. GF mice display lower anxiety-like behavior measured by more time spent in light versus dark boxes and more time spent in the open arms of the elevated plus mazes compared to SPF mice [50, 51]. Similarly, in open field tests, GF mice spent more time than SPF mice in the center of the field instead of the periphery, further indicating reduced anxiety-like behavior among GF animals [52]. Interestingly, the introduction of microbiota from SPF mice thirty days before mating normalized some behaviors of the progeny as compared to SPF mice; however, this was not the case in adult GF mice introduced to microbiota from SPF mice [50]. Depending on the mouse strain, the differences in anxiety-like behaviors between GF and SPF mice do not always hold true. For example, C57BL/6J mice that are SPF spent more time in the center of the open field test than GF at 4 weeks of age, but this was not the case at 12 weeks. Furthermore, in C57BL/6J mice there were no differences detected in the time spent in the open and closed arms of the elevated plus maze between GF and SPF mice at 4 or 12 weeks [53]. In regards to memory, the C57BL/6J SPF mice demonstrated greater spatial and learning memory than GF mice as measured by a Morris water maze (MWM) test and had better contextual memory as measured by fear conditioning [53]. The behavioral differences between GF and SPF mice are also accompanied by neurochemical and structural differences in the brain. For example, BDNF, which is neuroprotective and promotes synaptic growth, is reduced in the amygdala and hippocampus of GF mice as compared to SPF mice [48, 50, 52, 54, 55]. GF mice also display decreased brain volume and myelination as compared to SPF mice, indicating less brain maturation [53].

GF mice display higher BBB permeability as compared to SPF mice [56]. However, colonization of the gut of GF mice with a single strain of bacteria, *Clostridium tyrobutyricum,* whose main metabolite is butyrate, or *Bacteroides thetaiotaomicron,* whose main metabolites are acetate and propionate, improved BBB permeability. When treated specifically with the SCFA sodium butyrate, the GF mice displayed decreased BBB permeability and increased protein levels of occludin-5 in the frontal cortex and hippocampus [56].

The use of GF mice to study the relationship between the gut microbiota and the brain is popular, though this method comes with challenges, as maintaining GF mice is a labor-intensive and costly process requiring specialized equipment and well-trained personnel [57]. Therefore, many researchers focus on probiotic interventions or fecal microbiota transfer (FMT) to better understand the gut microbiota-brain interaction and potential therapeutics for neuropsychiatric and neurodegenerative disorders, including AD.

FECAL MICROBIOTA TRANSFER AND HUMANIZED GNOTOBIOTIC MICE

FMT is the transfer of fecal bacteria from one donor to another. In animal models, the FMT recipient is typically a GF mouse. This allows researchers to determine the effect that the gut microbiota can have on the host. In humans, FMT is frequently used to treat *Clostridium difficile* (CDI), which causes diarrhea and intestinal inflammation, as FMT is thought to help re-establish a healthy gut microbiota [58]. FMT is receiving further consideration as a treatment option for diseases such as irritable bowel syndrome, autism, psychiatric disorders, and Parkinson's disease, though reports of success vary [59–61]. Regarding Parkinson's disease specifically, a recent animal study demonstrated that a FMT from control mice to an induced Parkinson's disease mouse model improved bradykinesia and reflexes in the FMT-recipient mice. Conversely, control mice treated with a FMT from the Parkinson's disease mouse model displayed reduced movement and reflexes accompanied by lower levels of dopamine and serotonin in the striatum [61]. In the context of AD, a recent case study showed that an elderly man with AD had improved cognitive function when treated with a FMT for CDI at 2 and 6 months post treatment [62]. While this is a single case study, it displays great promise for the future treatment of AD with FMT.

Another relevant animal model is the humanized gnotobiotic mouse model which involves a FMT from humans to GF mice. Post-transplant, the mice incorporate the human microbiota into their systems, providing a powerful tool for researchers to study the human gut microbiota and various diet and therapeutic interventions [63]. The humanized gnotobiotic mouse model has been studied since the 1980s, but additional research must be conducted, as behavioral and cognitive function assessments with this animal model have only recently commenced [63, 64]. Specifically, in 2016, FMT recipient GF Kunming mice from patients with major depressive disorder displayed increased depressive-like behaviors as measured with a forced swim test as compared to FMT recipient GF Kunming from healthy con-

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trol volunteers [65]. More recently, FMT recipient C57BL/6J mice from patients with schizophrenia displayed impaired memory and recognition memory as measured by a Barnes maze and novel object recognition test. However, these were not GF mice but were treated with antibiotics, which depleted most of their gut microbiota [66].

PROBIOTICS

Probiotics are defined as "live microorganisms that confer a health benefit to the host when consumed in adequate amounts" [67]. Probiotic intervention involves the administration of live mono or mixed bacteria cultures to humans or animals, which can alter the host gut microbiota and/or produce substances that benefit the host. Several bacterial genera have been used as probiotics, including, but not limited to, *Lactobacillus, Bacteroides, Bifidobacterium, Bacillus,* and *Streptococcus* [68]. Such genera have been used extensively to treat various gastrointestinal conditions, such as diarrhea, constipation, and irritable bowel syndrome, supporting the notion that probiotic consumption is associated with improved immune health, bowel disease prevention, and antihypertensive effects [69]. Additional studies have found treatment of fibromyalgia patients with multispecies probiotics targeting gut microbial imbalances improved cognitive deficits (i.e., attention) associated with the syndrome [70]. The beneficial effects of probiotics are well established, and although their mechanism of action remains unknown, they provide a potential strategy to restore gut microbial communities and diminish inflammation induced by disease and environmental exposures [71].

One of the first *in vivo* studies to measure the effect of probiotics on behavior involved treating the drinking water of Sprague Dawley rats with a single strain of *Bifidobacteria infantis* for 14 days. The probiotic treatment did not elicit antidepressant effects, as no changes were found in forced swim test swimming behaviors. However, probiotic treatment caused an increase in blood plasma tryptophan levels, a precursor to serotonin (5-HT). Still, this increase in tryptophan failed to translate into an increase in 5-HT or any other monoamine measured in the brain (e.g., noradrenaline and dopamine) [72]. Alternatively, a 12-week clinical study with a randomized, double-blinded, and placebo-controlled intervention administering one probiotic strain, *Lactobacillus plantarum,* found reductions in stress and anxiety as measured by a total Depression, Anxiety, and Stress Scale (DASS-42) questionnaire in the probiotic group as compared to the placebo (maltodextrin) control. This change was accompanied by marginal differences in cortisol levels and significant reductions in pro-inflammatory cytokines. Furthermore, this study found that the probiotic group had heightened memory and cognition as measured by the CogState battery test (e.g., increased speed for social emotional cognition and verbal learning and memory) as compared to the placebo group. There were some sex-specific differences as women consuming the probiotic demonstrated marginal improvements in speed of the identification tasks, while men experienced marginal improvements in overall correct input. However, both women and men experienced enhanced cognition at some capacity [73].

In regards to multispecies probiotic intervention in animals, one of the first studies to measure the effects of probiotics on memory was in a diabetic rat model in 2013 [74]. In this study, the probiotic was dissolved in water and consisted of three strains of bacteria: *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus fermentum.* The diabetic rats lacking probiotic supplementation took the longest time to find the platform in the MWM and spent the shortest amount of time in the correct quadrant during the retrieval test of the MWM as compared to the non-diabetic rats. However, the diabetic rats supplemented with the probiotic experienced improved spatial memory almost to the point of non-diabetic rat memory capabilities [74]. A clinical study using a multispecies probiotic (*Lactobacillus casei W56, Lactobacillus acidophilus W22, Lactobacillus paracasei W20, Bifidobacterium lactis W51, Lactobacillus salivarius W24, Lactococcus lactis W19, Bifidobacterium lactis W52, Lactobacillus plantarum W62*, and *Bifidobacterium bifidum W23*), divided healthy participants into a probiotic, placebo, and no intervention group. Following the intervention, the probiotic group had improved self-reported behavioral measures of positive affect and cognitive reactivity. The probiotic group had altered behavior scores for depression and anxiety questionnaires by increasing positive affect and dampening vulnerability to depression (hopelessness and risk aversion measures). In addition, the probiotic group was found to have improved memory performance, altered brain activation patterns, and reduced decision changes for unpleasant stimuli in the emotional decision making task as compared to the placebo and no intervention control groups.

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Response accuracy was also found to be increased in the probiotic intervention. Lastly, during the emotional decision-making task, the researchers found significant differences in brain activation patterns between the three groups. Specifically, different activation patterns in the precuneus, mid cingulum, middle temporal gyrus, inferior parietal lobule, and paracentral lobule were detected. There were also significant increases in brain activity in the left anterior cingulum in the probiotic versus nonintervention group. These findings indicate that probiotics play a role in decision making processing and memory performance [75]. Furthermore, multispecies probiotic supplementation of depressive patients over a four-week intervention resulted in reduced IL-6 gene expression, supporting the role of probiotics in reducing inflammation among depressed individuals and potentially among other diseased groups as well [76].

PREBIOTICS AND BEYOND

Prebiotics are non-digestible dietary substances that can alter the composition of the gut microbiota and be fermented by its constituents to produce SCFAs. Prebiotics are typically comprised of carbohydrates, though they can also include flavanols [77]. Several studies have tested prebiotics as a possible therapeutic for gastrointestinal disease, though they have additional potential to improve cognition. In a clinical study, a prebiotic dietary fiber supplement, comprised mainly of polydextrose (PDX) was compared to maltodextrin (placebo). A group of women ($n = 18$) were administered the prebiotic or placebo over a four-week intervention period. This double-blind, randomized, crossover study revealed improved cognitive flexibility and sustained attention (via CANTAB battery test) in the PDX-treated group compared to controls, representing a moderate improvement in cognitive function. Still, the effects of PDX were limited, and further research on the impact of dietary fiber and prebiotics on gut microbial composition and cognitive function is necessary to better understand the role prebiotics as a supplement and possible therapeutic going forward [78].

Like prebiotics, antibiotics have been a proposed method of improving cognition by inhibiting microglial activation and modulating memory systems. Berens et al. [79] implemented minocycline in a clinical trial of 20 healthy male participants, but contrary to expectations, found reduced overall spatial memory performance. Additional research is needed to determine the potential, if any, for antibiotics to modulate cognitive functions, specifically in the memory space [80]. In essence, the gut-brain-axis is implicated in various disease phenotypes and cognitive outcomes.

MICROBIAL INVOLVEMENT IN COGNITION AND AD

Disruption of the gut microbiota is associated with AD development, and pathogenic microbe elicited neuroinflammation is a proposed etiological link between gut dysbiosis and AD progression. As previously mentioned, *APOE* genotypes are differentially associated with late-onset AD risk. A study conducted by Tran et al. [80] investigated the relationship between *APOE* genotypes and the composition of the human gut microbiota [80]. Fifty-six healthy participants ages 56 to 78 were selected according to *APOE* genotype to determine if there was a unique microbial signature associated with the *APOE2/E3, APOE3/E4,* or *APOE4/E4* genotypes. No significant differences in alpha or beta microbial diversity were found between genotypes; however, differences in relative abundances of bacterial taxa were established, and the *APOE* genotype was associated with specific gut microbial profiles in human and *APOE*-transgenic mice. Relative abundance of the phylum *Firmicutes* and order *Clostridiales* was heightened in participants with the *APOE2/E3* genotype compared to *APOE3/E4* or *APOE4/E4*. At the family level, abundance of *Ruminococcaceae*, a family of fermentative anaerobes linked to fiber break-down and SCFA production was higher in *APOE2/E3* than in *APOE3/E3*, *APOE3/E4*, or *APOE4/E4* and 3 genera within the family including *Clostridium* cluster IV, *Clostridium* cluster XIVa, and *Gemmiger*, were statistically significant and differentially abundant based on *APOE* genotypes. This study established an association between Prevotellaceae and Ruminococcaceae and various butyrate-producing genera with *APOE* genotypes [80]. The differences in taxa among *APOE* genotypes supports the association between genotypes and gut microbiota composition. Further investigation is necessary to ascertain whether the altered microbiota profile is responsible for exacerbated AD pathogenesis according to *APOE* genotype.

A recent study provides insight into the relationship between the at-risk *APOE* genotype (E4 allele) and cognitive function. Rodriguez et al. [81] investi-

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gated the effects of the at-risk *APOE* genotype (E4 allele) and mental work demands on cognitive decline in cognitively healthy adults older than 75 years from the Ageing, Cognition and Dementia in Primary Care Patients multi-centered population-based longitudinal cohort study in Germany. The Mini-Mental State Examination was used to determine cognitive functioning. Information pertaining to participants' occupational history was also gathered to determine mental demands at work and the relationship between demand level, *APOE* genotype, and cognitive decline. The researchers found heightened work demands were associated with better cognitive functioning at baseline and slower cognitive decline overtime. In terms of the *APOE4* allele, those carrying this allele experienced an accelerated difference in cognitive decline at follow-up. Furthermore, medium versus low work demands were associated with slower cognitive decline among *APOE4* carriers. This study better characterized the relationship cognitive decline and the influence of a high-risk genotype for late-onset AD [81].

Another research team utilized fecal samples to characterize the bacterial taxonomic composition of patients with AD diagnoses [16]. Using bacterial 16S rRNA genetic sequencing of DNA isolated from fecal samples, researchers characterized the gut microbial communities of participants with and without clinical diagnoses of dementia due to AD. Here, AD patients were found to possess reduced microbial diversity and maintained compositionally distinct microbiomes compared to age and sex-matched healthy individuals. Phylum through genus wide differences in AD participants' gut microbiome bacterial abundance were established, including reduced Firmicutes, heightened Bacteroidetes, and reduced Bifidobacterium (Table 1) [16]. AD patients experienced significantly reduced microbiome richness compared to control with respect to ACE and Chao1. Furthermore, Shannon Index and Faith's PD were significantly decreased in AD participants. In terms of beta diversity, Bray-Curtis dissimilarity and UniFrac analysis demonstrated compositional dissimilarity in the microbiomes of AD versus control groups. Fecal sample analysis revealed reduced abundance at the phylum level of Firmicutes and Actinobacteria, which exhibit anti-inflammatory properties and reduce intestinal permeability, while AD participants experienced increased abundance of Bacteroidetes compared to healthy controls (Table 1) [16]. Families within Firmicutes (*Ruminococcaceae*, *Turicibacteraceae*, *Peptostreptococcaceae*, *Clostridiaceae*, and *Mogibacteriaceae*) and the genera *SMB53* (family *Clostridiaceae)*, *Dialister*, *Clostridium*, *Turicibacter*, and *cc115* (family *Erysipelotrichaceae*) were less abundant, while the family *Gemellaceae* and genera *Blautia*, *Phascolarctobacterium*, and *Gemella* were increasingly abundant in AD participants (Table 1). Within Bacteroidetes, *Bacteroidaceae* and *Rikenellaceae* at the family level, and *Bacteroides* and *Alistipes* at the genus level were more abundant in AD participants (Table 1).

Table 1
Differences in bacteria taxa and metabolites among patients with MCI or AD and controls ($p < 0.05$)

Bacterial Taxa and Metabolites	Change in AD Patients versus Healthy Controls
Taxa	
Firmicutes	Decreased [16, 17]
Ruminococcaceae	Decreased [16, 17], increased [18]
Clostridiaceae	Decreased [16, 17]
Mogibacteriaceae	Decreased [16], increased [42*]
Lachnospiraceae	Decreased [17, 18]
Blautia	Decreased [17], increased [16, 45]
Lachnoclostridium	Decreased [18, 86]
Phascolarctobacterium	Increased [39*,42]
Actinobacteria	Decreased [16], increased [18]
Proteobacteria	Increased [17, 85]
Bacteroidetes	Decreased [18], increased [16]
Bacteroidaceae	Decreased [18], increased [16]
Bacteroides	Decreased [18, 45], increased [16, 86]
Bifidobacterium	Decreased [16], increased [45]
Alistipes	Decreased [45], increased [16, 86]
Metabolites	
Lipopolysaccharide	Increased [83]
Acetate	Increased [83]
Valerate	Increased [83]
Butyrate	Decreased [83]

*MCI patient versus Healthy controls.

The heightened levels of Bacteriodetes found among AD patients aligns with hypotheses related to LPS abundance, inflammation, and AD. Gram negative Bacteriodetes maintain an outer membrane made of LPS; thus, increased Bacteriodetes correlates with greater LPS abundance (Table 1) [82]. Increased presence of LPS in elderly individuals has been associated with heightened gut permeability, microbial translocation, inflammation, and amyloid pathology as LPS can trigger systemic inflammation and release of proinflammatory cytokines post-translocation from the gut to systemic circulation [16, 83]. Additional support for the interplay between gut microbial composition and AD pathology is supported by the negative correlation found between genera abundant in AD and cerebrospinal fluid (CSF) $A\beta_{42}/A\beta_{40}$ levels since reduced CSF $A\beta_{42/40}$ is suggestive of heightened amyloid deposition in the brain. Fur-

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thermore, positive correlations between bacterial abundance and CSF p-tau and p-tau/Aβ_{42} were established in this study [16, 84].

PROBIOTIC AND DIETARY INTERVENTIONS IN AD

The use of probiotics in animal and clinical studies of AD have elicited mixed findings. Two different rodent models of AD with distinct probiotic supplementations, were found to improve the disease phenotype. One study utilized a triple transgenic mouse model of AD, B6;129-Psen1tm1Mpm Tg (APPSwe,tauP301L)1Lfa/J (named 3xTg-AD), and the other used a rat model to induce AD via intraperitoneal injection of D-Galactose [87, 88]. Both rodent models displayed reduced cognition as measured by the MWM test and novel object recognition test and presented amyloid plaque burden in the hippocampus [87, 88]. The 3xTg-AD mice were treated with SLAB51, a probiotic comprised of 9 different bacterial strains ((*Streptococcus thermophilus, bifidobacteria* (*B. longum, B. breve, B. infantis*), and *lactobacilli* (*L. acidophilus*, *L plantarum, L. paracasei, L. delbrueckii subsp. bulgaricus, L. brevis*)) and the D-galactose induced AD rats were treated with *L. plantarum MTCC1325* [88]. In response to probiotic treatments, both rodent models experienced improved cognition and reduced plaque burden in the brain. More recently, researchers treated a third model of AD, App^{NL-G-F} with a probiotic cocktail of eight bacterial strains (*L. plantarum, L. delbrueckii subsp. Bulgaricus, L. paracasei, L. acidophilus, B. breve, B. longum, B. infantis, and S. salivarius subspecies, thermophiles)* commercially marketed as VSL#3. Probiotic-treated App^{NL-G-F} mice displayed reduced anxiety-like behaviors and increased c-Fos expression in the hippocampus, a common functional marker for neuronal activity [89, 90]. However, the probiotic cocktail failed to reduce plaque burden, astrogliosis, or microgliosis in the App^{NL-G-F} mice brains [89]. It should be noted that this failure to improve markers of AD in the brain was only evaluated in female App^{NL-G-F} and not male mice. A previous study with APPSWE/PS1L166P male and female mice treated with a cocktail of antibiotics found that only male mice had reduced Aβ plaques and changes in microglial phenotype [24]. These studies highlight the need for both male and female in AD research as the disease treatment may be sex-dependent.

A clinical study found results contrasting the findings from studies of AD rodent models supplemented with various probiotics. Leblhuber et al. [91] analyzed serum, fecal samples, and cognitive statuses via MMSE and clock Drawing test (CDT) of 18 patients with AD diagnoses (based on ICD-10, MRT, and routine lab testing) pre- and post-28-day multispecies probiotic supplementation of *Lactobacillus casei W56, Lactococcus lactis W19, Lactobacillus acidophilus W22, Bifidobacterium lactis W52, Lactobacillus paracasei W20, Lactobacillus plantarum W62, Bifidobacterium lactis W51, Bifidobacterium bifidum W23*, and *Lactobacillus salivarius W24* [91]. There were no significant changes in cognitive parameters nor differences in blood serum levels of tryptophan, phenylalanine, and tyrosine after the 28-day probiotic intervention. Though increases were found in serum levels of kynurenine, a metabolite of tryptophan and key regulator of the immune system, in probiotic supplemented individuals [92]. Still, in patients diagnosed with MCI, there is evidence that a probiotic intervention may be of benefit. Patients with MCI experience a unique intermediate period between healthy cognitive function and an AD diagnosis. Addressing symptoms at this time may provide a means of preventing the escalation of cognitive deficits associated with AD. In a 12-week, double-blind, placebo-controlled, randomized clinical trial, an 800 mg/day *Lactobaccillus plantarum* C29-fermented soybean intervention improved cognition among the probiotic-treated group [93]. Both probiotic and placebo groups were at least 90% compliant in following daily dosage prescriptions. Post-test, patients diagnosed with MCI based on DSM-5 criteria and treated with the probiotic experienced enhanced composite scores in cognitive function (attention, working memory, verbal memory) based on results from computerized neurocognitive function tests. Specifically, the probiotic group demonstrated significant score increases in attention. Lastly, the probiotic group experienced significant increases in the *Lactobacilli* bacterial population and serum BDNF levels which is positively associated with enhanced combined cognitive function scores [93]. Akbari et al. also offers support for the use of probiotics in combating cognitive decline [94]. After a 12-week study, cognitive functionality, assessed via MMSE scores, was improved in 30 AD patients treated with a probiotic cocktail containing *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum*, and *Lactobacillus fermentum*, compared to patients treated with control milk.

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MMSE scores improved by $27.90\% \pm 8.07$ among those treated with the probiotic cocktail, while scores declined by $5.03\% \pm 3.00$ among control participants ($p < 0.001$) [94]. This significant difference in cognitive function between treatment groups indicates probiotics may have a therapeutic application in ameliorating symptoms associated with AD [94]. A recent review by Arora et al. also highlights the use of probiotics as anti-inflammatory and antioxidant-containing agents and the potential limitations as a therapeutic [95].

Dietary intervention must be considered in addition to probiotics when discussing microbiome-based therapeutics as diet is proposed to influence the gut-brain axis via changes of the gut microbiota composition. The shared incidence between AD and illnesses that diet directly influences, including obesity, cardiovascular disease, type 2 diabetes, and metabolic syndrome point us to a connection between diet and AD pathology [96]. There is evidence to suggest that the use of certain nutrient combinations can combat the synaptic loss associated with Parkinson's disease [97]. A similar line of thinking may be applied to AD. Rather than supplementing the diet with individual nutrients, a more comprehensive approach shows promise in combating AD with diet. For example, uridine, docosahexaenoic acid (DHA), and choline are phospholipid precursors responsible for the production and maintenance of neuronal membranes, and their systemic availability has been found to be reduced in patients suffering from AD [96, 97]. By increasing the abundance of these nutrients and cofactors associated with phospholipid synthesis (i.e., vitamins B, C, E, and selenium) via dietary intervention, circulating levels in blood and CSF may be increased, resulting in heightened phospholipid production and maintenance of neuronal membrane integrity. In addition to individual nutrients, dietary fat intake may modify the risk of developing AD by altering the body's response to environmental neurotoxins, and preventative dietary interventions are thought to reduce colonic inflammation. Lastly, polyunsaturated fatty acids like docosahexaenoic acid have anti-inflammatory effects and may reduce reactive oxygen species and oxidative stress associated with AD [97].

Healthy dietary patterns, including those primarily comprised of plant-based foods, probiotics, antioxidants, soybeans, nuts, and omega-3 polyunsaturated fatty acids and minimally containing saturated fats, animal-derived proteins, and refined sugars have been shown to reduce neurocognitive impairment risk and eventual onset and diagnosis of AD [96]. Diets comprised of such rich foods have been shown to elicit a positive impact on the body's inflammatory and immune response and reduce insulin resistance and risk of neurocognitive impairments [96]. The Mediterranean diet, a cultural diet incorporating healthy dietary practices from countries surrounding the Mediterranean Sea (i.e., France, Greece, Italy, and Spain) and DASH (Dietary Approach to Stop Hypertension) have been researched for their vast applications in medicine, including their ability to reduce cardiovascular diseases and other risk factors [96, 98]. These diets are hypothesized to slow cognitive decline and have been found to elicit more benefits than single nutrient supplementation [96, 98]. In turn, MIND (Mediterranean-DASH diet Intervention for Neurodegenerative Delay) was developed as a hybrid of the Mediterranean and DASH diets. MIND focuses on brain protection by incorporating factors from both diets found to enhance cognitive performance and lower dementia and AD risk [96, 98]. Specifically, the MIND diet deviates from the others by emphasizing the value of berries over general fruit consumption and recommending one meal containing fish per week [98]. Further, the MIND diet highlights the importance of green leafy vegetables and plant-based foods and restricts consumption of animal products and those containing high levels of saturated fat [98]. Morris et al. conducted a study with participants from the Memory and Aging Project and found the MIND diet notably reduced cognitive decline over an average 4.7 years over the study duration. By scoring the dietary practices of each of the 960 participants on a scale, they determined that the difference in cognitive scores and function between the top and bottom tertiles was equivalent to being 7.5 years younger [98]. Additional studies support the substantial influence of the MIND diet on cognitive acuity; thus, the role of dietary practices in microbiota health, cognition, and AD must be studied further to understand the power of dietary intervention in mitigating AD [99–101].

FECAL MICROBIOTA TRANSFER AND HUMANIZED GNOTOBIOTIC MICE IN AD

The application of FMT as a therapeutic for AD patients remains unclear. However, a study of humanized gnotobiotic mice provides additional support for the use of FMT as a tool to study the gut-brain axis specifically in AD. In this study, FMT from a

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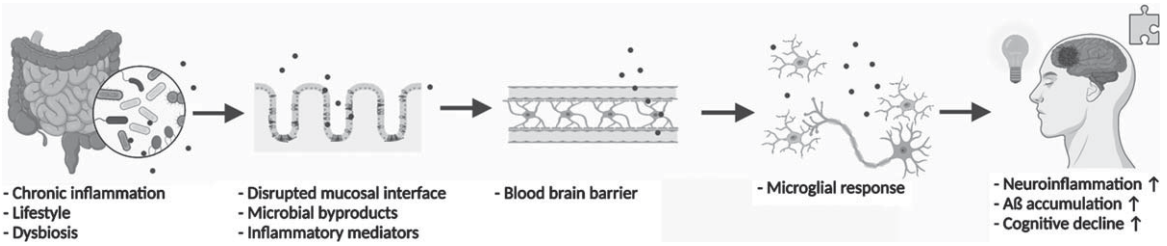


Fig. 1. The proposed pathogenesis of cognitive decline and AD development via gut microbiota disturbances and inflammation. Created with BioRender.com.

human AD donor to GF mice reduced cognitive function earlier than FMT from a healthy non-AD donor to GF mice. In fact, mice treated with FMT from human AD patients performed significantly worse on an object location test and object recognition test at 55 weeks of age as compared to mice with FMT from healthy non-AD donors [102]. There are limitations to the use of humanized gnotobiotic mice as the GI anatomy, physiology, and diet between mice and humans are different [103]. There is also low engraftment from human donor samples to GF recipient mice and the gut microbiota of humanized mice only partially resembles that of the human donor [104, 105].

SHORT CHAIN FATTY ACIDS IN AD

SCFAs are carboxylic acids 2–6 carbons long and are the metabolites produced by gut bacteria during the fermentation of fiber [37]. The main SCFAs produced from gut bacteria metabolism of carbohydrates are acetate, butyrate, and propionate [106]. In the context of AD, butyric and propionic acid have the ability to disrupt the formation of Aβ oligomers *in vitro* and AD mice have been shown to exhibit lower levels of total SCFA in the brain as compared to WT mice [107, 108]. Additionally, when a late disease stage mouse model of AD was treated with sodium butyrate, animals experienced enhanced associated memory, though plaque load in the brain remained the same. The improved associated memory in AD mice treated with sodium butyrate was accompanied by an increase in the expression of genes related to memory consolidation in the hippocampus [109]. In an alternate mouse model of AD, 5XFAD, animals were treated with sodium butyrate in their food at an early stage of disease progression (2 months) rather than a late stage (14-15 months). This 12-week intervention, resulted in a 40% reduction in brain Aβ levels and significantly improved associative learning and cognitive function [110]. Additionally, cognitively impaired mice (LPS-induced and APP/PS1 transgenic) treated with a specific bacterium, *Agathobaculum butyriciproducens,* which mainly produces butyric acid showed improved memory recognition and spatial memory as measured by a novel object test and a spontaneous Y-maze alternation test. The *A. butyriciproducens*-treated LPS and APP/PS1 mice also displayed reduced microglial activation and in the APP/PS1 treated mice they displayed reduced Aβ plaque deposition [111].

As exciting as the possibility is to use SCFAs as a therapeutic for AD, contradicting research exists. In a recently published study by Colombo [112], GF APP/PS1 mice presented with lower plaque burden and improved memory as compared to the re-conventionalized GF AD and SPF AD mice. Further evidence shows the SCFA treatment, consisting of sodium propionate, sodium butyrate, and sodium acetate, in the SPF and GF AD mice caused a larger plaque burden in the brain as compared to GF AD mice [112]. Additionally, treatment of a GF Parkinson's disease mouse model with SCFAs increased the motor dysfunction of these mice and further increasing the controversy surrounding the benefit of SCFA use [113, 114]. However, these data should also be examined with appropriate limitations. As previously described, GF mice have altered brain development and are behaviorally and neurochemically different from conventional mice [50–52]. GF mice also have immature immune systems and in the context of the brain they have naïve microglia with decreased responses to viral infections [115]. Furthermore, GF mice have been shown to lack a working and non-spatial memory as measured by a T-Maze and novel object recognition test [116]. Therefore, care should be exercised in the examination of potential therapeutics with a GF mouse model.

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CONCLUSIONS

Significant progress has been made in understanding the relationship between the gut microbiota and the brain; the potential to mitigate gut dysbiosis and its associated diseases exists via a variety of intervention strategies. Figure 1 highlights the current understanding of the factors influencing gut dysbiosis and the associated pathogenesis of cognitive decline and AD. Considerable research must still be conducted in order to understand how the gut microbiota may be leveraged to treat cognitive disorders, including AD.

Regarding the use of rodent models, we suggest:

1. The use of more and varied rodent models of AD with probiotic, FMT, or SCFA treatment before symptomatic AD, during mild cognitive impairment period, and at the late stage of cognitive impairment. Special attention should be paid to the sex and the microbial state (GF versus SPF) of the animal models used, as the effects of any treatment could vary based on these factors. Various outcomes are expected, but this is a necessary strategy to reach a consensus and to establish which (if any) therapeutics are worth further study and at what stage of the disease.
2. The use of different probiotics containing single or multiple strains and different concentrations. The use of single and multiple strains of bacteria in a probiotic supplementation would aid in identifying useful strains and whether any work synergistically to the benefit of the host.
3. The use of humanized gnotobiotic mice with humanized gut microbiotas. To better understand the interactions between the gut microbiota and brain in the context of human health and disease while maintaining the flexibility of an animal model, it will be necessary to use humanized gnotobiotic mice. This animal model provides more clinical relevance and translation to human health.

For clinical studies, we recommend the increased abundance and variation of interventions such as different durations, probiotic compositions, and stages (pre-clinical, mild, late) of AD and potentially FMTs. This will allow researchers to better characterize strategies to mitigate the presence and progression of this widespread and devastating disease.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Relationship Between Plasma Lipopolysaccharides, Gut Microbiota, and Dementia: A Cross-Sectional Study

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Abstract.
Background: Previous studies have demonstrated associations between gut microbiota, microbial metabolites, and cognitive decline. However, relationships between these factors and lipopolysaccharides (LPS; molecules of the outer membrane of gram-negative bacteria) remain controversial.
Objective: To evaluate associations between plasma LPS, gut microbiota, and cognitive function.
Methods: We performed a cross-sectional sub-analysis of data of 127 participants (women: 58%, mean age: 76 years) from our prospective cohort study regarding the relationship between gut microbiota and cognitive function. We enrolled patients who visited our memory clinic and assessed demographics, dementia-related risk factors, cognitive function, brain imaging, gut microbiomes, and microbial metabolites. We evaluated relationships between cognitive decline and plasma LPS using multivariable logistic regression analyses.
Results: Plasma LPS concentration increased with increasing degree of cognitive decline and total cerebral small vessel disease (SVD) score (Kruskal-Wallis test; $p = 0.016$ and 0.007, respectively). Participants with high plasma LPS concentrations tended to have lower concentrations of gut microbial metabolites, such as lactic acid and acetic acid, and were less likely to

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consume fish and shellfish (44.7% versus 69.6%, $p = 0.027$) than those with low plasma LPS concentrations. Multivariable analyses revealed that plasma LPS concentration was independently associated with the presence of mild cognitive impairment in participants without dementia (odds ratio: 2.09, 95% confidence interval: 1.14–3.84, $p = 0.007$).
Conclusion: In this preliminary study, plasma LPS concentration was associated with both cognitive decline and cerebral SVD and significantly correlated with beneficial gut microbial metabolites. Plasma LPS may be a risk factor for cognitive decline.

Keywords: Biomarkers, cerebral small vessel disease, cognitive decline, dementia, gut microbiota

INTRODUCTION

Dementia is a substantial healthcare problem; in 2015, 47 million individuals worldwide were living with dementia [1]. The number of individuals with dementia is expected to increase to 66 million by 2030 and nearly 130 million by 2050 [2]. Furthermore, the global cost of dementia was estimated to be $820 billion (USD) in 2015, and this cost has continued to increase [1, 2]. Japan is also facing a similar healthcare problem because of the increasing proportion of individuals with dementia; therefore, a comprehensive strategy for dementia research has been introduced in Japan to address this issue [3].

Recently, there has been a focus on the associations between gut microbiota and cognitive decline [4–6], as researchers suspect they may reveal the mechanisms underlying the onset of dementia. Previous studies have suggested that disruption of the neuroinflammatory system [7], vascular inflammation [8], and remote relationships driven by various metabolites [9] are mechanisms underlying cognitive decline that are caused by the gut microbiome. Furthermore, multiple direct (e.g., vagus nerve) and indirect (e.g., short-chain fatty acids, cytokines, and dietary amino acids) gut–brain pathways may be involved in the impairment of cognitive function, and the gut–brain axis is modulated by the gut microbiota via these pathways [6]. The degree of gut dysbiosis worsens with the progression of the disease from mild cognitive impairment (MCI), predementia stage, to dementia [10]. Moreover, the fecal microbial composition of patients with Alzheimer's disease (AD) is altered, characterized by the depletion of gut microbial metabolite-producing and enriched inflammation-promoting bacteria, which correlates with the severity of AD [11]. Thus, microbiome analysis contributes to the classical predictors of dementia severity [11]. However, knowledge regarding the effects of gut microbiota on cognitive function and the associations regarding blood biomarkers remains limited.

Lipopolysaccharides (LPS), which are molecules of the outer membrane of gram-negative bacteria, induce the release of critical pro-inflammatory cytokines that are necessary to activate a potent immune response, and this increases amyloid-β (Aβ) production and accumulation and the hyperphosphorylation of the tau protein [12]. Bacterial LPS in the brain lysates from the hippocampus and superior temporal lobe neocortex of AD brains [13]. Additionally, LPS progressively accumulate in neuronal parenchyma and appear to preferentially associate with the periphery of neuronal nuclei in patients with sporadic AD [14]. Therefore, LPS are useful as a surrogate marker for cognitive decline, neurodegeneration, and gut microbial dysbiosis.

We conducted an observational study that was originally designed to investigate the relationship between gut microbiota and cognitive function. In our previous study, gut microbial dysregulation was revealed to be associated with cognitive decline [15, 16], vascular risk factors [17], and white matter hyperintensity (WMH) [18]. In addition, gut microbial metabolites have been shown to be associated with both cognitive decline [19] and adherence to a Japanese-style diet [20]. These factors indicate the presence of a diet–microbiome–dementia cascade. However, we previously found that plasma neurofilament light chain (NfL), which is a disease-non-specific marker of neural damage, is not significantly correlated with gut microbial metabolites [21]. NfL is an emerging biomarker of neuronal degradation that is receiving increasing attention [22]. NfL is an integral component of axons and is released into the bloodstream and cerebrospinal fluid during neurodegeneration [22]. Examining another biomarker in addition to NfL, such as LPS, that indicates both dysbiosis and neural damage, and identifying links between the biomarker, gut microbiota, and cognitive function may clarify the unidentified pathway of the gut–brain axis.

Thus, the present study aimed to evaluate the relationships between LPS, gut microbiota, and cognitive

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function by performing a sub-analysis of the data from our ongoing clinical study. We hypothesized that in our cohort data, plasma LPS would be associated with cognitive decline, and there would be an association between plasma LPS, the gut microbiome, and microbial metabolites.

MATERIALS AND METHODS

Study design

We performed a cross-sectional sub-analysis of data from a hospital-based prospective cohort study, the Gerontological Investigation of Microbiome: a Longitudinal Estimation Study (the Gimlet study), conducted at the National Center for Geriatrics and Gerontology (NCGG) in Japan [15]. Briefly, patients who visited the Memory Clinic at the NCGG and agreed to undergo both a medical assessment of their cognitive function and a fecal examination were enrolled. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the NCGG (no. 1191-3). Written informed consent was obtained from all patients and their families before their participated in the study. The Gimlet study is registered on the UMIN Clinical Trials Registry (UMIN000031851). Detailed information is provided in the Supplementary Materials.

Participants

We enrolled 128 patients (women: 59%, mean age: 74.2 ± 8.7 years) from the Gimlet study who had subjective memory impairment and visited the Memory Clinic at the NCGG between March 2016 and March 2017. Participants in the Gimlet study were eligible for this sub-study if they met the following criteria: 1) able to undergo brain magnetic resonance imaging (MRI); 2) blood samples were successfully preserved; and 3) provided written informed consent. One participant was excluded from the current sub-analysis because of a lack of blood sample for the measurement of LPS, which resulted in a total of 127 participants in the analysis.

Assessments

All participants underwent a comprehensive geriatric assessment based on the following features: 1) demographic characteristics; 2) risk factors, such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, and a history of stroke; 3) activities of daily living; 4) global cognitive function, which was assessed using the Mini-Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) scales; 5) neuropsychological testing using evaluations, such as the Alzheimer's Disease Assessment Scale-Cognitive Subscale and Frontal Assessment Battery; 6) behavioral and psychological symptoms assessed using the Dementia Behavior Disturbance Scale (DBDS); 7) burden on caregivers; 8) depression status, which was assessed using the Geriatric Depression Scale; 9) laboratory parameters, such as apolipoprotein E $\varepsilon 4$, plasma NfL, and C-reactive protein; 10) ankle brachial index and pulse wave velocity as indicators of arteriosclerosis [23] and the 'impact' of pulse [24], respectively; 11) results of brain imaging, such as MRI and single-photon emission-computed tomography (SPECT); and 12) dietary assessments based on the Japanese Diet Index [20, 25]. Clinical samples and data were provided by the NCGG Biobank, which collects clinical data for research. In this sub-analysis, we used a single dataset that was obtained at enrollment in the Gimlet study.

Brain imaging

Participants underwent a 1.5-T brain MRI (Philips Ingenia, Eindhoven, Netherlands). The presence of cerebral small vessel disease (SVD) and its components, such as silent lacunar infarcts (SLIs), WMH, cerebral microbleeds (CMBs), and enlarged perivenricular space, was assessed. Following previous studies [18, 26], we rated the total MRI burden of SVD on an ordinal scale from 0 to 4 by summing the presence of each of these four features. The voxel-based specific regional analysis system for Alzheimer's Disease (VSRAD) software (Eisai Co., Ltd., Tokyo, Japan) was used to quantify cortical and hippocampal atrophy. In detail, medial temporal structures involving the entire region of the entorhinal cortex, hippocampus, and amygdala show significant atrophy in the patients with very mild AD and are specifically determined by VSRAD software program [27]. Thus, VSRAD is a useful indicator of early AD [27]. In addition, previous studies show that VSRAD can distinguish between AD and MCI [28] and between AD and dementia with Lewy bodies [29]. Participants also underwent N-isopropyl-p-[^{123}I]-iodoamphetamine SPECT, in which low blood flow in the area of the posterior cingulate gyrus and/or

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precuneus was regarded as a surrogate marker of AD [30].

Classification of cognitive function

Dementia was defined as an MMSE score of < 20 and/or a CDR score of ≥ 1 [15]. Participants who did not have dementia were further categorized as having either MCI or normal cognition (NC). MCI was defined as an MMSE score of ≥ 20 and a CDR score of 0.5, which indicated possible, very mild dementia and a higher risk of developing dementia [16]. NC was defined as an MMSE score of ≥ 20 and a CDR score of 0.

Lipopolysaccharides

Blood samples were collected, processed onsite to isolate plasma, aliquoted, and frozen at –81°C in the NCGG Biobank. Plasma LPS concentration was measured using a Limulus amebocyte lysate assay (No. K50-643J; Lonza Inc., Basel, Switzerland) according to manufacturer instructions. The plasma was diluted 10-fold in pyrogen-free water and inactivated for 15 min at 90°C. LPS measurements were performed in pyrogen-free glass tubes, Eppendorf tubes, and plates [31]. All samples were measured blinded.

Gut microbiome

Fecal samples were collected, frozen, and stored at –81°C in the NCGG Biobank. The gut microbiome of each participant was analyzed by Techno Suruga Laboratory (Shizuoka, Japan) using terminal restriction fragment length polymorphism (T-RFLP) analysis [32] to classify gut microbes. By referencing the human fecal microbiome T-RFLP profile, each gut microbiome was categorized as representing one of three enterotypes: enterotype I, which comprised *Bacteroides* at > 30%; enterotype II, which comprised *Prevotella* at > 15%; and enterotype III, which comprised other combinations of microorganisms. The Firmicutes/Bacteroidetes (F/B) ratio was also calculated because a high ratio is indicative of dysbiosis [33].

Analysis of microbial metabolites in feces

Fecal concentrations of organic acids, short-chain fatty acids, ammonium ions, indoles, phenol, skatole, and p-cresol were measured, as previously described [19]. The concentrations of organic acids and short-chain fatty acids (acetic, propionic, butyric, iso-butyric, succinic, lactic, formic, valeric, and isovaleric acids) were measured using high-performance liquid chromatography. Ammonium ion concentration was quantified using ion chromatography, and fecal concentrations of indoles, phenol, skatole, and p-cresol were quantified using gas chromatography/mass spectrometry.

Statistical analysis

Continuous, ordinal, and categorical variables are expressed as means ± standard deviations, medians and interquartile ranges, or frequencies and proportions (percentage), respectively. Data were compared using Student's unpaired *t*-tests, Wilcoxon rank-sum tests, and χ^2 tests, respectively. Natural logarithm-transformed plasma LPS concentrations were occasionally used to reduce the rightward skew of plasma LPS concentrations for subsequent analysis as well as for plasma NfL. Firstly, we compared clinical characteristics and plasma LPS concentrations between participants with dementia and those without dementia, and between participants with MCI and those with NC. Subsequently, we compared clinical characteristics between participants with high concentrations of plasma LPS and those with low concentrations, who were divided according to the median value of plasma LPS concentration. Furthermore, we compared the dietary composition between these two groups. Thirdly, relationships between the gut microbiome, microbial metabolites, and plasma LPS concentration were evaluated. Finally, multivariable logistic regression models were used to identify independent associations between the presence of dementia (or MCI among those who did not have dementia) and plasma LPS concentration, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All comparisons were two-tailed, and $p < 0.05$ represented statistical significance. Data were analyzed using the JMP 14.3 software package (SAS Institute Inc., Cary, NC, USA).

RESULTS

Participant characteristics

The number of participants who were classified into NC, MCI, and dementia were as follows: NC ($n = 23$), MCI ($n = 71$), and dementia ($n = 33$). Women were more likely than men to have a lower MMSE score (women versus men; 24 versus 26, respectively,

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$p = 0.018$), body mass index (22.2 versus 23.2, respectively, $p = 0.045$), and MNA-SF score (12 versus. 13, respectively, $p = 0.046$) and were less likely to have a smoking habit (4.1% versus 54.7%, respectively, $p < 0.001$) and alcohol consumption (21.6% versus 62.3%, respectively, $p < 0.001$). However, there were no significant differences between women and men in age (median age; 76 versus 75 years, respectively, $p = 0.227$), plasma NfL concentration (median value: 26.1 versus 23.0 pg/mL, respectively, $p = 0.141$), or plasma LPS concentration (median value: 4.5 versus 4.4 EU/mL, respectively, $p = 0.207$, Supplementary Table 1).

Dementia versus non-dementia

Compared with participants without dementia, participants with dementia tended to be women (84.9% versus 48.9%, respectively, $p < 0.001$), be apolipoprotein E $\varepsilon 4$ carriers (54.6% versus 21.3%, respectively, $p < 0.001$), not be enterotype I (15.2% versus 44.7%, respectively, $p = 0.003$), have cerebral SVD, such as SLIs (24.2% versus 5.3%, respectively, $p = 0.005$) and CMBs (39.4% versus 16.0%, respectively, $p = 0.008$), have higher F/B ratio (median value: 2.05 versus 1.38, respectively, $p = 0.019$), have higher plasma NfL (median value: 33.2 versus 21. pg/mL, respectively, $p < 0.001$), and have higher scores in the VSRAD (median score: 2.0 versus 0.9, respectively, $p < 0.001$). Plasma LPS concentrations in participants with dementia were slightly higher than those in participants without dementia; however, the difference was not significant (median score: 4.95 versus 4.40 EU/mL, respectively, $p = 0.272$, Table 1).

MCI versus NC

Compared with participants with NC, participants with MCI tended to be older (MCI versus NC: 77 versus 69 years, $p < 0.001$), be hypertensive (64.8% versus 39.1%, $p = 0.050$), be enterotype I (52.1% versus 21.7%, $p = 0.0028$), have WMH (33.8% versus 4.4%, $p = 0.006$), have higher plasma NfL (median value: 23.4 versus 18.2 pg/mL, $p < 0.001$), and have higher scores in the VSRAD (median score: 1.0 versus 0.6, $p = 0.015$). Plasma LPS concentrations in participants with MCI were significantly higher than those in participants with NC (median value: 4.66 versus 4.04 EU/mL, $p = 0.007$, Table 1).

Clinical data and plasma LPS

Plasma LPS concentration significantly increased with increasing degree of cognitive decline (median values of 4.04 EU/mL for NC participants, 4.66 for MCI participants, and 4.94 for dementia participants; $p = 0.016$; Fig. 1A). Compared with participants with low concentrations of plasma LPS, those with high concentrations tended to have lower cognitive function (high versus low LPS concentrations: median

Table 1
Comparison of clinical biomarkers between participants with and without dementia, participants with enterotype I and those with other enterotypes, and participants with mild cognitive impairment (MCI) and normal cognition (NC)

	Dementia (+) ($n = 33$)	Dementia (–) ($n = 94$)	p
Plasma LPS, EU/mL	4.95, 3.94–5.89	4.40, 3.83–5.25	0.272
Plasma NfL, pg/mL	33.2, 26.5–49.4	21.3, 15.5–27.2	< 0.001
Enterotype I, *n* (%)	5 (15.2)	42 (44.7)	0.003
F/B ratio	2.05, 1.24–2.85	1.38, 0.81–2.25	0.019
	Enterotype I (+) ($n = 47$)	Enterotype I (–) ($n = 80$)	p
Plasma LPS, EU/mL	4.39, 3.93–5.23	4.47, 3.76–5.47	0.747
Plasma NfL, pg/mL	23.4, 17.9–36.4	24.3, 19.0–34.0	0.621
Dementia, *n* (%)	5 (10.6)	28 (35.0)	0.003
F/B ratio	0.77, 0.60–1.19	2.12, 1.51–3.12	< 0.001
	MCI ($n = 71$)	NC ($n = 23$)	p
Plasma LPS, EU/mL	4.66, 4.01–5.35	4.04, 3.49–4.41	0.007
Plasma NfL, pg/mL	23.4, 17.6–33.3	18.2, 12.8–20.8	< 0.001
Enterotype I, *n* (%)	37 (52.1)	5 (21.7)	0.015
F/B ratio	1.25, 0.70–2.25	1.65, 1.11–2.26	0.082

Data are represented as medians and interquartile ranges. Wilcoxon rank-sum test and χ^2 tests were used. Enterotype I: *Bacteroides* > 30%. F/B ratio, Firmicutes/Bacteroidetes ratio; NC, normal cognition; NfL, neurofilament light chain; LPS, lipopolysaccharides; MCI, mild cognitive impairment.

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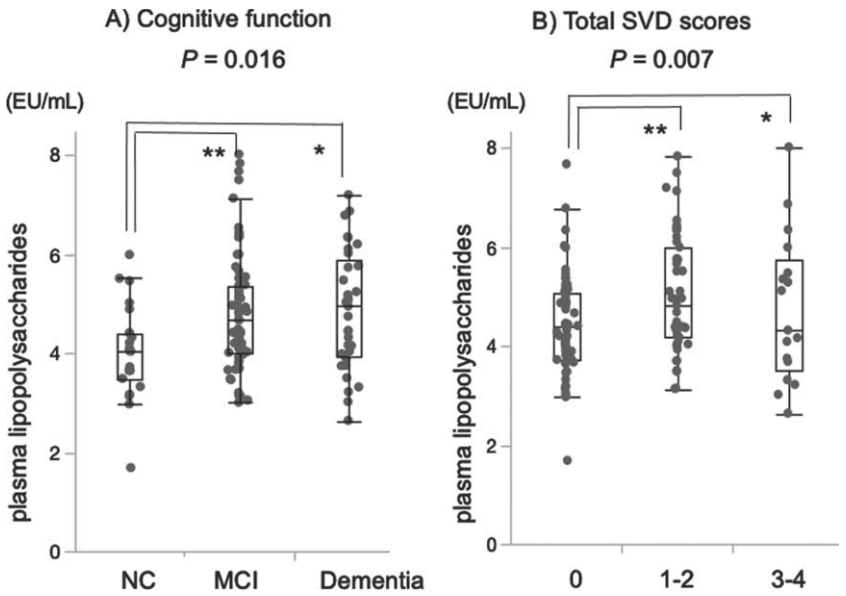


Fig. 1. Comparisons of plasma lipopolysaccharides (LPS) concentration and (A) cognitive function and (B) total cerebral small vessel disease (SVD) scores The *x*-axes show (A) cognitive function stratified into normal cognition (NC), mild cognitive impairment (MCI), and dementia groups; (B) total cerebral SVD scores stratified as scores of 0, 1–2, or 3–4 in participants without dementia. The *y*-axes show plasma LPS concentrations. Plasma LPS concentration increased with increasing degree of cognitive decline and total cerebral SVD score (Kruskal-Wallis test). *Post hoc* analysis was performed using Dunnett's test. $^{*}p<0.05$; $^{**}p<0.01$.

MMSE score: 24 versus 25, $p=0.035$; median CDR-sum of boxes (SB) score: 2.0 versus 1.5, $p=0.070$; median Logical Memory subtest I of the Wechsler Memory Scale-Revised (LM-WMSR I) score: 7 versus 10, $p=0.031$), higher concentrations of plasma NfL (median value: 26.3 versus 21.9 pg/mL $p=0.096$), and higher scores in the VSRAD (median score: 1.20 versus 0.87, $p=0.055$, Supplementary Table 2). In participants without dementia, plasma LPS concentrations were significantly higher in participants with cerebral SVD than in those without cerebral SVD (median values [cerebral SVD score]: 4.20 EU/mL [0], 4.75 [1–2], 5.28 [3–4], $p=0.007$, Fig. 1B). In participants without dementia, compared with participants with low concentrations of plasma LPS, those with high concentrations tended to have MCI (high versus low LPS concentrations: 88.4% versus 64.7%, $p=0.009$), instrumental activities of daily living (IADL) impairment (50.0% versus 26.8%, $p=0.017$), higher scores in the DBDS (median score: 11 versus 6, $p=0.016$), and slightly higher concentrations of plasma NfL (median value: 24.9 versus 21.1 pg/mL, $p=0.090$, Supplementary Table 3). Natural logarithm-transformed plasma LPS was significantly correlated with natural logarithm-transformed plasma NfL ($\beta=0.419$, 95% CI: 0.043–0.795, $p=0.029$).

Dietary assessment and LPS

Compared with participants with high concentrations of plasma LPS, those with low concentrations tended to be more likely to consume fish and shellfish (high versus low LPS concentrations: greater intake: 44.7% versus 69.6%, $p=0.027$; Supplementary Table 4). Similarly, in participants without dementia, compared with participants with high concentrations of plasma LPS, those with low concentrations tended to be more likely to consume fish and shellfish (high versus low LPS concentrations: greater intake: 46.4% versus 73.2%, $p=0.042$; Supplementary Table 5).

Gut microbiota, metabolites, and LPS

There were no significant differences in plasma concentrations of both LPS and NfL between participants with enterotype I and those with other enterotypes (Table 1). Regarding the gut microbial metabolites, concentrations of both lactic acid and acetic acid in participants with high LPS concentrations tended to be lower than those in participants with low LPS concentrations (high versus low: median value [25–75th percentile]: lactic acid 0.03 [0.03–0.03] versus 0.03 [0.03–8.97] mg/g, $p=0.002$ and acetic acid 2.25 [0.03–5.02] versus

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3.58 [0.44–9.24] mg/g, $p = 0.048$). In participants without dementia, concentrations of both lactic acid and acetic acid in participants with high LPS concentrations also tended to be lower than those in participants with low LPS concentrations (high versus low: median value [25–75th percentile]: lactic acid 0.03 [0.03–0.03] versus 0.03 [0.03–8.97] mg/g, $p = 0.002$ and acetic acid 2.26 [0.03–4.96] versus 3.58 [0.83–9.24] mg/g, $p = 0.071$; Supplementary Table 6).

Multivariable analyses

Multivariable logistic regression analyses did not reveal significant associations between plasma LPS and dementia (univariate analysis: OR: 1.18, 95% CI: 0.85–1.66, $p = 0.139$; adjusted for age and sex: OR: 1.12, 95% CI: 0.78–1.63, $p = 0.537$; Table 2A). In participants without dementia, multivariable analyses revealed that plasma LPS was independently associated with the presence of MCI (univariate analysis: OR: 2.14, 95% CI: 1.22–3.75, $p = 0.003$; multivariate analysis: OR: 2.09, 95% CI: 1.14–3.84, $p = 0.007$; Tables 2B and 3). Furthermore, plasma NfL (OR: 1.12), the presence of enterotype I (OR: 3.78), and apolipoprotein E ε4 carrier (OR: 4.22) were also associated with the presence of MCI (Table 3). The plasma LPS cutoff value calculated according to the Youden index for the detection of MCI was 4.41 EU/mL, with 56% sensitivity and 78% specificity (area under the curve = 0.69, $p = 0.003$). Compared with participants with low plasma LPS concentrations, those with high concentrations had a higher OR (OR: 3.96, 95% CI: 1.20–13.1, $p = 0.017$) calculated in the stepwise multivariable logistic regression analyses after adjusting for other confounding covariables (Table 2B).

Table 2
Multivariable logistic regression analyses for the presence of (A) dementia and (B) mild cognitive impairment (MCI)

(A)Dementia	OR	95% CI	*p*
Plasma LPS, per 1 pg/mL			
Model 1	1.18	0.85–1.66	0.139
Model 2	1.12	0.78–1.63	0.537
High versus low concentration of plasma LPS*			
Model 1	1.82	0.81–4.09	0.141
Model 2	1.66	0.70–3.92	0.248
(A) The dependent variable was the presence of dementia.			
(B) MCI	OR	95% CI	*p*
Plasma LPS, per 1 EU/mL			
Model 1	2.14	1.22–3.75	0.003
Model 2	1.90	1.06–3.43	0.019
Model 3	1.91	1.05–3.45	0.019
Model 4	1.93	1.06–3.51	0.032
High versus low concentration of plasma LPS*			
Model 1	4.15	1.39–12.40	0.006
Model 2	3.92	1.21–12.70	0.016
Model 3	3.96	1.20–13.08	0.017
Model 4	2.63	0.77–8.96	0.122

(B)The dependent variable was the presence of MCI. *Participants were allocated to two groups according to plasma LPS concentrations: high LPS group if the LPS value was above the median or the low LPS group if the LPS value was below the median. Model 1: univariate analyses. Model 2: adjusted for age and sex. Model 3: backward stepwise multivariable logistic regression analyses adjusted for model 2 factors, years of education, and risk factors, such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking, alcohol consumption, and apolipoprotein E ε4 carrier. Model 4: backward stepwise multivariable logistic regression analyses adjusted for model 3 factors, enterotype I (*Bacteroides* > 30%), Firmicutes/Bacteroidetes ratio, neurofilament light chain, SLIs, WMH, CMBs, enlarged perivascular space (EPVS), score of the voxel-based specific regional analysis system for Alzheimer's disease, single-photon emission-computed tomography findings (presence or absence of low blood flow in the area of the posterior cingulate gyrus and/or precuneus). CI, confidence interval; LPS: lipopolysaccharides; OR, odds ratio.

Table 3
Multivariable logistic regression analyses for the presence of mild cognitive impairment (MCI)

	OR	95% CI	*p*
Plasma LPS, per 1 EU/mL	2.09	1.14–3.84	0.007
Plasma NfL, per 1 pg/mL	1.12	1.03–1.22	0.001
Enterotype I	3.78	1.11–12.8	0.025
Apolipoprotein E ε4 carrier	4.22	0.88–20.1	0.050

The dependent variable was the presence of MCI. Backward stepwise multivariable logistic regression analyses adjusted for age, sex, years of education, risk factors (e.g., hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking, alcohol consumption, and apolipoprotein E ε4 carrier), enterotype I (*Bacteroides* > 30%), Firmicutes/Bacteroidetes ratio, and plasma concentration of neurofilament light chain (NfL). CI, confidence interval; LPS: lipopolysaccharides; OR, odds ratio.

DISCUSSION

The main finding of the present study was that plasma LPS concentration significantly increased with increasing degrees of cognitive decline and cerebral SVD. Moreover, plasma LPS concentration was independently associated with the presence of MCI in participants without dementia. Furthermore, a low concentration of plasma LPS was significantly associated with a greater intake of fish and shellfish and a lower concentration of microbial metabolites, such as lactic acid and acetic acid. However, plasma LPS concentration was not independently associated with

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the presence of dementia in participants who were enrolled in the present sub-analysis.

LPS are the major outer membrane components of gram-negative bacteria and are capable of triggering systemic inflammation and the release of pro-inflammatory cytokines [34]. Recently, Andreadou et al. reported that levels of LPS are increased in the serum and cerebrospinal fluid of patients with AD and the serum of patients with MCI [12], which is consistent with our data. Furthermore, we revealed associations between gut microbial metabolites and the level of plasma LPS. This is the first report that demonstrates associations between plasma LPS concentration, gut microbial metabolites, and cognitive decline. However, Voigt et al. reported that the LPS-binding protein, a marker of intestinal barrier integrity, is not associated with incident AD or MCI [35]. This discrepancy may be attributed to the different factors measured between the studies: LPS and LPS-binding protein. Thus, this issue should be investigated in future studies.

We found that plasma LPS concentration was significantly correlated with gut microbial metabolites, such as lactic acid and acetic acid. These metabolites are products caused by *Bifidobacterium*, which is a healthy bacterium that has probiotic effects on human health, such as the suppression of neuroinflammation [36]. Therefore, a lower concentration of plasma LPS is expected to be associated with a higher concentration of beneficial microbial metabolites. Recently, we reported that several gut microbial metabolites are associated with dementia [19]; we found that several metabolites are positively associated with dementia, whereas others are inversely associated with dementia. Furthermore, previous studies have shown that some microbial metabolites, such as indole and propionic acid, promote brain health [37], and microbiota-derived short-chain fatty acids are critical mediators along the gut–brain axis and promote Aβ deposition [38]. Alkasir et al. reported the presence of a brain–gut link and proposed a novel strategy for the management of dementia by modulating microbiota [39]. These findings suggest a multiplex linkage among the gut microbiome, microbial metabolites, and cognitive function.

In our previous study, plasma NfL was significantly associated with cognitive decline [21]. In the present sub-analysis, we similarly found that both plasma LPS and NfL concentrations were significantly associated with MCI, independent of enterotype, apolipoprotein E ε4, and other dementia-related risk factors, such as age and years of education. Furthermore, plasma LPS concentration was positively correlated with plasma NfL concentration. Thus, plasma LPS concentration may be a novel risk factor for MCI. However, the relationship between the level of LPS and the presence of dementia was not statistically significant in this study. We speculate that this discrepancy is because the level of plasma LPS had already reached a high level at the stage of early cognitive decline, indicated by MCI, which resulted in a non-significant difference in plasma LPS level between participants with MCI and those with dementia. Previous reports also suggested that patients with AD and MCI have reduced microbial diversity, changes in gut microbiota can be detected in the early stages of AD, and that dysbiosis can occur in the MCI stage [40]. In addition, the criteria of dementia and MCI in our study were dependent on the CDR score. A CDR score of 0.5 includes patients at the very early stage of dementia [41]. Thus, the difference in plasma LPS concentration between our participants with dementia (CDR score of ≥ 1) and those with MCI (CDR score of 0.5) was likely small. Even after the early cognitive decline stage, levels of NfL can continue to increase according to the progression of neurodegeneration and/or neural damage.

Plasma LPS concentrations did not significantly differ between the different categories of gut microbiomes, which were categorized according to enterotypes. This negative finding may be a result of the methodological limitations of our T-RFLP analysis. Furthermore, there was no significant difference F/B ratio, an indicator of dysbiosis, between groups with high and low concentrations of plasma LPS. We speculate that F/B ratio is not specifically associated with the presence of plasma LPS because F/B ratio simply indicates the ratio of the presence of *Firmicutes* to *Bacteroidetes*. The use of next-generation sequencing can reveal more detailed microbial data than can be revealed by the T-RFLP method, and such assessments will likely clarify the presence of the gut–microbiome–brain axis.

Another notable result of the present study was that greater consumption of fish and shellfish was associated with a lower concentration of plasma LPS and thus may protect against cognitive decline. Previously, we revealed that a healthier diet, as measured by the Japanese-style diet index, was associated with a lower concentration of plasma NfL [21]. Greater consumption of fish and shellfish was associated with a lower concentration of plasma LPS, which indicated that these foods alter the gut microbiota, increase beneficial microbial metabolites, and suppress

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neurodegeneration and/or neural injury reflected by the increase in plasma NfL, resulting in the preservation of cognitive function. Previously, Otsuka et al. showed that a moderately high level of serum docosahexaenoic acid, accompanied by greater consumption of fish, prevents cognitive decline among community-dwelling older adult individuals [42]. Our data are in line with this previous study, although we did not assess the level of serum docosahexaenoic acid in this sub-analysis. This mechanism will be investigated in more detail in future studies.

The present study has several strengths. Firstly, we revealed novel relationships among plasma LPS concentration, gut microbial metabolites, and cognitive decline. Specifically, plasma LPS concentration was strongly associated with MCI and plasma NfL concentration, the latter of which is a robust biomarker of neurodegeneration. Secondly, we also revealed that LPS level was correlated with several gut microbial metabolites. Although we did not observe a positive association that included participants with dementia, this is the first study to analyze such associations and thus, our findings offer new research directions in this emerging field. Thirdly, we confirmed the utility of plasma LPS concentration for detecting the presence of MCI. Although the sensitivity of plasma LPS was not high, it was statistically significant. Finally, we systematically evaluated cognitive function using a comprehensive geriatric assessment and a range of neuropsychological tests. Our findings encourage further explorations of the relationships between blood biomarkers and cognitive function through examinations of the gut microbiome.

The present study has several limitations. A causal relationship between plasma LPS concentration and cognitive decline could not be established owing to the cross-sectional design. Moreover, we did not assess Aβ or tau in the present study because the Gimlet study did not perform cerebrospinal fluid testing or positron emission tomography to identify these biomarkers, as previously reported [21]. We did not discuss in detail the dementia subtypes because of the small number of participants with dementia. Furthermore, the small number of participants and the large number of potential variables may have statistically underpowered our study. However, because this was a sub-analysis, we did not perform sample size calculation. Selection bias may exist because this was a single hospital-based cohort study. In addition, we did not perform any assessment of bile acids and gastrointestinal infections. Bile acids are known as microbe-derived neuroactive molecules [43], and recurring gastrointestinal infections affect the nervous system and can increase the risk of developing dementia [44]. Physical activity, which we did not assess in this study, can also significantly modulate the gut microbiome [45]. A systematic assessment including other biomarkers, such as the amyloid-β protein precursor and non-neurodegenerative inflammatory biomarkers, may also be useful given their association with the risk of the development of cognitive impairment [46].

Although this sub-study was a preliminary analysis, we provided evidence for the relationships between plasma LPS, gut microbial metabolites, and cognitive function. Detailed assessments of these relationships should be conducted in future studies to determine the underlying mechanisms.

Conclusions

High plasma LPS concentration was independently associated with MCI in participants without dementia. Plasma LPS concentration was also associated with cerebral SVD in participants without dementia and significantly correlated with gut microbial metabolites, such as lactic acid and acetic acid. Furthermore, a low concentration of plasma LPS was significantly correlated with a greater intake of fish and shellfish. Further studies are warranted to examine these associations in relation to the microbiome–gut–brain axis.

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SUPPLEMENTARY MATERIAL

Relationship Between Plasma Lipopolysaccharides, Gut Microbiota, and Dementia: A Cross-Sectional Study

SUPPLEMENTARY METHODS

Outline of the gimlet study

Subjects

Between March 2016 and March 2017, we enrolled consecutive patients visiting the memory clinic at the National Center for Geriatrics and Gerontology (NCGG) in Japan who agreed to undergo a medical assessment of their cognitive function and a fecal examination.

Patients were eligible for the Gimlet study if they met the following criteria: 1) were able to undergo a brain magnetic resonance imaging (MRI); 2) provided informed consent in writing; 3) provided informed consent for the NCGG Biobank to store their clinical data, blood, and fecal samples; and 4) were accompanied by a study partner who could assess the patient's condition. Patients were excluded if they met the following criteria: 1) were unable to undergo an MRI examination or the MRI was unable to be evaluated because of movement; 2) had local lesions, such as cerebral infarctions, that were detected by MRI before enrolment and might significantly affect cognitive function; 3) had a history of a major psychological disorder or current, serious, or unstable alcohol or drug abuse; 4) had $\leq$6 years of education; 5) had a history of cancer of the digestive tract; or 6) were judged by an investigator to be ineligible to participate as a study subject (e.g., recent use of antibiotics, digestive disorder such as liver cirrhosis or inflammatory bowel disease, neurological disorder such as brain tumor, encephalitis/meningitis, normal pressure hydrocephalus, or Huntington's disease).

Baseline assessment

All participants underwent a comprehensive geriatric assessment [1] using the following: 1) demographic characteristics; 2) risk factors, such as hypertension, dyslipidemia, diabetes mellitus, ischemic heart disease, chronic kidney disease, smoking habits, or a history of stroke and alcohol consumption; 3) basic and instrumental activities of daily living scales, assessed using the Barthel Index [2] and Lawton and Brody scale [3]; 4) global cognitive function, assessed using the Mini-Mental State Examination [4] and Clinical Dementia Rating [5]; 5) neuropsychological testing, using the Alzheimer's Disease Assessment Scale–Cognitive Subscale [6], Raven's Coloured Progressive Matrices [7], Frontal Assessment Battery [8], and Logical Memory subtests of the Wechsler Memory Scale-Revised [9]; 6) behavioral and psychological symptoms, assessed using the Dementia Behavior Disturbance Scale [10]; 7) burden of caregivers, assessed by the Zarit Caregiver Burden Interview [11]; 8) depressive status, assessed by the Geriatric Depression Scale [12]; 9) laboratory variables, including *APOE* ε4 as a risk factor for Alzheimer's disease (AD); 10) ankle brachial index and pulse wave velocity, as indicators of arteriosclerosis [13] and the 'impact' of pulse [14]; 11) brain imaging, such as MRI and single-photon emission computed tomography (SPECT); 12) an assessment of other factors, such as the presence of frailty [15] and subjective hearing loss; and 13) an assessment of social and lifestyle factors, such as the Mini-Nutritional Assessment-Short Form to assess nutritional status [16]. All clinical samples and data were provided by the NCGG Biobank, which collects clinical data for research.

Risk factors

Hypertension was defined by a systolic blood pressure of $\geq$140 mmHg or a diastolic blood pressure of $\geq$90 mmHg, and/or the use of antihypertensive drugs. Dyslipidemia was defined by a serum low-density lipoprotein cholesterol concentration of $\geq$140 mg/dL, a serum high-density lipoprotein cholesterol concentration of <40 mg/dL, a serum triacylglycerol concentration of $\geq$150 mg/dL, and/or the use of statins. Diabetes mellitus was defined by a hemoglobin A1c concentration of $\geq$6.5%, and/or the use of oral hypoglycemic drugs or insulin, and/or a fasting serum glucose concentration of $\geq$69.9 mol/L (126 mg/dL). Ischemic heart disease was defined by a history of physician-diagnosed angina pectoris and/or evidence of a prior myocardial infarction or coronary revascularization procedure (percutaneous coronary intervention or coronary artery bypass surgery). Serum creatinine was measured and the estimated glomerular filtration rate (eGFR) was determined using the equation proposed by the Japanese Society of Nephrology, as follows: eGFR (mL/min/1.73 m^2) = 194 $\times$ (serum creatinine [mg/dL])$^{-1.094}$ $\times$ (age [years])$^{-0.287}$ ($\times$0.739 if female). Chronic kidney disease was defined by an eGFR of <60 mL/min/1.73

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m^2. We performed blood count and biochemical examination.

MRI imaging and SPECT imaging

The participants underwent a 1.5-T MRI of their brains (Philips Ingenia, Eindhoven, the Netherlands). MRI scans were obtained, including diffusion-weighted images, fluid-attenuated inversion recovery images, T2-weighted images, T2*-weighted gradient-echo images, three-dimensional T1-weighted sagittal and axial coronal views, and 3D time-of-flight magnetic resonance angiography scans. The presence and components of cerebral small vessel disease, such as silent lacunar infarct (SLI), white matter hyperintensity (WMH), cerebral microbleeds (CMB), and enlarged periventricular space (EPVS), were categorized using previously published standards for reporting vascular changes on neuroimaging [17]. The voxel-based specific regional analysis system for Alzheimer's disease (VSRAD) software (Eisai Co., Ltd., Tokyo, Japan) was used to quantify cortical and hippocampal atrophy, using standardized z-scores [18]. A high VSRAD score suggests the presence of AD because this score reflects hippocampal atrophy, which is one of the characteristics of the brain of a patient with AD. The participants also underwent N-isopropyl-p-[^{123}I]-iodoamphetamine-SPECT, in which the presence of low blood flow in the area of the posterior cingulate gyrus and/or the precuneus was regarded as a surrogate marker of AD [19].

Total small vessel disease (SVD) score

Based on a previous study [20], we rated the total MRI burden of SVD on an ordinal scale from 0 to 4, by summing the presence of each of four features of cerebral SVD. This score consists of the following: 1) SLI (1 point if present); 2) CMB (1 point if present); 3) EPVSs (1 point if moderate to severe EPVSs are present in the basal ganglia [grades 2–4]); and 4) WMH (1 point for the presence of either [early] confluent deep WMH [Fazekas score 2 or 3] or irregular periventricular WMH extending into the deep white matter [Fazekas score 3]).

Dietary assessments

The questionnaire regarding dietary composition consisted of 12 items. All the components of a typical Japanese diet were grouped on the basis of the definitions used in the Japanese National Health and Nutrition Survey and the previous studies [21]. We evaluated the Japanese dietary index (JDI) based on the items shown in the above survey. In detail, JDI_{12} was defined recently [22] and comprises 10 beneficial components (rice, miso, fish and shellfish, green and yellow vegetables, seaweed, pickles, green tea, soybeans and soybean-derived foods, fruit, and mushrooms) and two less beneficial components (beef and pork, and coffee). The participants were assigned one point if their daily beneficial intake was equal to or greater, and their daily less beneficial intake was below the sex-specific median intake.

Plasma neurofilament light (NfL) chain

Blood samples were collected, processed onsite to isolate plasma, aliquoted, and frozen at –81°C in the NCGG Biobank. Plasma NfL concentrations were measured according to manufacturer instructions using the NF-Light Advantage Kit on a highly sensitive single-molecule array assay Simoa HD-1 platform (Quanterix, Lexington, MA, USA) [27, 28]. Plasma samples were measured at a 1:4 dilution and were run in duplicate by a trained technician. All samples were measured blinded. All NfL values were within the linear ranges of the assays.

Sample collection

Patients or their family members used a fecal sampler to collect a fecal sample as soon as possible after the patient's bowel movement. The sample was placed in a specimen container. Patients collected fecal samples on the day of their hospital consultation, and the samples were presented to the clinical laboratory center of the NCGG (preferably within 4 h of the bowel movement). Because one of the inclusion criteria for our study was that patients presented with study partners (family members), all the demented patients were able to be supported if they needed support about excretion. Furthermore, when we received the fecal samples, we only accepted appropriate samples as per the analyzing company's criteria, while inadequate samples were disposed of and resubmission was requested. The samples were frozen and stored at –81°C in the NCGG Biobank. After all samples had been collected, they were transported (frozen) to TechnoSuruga Laboratory (Shizuoka, Japan).

Gut microbiome

Fecal samples were collected at home, just after a bowel evacuation, by patients or their family members. The samples were collected using scoop collection tubes, and patients consumed their usual

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diets before and after sample collection. Samples were frozen and preserved at –81°C at the NCGG Biobank. After all samples had been collected, the gut microbiomes were analyzed by TechnoSuruga Laboratory (Shizuoka, Japan) using T-RFLP analysis [23]. T-RFLP analysis is one of the most well-established and reliable 16 S ribosomal RNA-based methods, especially when considering its high throughput and reproducibility. First, T-RFLP was used to classify gut microbes into the following 10 groups: Prevotella, Bacteroides, Lactobacillales, Bifidobacterium, Clostridium cluster IV, Clostridium subcluster XIVa, Clostridium cluster IX, Clostridium cluster XI, Clostridium cluster XVIII, and 'others'. Second, by referencing the Human Fecal Microbiome T-RFLP profile [24,25], the gut microbiome was stratified into three enterotypes: enterotype I included Bacteroides at >30%, enterotype II included *Prevotella* at >15%, and enterotype III included the remaining bacteria. Third, the F/B ratio was examined, because an increase in F/B ratio indicates dysbiosis [26]. The phylum Firmicutes included the Lactobacillales and *Clostridium* clusters, and the phylum Bacteroidetes included *Bacteroides* and *Prevotella*.

Terminal restriction fragment length polymorphisms

Fecal samples (approximately 10 mg each) were suspended in 900 μL of a solution containing 100 mM tris-HCl (pH 9.0), 40 mM ethylenediaminetetraacetic acid, 4 M guanidine thiocyanate, and 0.001% bromothymol blue. Fecal solids in the suspension were broken down using a FastPrep FP100A Instrument (MP Biomedicals; CA, USA) and zirconia beads at 5 m/s for 2 min. DNA was then extracted from 200 μL of suspension using an automatic nucleic acid extractor and MagDEA® DNA 200 (Precision System Science, Chiba, Japan). PCR was performed using a Takara Thermal Cycler Dice TP650 (Takara Bio, Shiga, Japan) in a reaction mixture (20 μl) containing 1 × PCR buffer, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.2 μM, 10 ng of fecal DNA and 0.2 U of HotStar Taq DNA polymerase (Qiagen, Hilden, Germany). The primers used were 5' fluorescein amidite-labeled 516f (5′-TGC- CAGCAGCCGCGGTA-3′; *Escherichia coli* positions 516–532) and 1510r (5′-GGTTACCTTGTTACGA- CTT-3′; *E. coli* positions 1510–1492). The amplification program used was as follows: preheating at 95°C for 15 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s; and finally, a terminal extension at 72°C for 10 min. The size of the amplicon was verified by electrophoresis, and it was purified using a MultiScreen PCR96 Filter Plate (Millipore, Billerica, MA, USA). The purified 16 S rDNA amplicons were treated with 10 U of FastDigest *BseLI* (Thermo Fisher Scientific., MA, USA) for 10 min. An ABI PRISM 3130 × l genetic analyzer (Thermo Fisher Scientific) was used to analyze the resultant fluorescence-labeled terminal restriction fragments (T-RFs), and GeneMapper software (Thermo Fisher Scientific) was used to determine T-RF length and peak area for each sample. T-RFs were divided into 29 operational taxonomic units (OTUs). The OTUs were quantified as the percentage of each OTU of the total OTU area and expressed as the percentage of the area under the curve (% AUC). The reference database, Human Fecal Microbiota T-RFLP profiling (http://www.tecsrg-lab.jp/), was used to putatively identify the bacteria in each classification unit and the corresponding OTU.

Analysis of metabolites in feces

To determine metabolite levels, we measured fecal levels of organic acids, SCFAs, ammonium ions, indoles, phenol, skatole, and p-cresol. The organic acids and SCFAs, such as acetic acid, propionic acid, butyric acid, iso-butyric acid, succinic acid, lactic acid, formic acid, valeric acid, and iso-valeric acid, were measured using high-performance liquid chromatography (Prominence, Shimadzu, Kyoto, Japan) with a detector (CDD-10A, Shimadzu, Kyoto, Japan), two tandemly-arranged columns (Shim-pack SCR-102(H), 300 mm × 8 mm ID, Shimadzu), and a guard column (Shim-pack SCR-102(H), 50 mm × 6 mm ID, Shimadzu). We measured ammonium ion concentrations using an ion chromatography system (ICS-1000, DIONEX, Thermo Fisher Scientific K.K., Tokyo, Japan) with a column (IonPac CS12A, 4 mm × 250 mm, DIONEX) and a guard column (IonPac CG12A, 4 mm × 50 mm, DIONEX). Indoles, phenol, skatole, and p-cresol levels were measured using gas chromatography/mass spectrometry (QP-2010, Shimadzu) and a capillary column (Inert cap WAX, 30 m × 0.25 mm × 0.25 μm, GL Sciences, Tokyo, Japan).

Organic acids. To determine the presence of organic acids, 0.1 g of feces was placed into a 2.0-mL

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tube with zirconia beads and suspended in MilliQ water. Samples were heated at 85°C for 15 min, vortexed at 5 m/s for 45 s using FastPrep 24 (MP Biomedicals, CA, USA), and centrifuged at 15,350× *g* for 10 min. The supernatant was filtered using a 0.2-μm filter. Organic acids (acetic acid, propionic acid, butyric acid, iso-butyric acid, succinic acid, lactic acid, formic acid, valeric acid, and iso-valeric acid) in feces were measured using high-performance liquid chromatography (Prominence, Shimadzu) with a detector (CDD-10A, Shimadzu), two tandemly-arranged columns (Shim-pack SCR-102(H), 300 mm × 8 mm ID, Shimadzu), and a guard column (Shim-pack SCR-102(H), 50 mm × 6 mm ID, Shimadzu). A 5 mM p-toluenesulfonic acid solution was used in the mobile phase and a mixture (5 mM p-toluenesulfonic acid, 100 μM EDTA, and 20 mM Bis-Tris) was used as the reaction solution. The flow rate and oven temperature were 0.8 mL/min and 45°C, respectively. The detector cell temperature was maintained at 48°C. The measurements were performed with an absolute calibration curve (range 5–1,000 mg/L).

Ammonia. To measure ammonium ion concentrations, 0.1 g of feces was suspended in 0.9 mL MilliQ water, heated at 85°C for 15 min, and filtered through a 0.20-μm filter. Samples were diluted 200-fold in MilliQ water. Ammonium ion concentrations were then measured using an ion chromatography system (ICS-1000, DIONEX) with a column (IonPac CS12A, 4 mm × 250 mm, DIONEX) and a guard column (IonPac CG12A, 4 mm × 50 mm, DIONEX). In the mobile phase, a 10.7 mM sulfuric acid solution was used, and the flow rate was 1.0 mL/min. The detector temperature was maintained at 35°C. The measurements were performed with an absolute calibration curve (range 0.025–2.0 mg/L).

Indole, phenol, skatole, and p-cresol. A total of 0.1 g of feces was suspended in 2.5 mL phosphate buffer, including 0.4 mg/L 4-isopropylphenol as an internal standard. The sample was heated at 85°C for 15 min, mixed with 2.5 mL acetonitrile and 1 g NaCl, shaken for 30 min, and centrifuged at 1,300× *g* for 10 min. Next, 1 mL of the supernatant was dehydrated and purified using a sodium sulfate drying cartridge (Bond Elut LRC, Agilent Technologies, USA), C18 cartridge (Smart SPE C18-30, AiSTI SCIENCE, Japan), and PSA cartridge (Smart SPE PSA-30, AiSTI SCIENCE), and placed into a vial.

Indoles and phenols were measured using gas chromatography/mass spectrometry (QP-2010, Shimadzu) and a capillary column (Inert cap WAX, 30 m × 0.25 mm × 0.25 μm, GL Sciences). Helium was used as the carrier gas at 1.11 mL/min. The injector and interface temperatures were maintained at 240°C and 230°C, respectively. The oven temperature program was as follows: 70°C for 2 min; rose by 20°C/min to 200°C, held for 3 min; rose by 10°C/min to 240°C; and finally held for 16 min at 240°C. One microliter of the extract was injected in the splitless mode. The mass spectrometer was operated in electron impact ionization mode at 70 eV. The measurements were performed with an absolute calibration curve (range 0.02–5.0 mg/L). Data acquisition was performed in selected ion monitoring mode for quantification.

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Supplementary Table 1
Comparisons of participant characteristics between women and men

	Women ($n = 74$)	Men ($n = 53$)	p
Demographic factor			
Age, y	76, 73–81	75, 67–80	0.227
Education, y	12, 9–12	12, 9–16	0.005
Body mass index, kg/m^2	22.2, 20.1–24.2	23.2, 21.4–24.8	0.045
Systolic BP, mmHg	143, 127–157	151, 125–168	0.225
Diastolic BP, mmHg	77, 68–86	84, 72–91	0.037
JDI_{12}, points	7, 6–9	8, 8–9	0.040
$rJDI_{12}$, points	8, 6–10	9, 8–10	0.049
Risk factor			
Hypertension, *n* (%)	45 (60.8)	34 (64.2)	0.715
Diabetes mellitus, *n* (%)	12 (16.2)	8 (15.1)	1.000
Dyslipidemia, *n* (%)	39 (52.7)	20 (37.7)	0.107
Ischemic heart disease, *n* (%)	5 (6.8)	7 (13.2)	0.236
Stroke, *n* (%)	5 (6.8)	6 (11.3)	0.524
CKD, *n* (%)	22 (29.7)	19 (35.9)	0.564
Smoking habit, *n* (%)	3 (4.1)	29 (54.7)	<0.0001
Alcohol consumption, *n* (%)	16 (21.6)	33 (62.3)	<0.0001
Apolipoprotein E ε4 carrier, *n* (%)	27 (36.5)	11 (20.8)	0.077
Dementia, *n* (%)	28 (37.8)	5 (9.4)	<0.001
Comprehensive geriatric assessment			
Barthel Index	100, 100–100	100, 100–100	0.417
IADL impairment, *n* (%)	37 (50.0)	21 (39.6)	0.281
DBDS	9.5, 4–16.5	8.5, 4–14	0.530
GDS	3, 1–5	3, 1–4.5	0.745
ZBI	13, 3–23	10, 5–20	0.668
MNA-SF	12, 11–13	13, 12–13	0.046
Cognitive function			
MMSE	24, 19–28	26, 22–29	0.018
CDR-global score			0.066
0, *n* (%)	10 (13.5)	13 (24.5)	
0.5, *n* (%)	(66.2)	36 (67.9)	
>1, *n* (%)	15 (20.3)	4 (7.6)	
CDR-sum of boxes	2.5, 1.0–4.0	1.0, 0.5–2.5	0.001
ADAS-cog	11.3, 5.3–15.4	8.3, 5.9–13.3	0.336
RCPM	28, 23–31	29, 24–33	0.302
FAB	10, 9–13	11, 10–14	0.242
LM-WMSR I	7, 3–13	11, 4–18	0.021
LM-WMSR II	1, 0–5	3, 0–11	0.019
Brain MRI finding			
SLI, *n* (%)	6 (8.1)	7 (13.2)	0.385
WMH, *n* (%)	23 (31.1)	11 (20.8)	0.227
CMB, *n* (%)	16 (21.6)	12 (22.6)	1.000
EPVS, *n* (%)	54 (73.0)	42 (79.3)	0.531
VSRAD	1.03, 0.65–2.05	0.99, 0.62–1.52	0.528
SPECT			
Low blood flow*	49 (70.0)	36 (72.0)	0.841
Arterial stiffness			
Ankle brachial index	1.11, 1.05–1.15	1.09, 1.05–1.15	0.906
Pulse wave velocity, m/s	19.3, 16.2–22.1	18.2, 15.8–21.7	0.504
Laboratory finding			
Total protein, g/dL	7.3, 6.9–7.6	7.3, 6.9–7.5	0.984
Albumin, g/dL	4.2, 4.0–4.4	4.2, 4.0–4.4	0.477
White blood cells	58.5, 47.0–73.3	57.0, 49.0–66.0	0.649
CRP, mg/dL	0.05, 0.02–0.11	0.06, 0.03–0.17	0.294
eGFR, mL/min/1.73 m^2	70.5, 53.4–74.8	70.2, 56.0–79.0	0.642

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Supplementary Table 1
(*Continued*)

	Women ($n = 74$)	Men ($n = 53$)	*p*
Hemoglobin A1c, %	5.8, 5.6–6.1	5.7, 5.5–6.2	0.276
NfL, pg/mL	26.1, 19.4–34.5	23.0, 15.7–33.7	0.141
LPS, EU/mL	4.47, 4.00–4.47	4.36, 3.66–5.30	0.207

Data are represented as medians, interquartile ranges, or numbers of patients (%). Wilcoxon rank-sum tests and χ^2 tests were used. *Blood flow reduction in the posterior cingulate gyrus and/or precuneus, as seen in SPECT images. ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; BP, blood pressure; CDR, Clinical Dementia Rating; CKD, chronic kidney disease; CMB, cerebral microbleed; CRP, C-reactive protein; DBDS, Dementia Behavior Disturbance Scale; eGFR, estimated glomerular filtration rate; EPVS, enlarged periventricular space; FAB, Frontal Assessment Battery; GDS, Geriatric Depression Scale; IADL, instrumental activities of daily living; JDI, Japanese diet index; LM-WMSR, Logical Memory subtests I and II of the Wechsler Memory Scale-Revised; LPS, lipopolysaccharides; MMSE, Mini-Mental State Examination; MNA-SF, Mini-Nutritional Assessment-Short Form; MRI, magnetic resonance imaging; NfL, neurofilament light chain; RCPM, Raven's Coloured Progressive Matrices; SLI, silent lacunar infarct; SPECT, single-photon emission computed tomography; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease; WMH, white matter hyperintensity; ZBI, Zarit Caregiver Burden Interview.

Supplementary Table 2
Comparisons of participant characteristics between participants with high plasma lipopolysaccharides (LPS) concentrations and those with low plasma LPS concentrations

	High LPS ($n = 63$)	Low LPS ($n = 64$)	*p*
Demographic factor			
Age, y	77, 70–81	75, 68–79.8	0.331
Female sex, *n* (%)	40 (63.5)	34 (53.1)	0.282
Education, y	12, 9–12	12, 9–13	0.666
Body mass index, kg/m^2	22.5, 20.6–24.2	22.7, 20.8–25.7	0.456
Systolic BP, mmHg	143, 125–156	147.5, 125.5–162	0.334
Diastolic BP, mmHg	79, 69–87	81, 71.3–88.5	0.510
JDI_{12}, points	8, 6–9.3	8, 7–9	0.498
$rJDI_{12}$, points	8, 6–9.3	8.5, 7–10	0.245
Risk factor			
Hypertension, *n* (%)	39 (61.9)	40 (62.5)	1.000
Diabetes mellitus, *n* (%)	11 (17.5)	9 (14.1)	0.634
Dyslipidemia, *n* (%)	30 (47.6)	29 (45.3)	0.860
Ischemic heart disease, *n* (%)	7 (11.1)	5 (7.8)	0.560
Stroke, *n* (%)	5 (7.9)	6 (9.4)	1.000
CKD, *n* (%)	20 (31.8)	21 (32.8)	1.000
Smoking habit, *n* (%)	15 (23.8)	17 (26.6)	0.839
Alcohol consumption, *n* (%)	23 (36.5)	26 (40.6)	0.716
Apolipoprotein E ε4 carrier, *n* (%)	18 (28.6)	20 (31.3)	0.847
Dementia, *n* (%)	20 (31.8)	13 (20.3)	0.161
Comprehensive geriatric assessment			
Barthel Index	100, 100–100	100, 100–100	0.807
IADL impairment, *n* (%)	28 (44.4)	41 (64.1)	0.033
DBDS	11, 4–15.3	7, 3–14	0.041
GDS	2, 1–5	3, 1–5	0.414
ZBI	12, 5–23	7.5, 3–19.8	0.246
MNA-SF	12, 11–13	12.5, 11–13	0.429
Cognitive function			
MMSE	24, 19–27	25, 22–29	0.035
CDR-global score			0.013
0, *n* (%)	5 (7.9)	18 (28.1)	
0.5, *n* (%)	47 (74.6)	(59.4)	
>1, *n* (%)	11 (17.5)	8 (12.5)	
CDR-sum of boxes	2.0, 1.0–4.0	1.5, 0.5–3.5	0.070
ADAS-cog	9.7, 5.6–16.5	9.3, 5.7–13.3	0.441
RCPM	27, 22–31	28.5, 24–32	0.294

(*Continued*)

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Supplementary Table 2
(*Continued*)

	High LPS ($n=63$)	Low LPS ($n=64$)	*p*
FAB	10, 9–13	11, 9.3–14	0.109
LM-WMSR I	7, 3–11	10, 4–18	0.031
LM-WMSR II	1, 0–7	3, 0–10	0.146
Brain MRI finding			
SLI, *n* (%)	5 (7.9)	8 (12.5)	0.560
WMH, *n* (%)	17 (27.0)	17 (26.6)	1.000
CMB, *n* (%)	12 (19.1)	16 (25.0)	0.522
EPVS, *n* (%)	44 (69.8)	52 (81.3)	0.152
VSRAD	1.20, 0.72–2.08	0.87, 0.57–1.65	0.055
SPECT			
Low blood flow*	45 (77.6)	40 (64.5)	0.159
Arterial stiffness			
Ankle brachial index	1.10, 1.05–1.15	1.10, 1.05–1.15	0.789
Pulse wave velocity, m/s	19.8, 16.2–22.8	17.8, 15.8–21.3	0.230
Laboratory finding			
Total protein, g/dL	7.3, 6.9–7.6	7.2, 6.9–7.5	0.192
Albumin, g/dL	4.2, 4.0–4.4	4.2, 4.0–4.4	0.816
White blood cells	59.0, 49.0–71.0	56.5, 46.0–67.3	0.186
CRP, mg/dL	0.06, 0.03–0.13	0.05, 0.02–0.09	0.184
eGFR, mL/min/1.73 m^2	70.2, 53.8–81.2	70.7, 56.3–73.8	0.750
Hemoglobin A1c, %	5.8, 5.6–6.2	5.7, 5.5–6.0	0.178
NfL, pg/mL	26.3, 18.5–36.3	21.9, 18.1–31.4	0.096

Data are represented as medians, interquartile ranges, or numbers of patients (%). Wilcoxon rank-sum tests and χ^2 tests were used. *Blood flow reduction in the posterior cingulate gyrus and/or precuneus, as seen in SPECT images. ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; BP, blood pressure; CDR, Clinical Dementia Rating; CKD, chronic kidney disease; CMB, cerebral microbleed; CRP, C-reactive protein; DBDS, Dementia Behavior Disturbance Scale; eGFR, estimated glomerular filtration rate; EPVS, enlarged periventricular space; FAB, Frontal Assessment Battery; GDS, Geriatric Depression Scale; IADL, instrumental activities of daily living; LM-WMSR, Logical Memory subtests I and II of the Wechsler Memory Scale-Revised; MMSE, Mini-Mental State Examination; MNA-SF, Mini-Nutritional Assessment-Short Form; MRI, magnetic resonance imaging; NfL, neurofilament light chain; RCPM, Raven's Coloured Progressive Matrices; SLI, silent lacunar infarct; SPECT, single-photon emission computed tomography; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease; WMH, white matter hyperintensity; ZBI, Zarit Caregiver Burden Interview.

Supplementary Table 3
Comparisons of participant characteristics between participants with high plasma lipopolysaccharides (LPS) concentrations and those with low plasma LPS concentrations in participants without dementia

	High LPS ($n=52$)	Low LPS ($n=56$)	*p*
Demographic factor			
Age, y	77, 68.3–81	75, 68–79	0.125
Female sex, *n* (%)	30 (57.7)	29 (51.8)	0.567
Education, y	12, 9–13	12, 9–12.8	0.824
Body mass index, kg/m^2	22.8, 20.9–24.5	22.5, 20.3–24.2	0.671
Systolic BP, mmHg	142, 125–157	149, 126–166	0.239
Diastolic BP, mmHg	79, 68–87	82, 73–89	0.260
JDI_{12}, point	8, 7–10	9, 7–9	0.733
$rJDI_{12}$, point	9, 7–10	9, 8–10	0.442
Risk factor			
Hypertension, *n* (%)	32 (61.5)	32 (57.1)	0.698
Diabetes mellitus, *n* (%)	10 (19.2)	5 (8.9)	0.166
Dyslipidemia, *n* (%)	24 (46.2)	23 (41.1)	0.698
Ischemic heart disease, *n* (%)	6 (11.5)	3 (5.4)	0.308
Stroke, *n* (%)	5 (9.6)	3 (5.4)	0.478
CKD, *n* (%)	16 (30.8)	15 (26.8)	0.676
Smoking habit, *n* (%)	15 (28.9)	15 (26.8)	0.833
Alcohol consumption, *n* (%)	21 (40.4)	22 (39.3)	1.000

(*Continued*)

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Supplementary Table 3
(*Continued*)

	High LPS ($n=52$)	Low LPS ($n=56$)	p
Apolipoprotein E ε4 carrier, *n* (%)	11 (21.2)	16 (28.6)	0.505
MCI, *n* (%)	38 (88.4)	33 (64.7)	0.009
Comprehensive geriatric assessment			
Barthel Index	100, 100–100	100, 100–100	0.214
IADL impairment, *n* (%)	26 (50)	15 (26.8)	0.017
DBDS	11, 4–16	6, 3–10	0.016
GDS	3, 1–5	3, 1–5	0.926
ZBI	12, 5–21.8	7, 2.3–17.8	0.162
MNA-SF	13, 11–13	13, 11–13	0.912
Cognitive function			
MMSE	25.5, 21.3–28	27, 23–29	0.110
CDR-global score			0.005
0, *n* (%)	5 (9.6)	18 (32.1)	
0.5, *n* (%)	47 (90.4)	38 (67.9)	
CDR-sum of boxes	2.0, 0.5–3.0	1.0, 0.5–2.5	0.118
ADAS-cog	8.2, 5–13.5	7.8, 5.3–12.7	0.915
RCPM	28, 23–32	29, 25.3–32	0.309
FAB	11, 9–13	12, 10–14	0.210
LM-WMSR I	8, 4–12.5	12, 6–21	0.072
LM-WMSR II	3, 0–8	4, 0–13	0.162
Brain MRI finding			
SLI, *n* (%)	4 (7.7)	4 (7.1)	1.000
WMH, *n* (%)	14 (26.9)	12 (21.4)	0.653
CMB, *n* (%)	9 (17.3)	9 (16.1)	1.000
EPVS, *n* (%)	17 (32.7)	8 (14.3)	0.039
VSRAD	1.03, 0.69–1.57	0.80, 0.56–1.43	0.101
SPECT			
Low blood flow*	36 (76.6)	34 (63.0)	0.194
Arterial stiffness			
Ankle brachial index	1.10, 1.05–1.15	1.10, 1.05–1.15	0.819
Pulse wave velocity, m/s	20.3, 16.3–23.9	17.5, 15.8–21.4	0.113
Laboratory finding			
Total protein, g/dL	7.3, 6.9–7.6	7.3, 7.0–7.5	0.531
Albumin, g/dL	4.1, 3.9–4.3	4.2, 4.0–4.4	0.257
White blood cells	58.0, 49.0–68.8	56.5, 46.3–67.3	0.415
CRP, mg/dL	0.06, 0.03–0.13	0.04, 0.02–0.09	0.087
eGFR, mL/min/1.73 m^2	70.3, 55–81.2	71, 58.8–74.0	0.919
Hemoglobin A1c, %	5.9, 5.6–6.2	5.7, 5.5–5.9	0.020
NfL, pg/mL	24.9, 17.0–35.9	21.1, 16.1–26.0	0.090

Data are represented as medians, interquartile ranges, or numbers of patients (%). Wilcoxon rank-sum tests and χ^2 tests were used. *Blood flow reduction in the posterior cingulate gyrus and/or precuneus, as seen in SPECT images. ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; BP, blood pressure; CDR, Clinical Dementia Rating; CKD, chronic kidney disease; CMB, cerebral microbleed; CRP, C-reactive protein; DBDS, Dementia Behavior Disturbance Scale; eGFR, estimated glomerular filtration rate; EPVS, enlarged periventricular space; FAB, Frontal Assessment Battery; GDS, Geriatric Depression Scale; IADL, instrumental activities of daily living; LM-WMSR, Logical Memory subtests I and II of the Wechsler Memory Scale-Revised; MMSE, Mini-Mental State Examination; MNA-SF, Mini-Nutritional Assessment-Short Form; MRI, magnetic resonance imaging; NfL, neurofilament light chain; RCPM, Raven's Coloured Progressive Matrices; SLI, silent lacunar infarct; SPECT, single-photon emission computed tomography; VSRAD, voxel-based specific regional analysis system for Alzheimer's disease; WMH, white matter hyperintensity; ZBI, Zarit Caregiver Burden Interview.

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Supplementary Table 4
Comparison of dietary composition between participants with high plasma lipopolysaccharides (LPS) concentrations and those with low plasma LPS concentrations

	High LPS ($n=38$)	Low LPS ($n=46$)	p
Food			
Rice, *n* (%)	38 (100.0)	44 (95.7)	0.499
Bread, *n* (%)	25 (65.8)	32 (69.6)	0.816
Noodles, *n* (%)	15 (39.5)	14 (30.4)	0.490
Miso, *n* (%)	23 (60.5)	32 (69.6)	0.490
Fish and shellfish, *n* (%)	17 (44.7)	32 (69.6)	0.027
Vegetables, *n* (%)	36 (94.7)	45 (97.8)	0.587
Seaweed, *n* (%)	17 (44.7)	25 (54.4)	0.511
Pickles, *n* (%)	24 (63.2)	28 (60.9)	1.000
Mushrooms, *n* (%)	18 (47.4)	27 (58.7)	0.380
Fruits, *n* (%)	28 (73.7)	41 (89.1)	0.088
Beef and pork, *n* (%)	23 (60.5)	35 (76.1)	0.157
Chicken, *n* (%)	19 (50.0)	30 (65.2)	0.187
Soybeans and soybean-derived foods, *n* (%)	31 (81.6)	39 (84.8)	0.773
Milk and dairy products, *n* (%)	30 (79.0)	38 (82.6)	0.782
Green tea, *n* (%)	28 (73.7)	29 (63.0)	0.353
Coffee, *n* (%)	24 (63.2)	36 (78.3)	0.151
Cooking method			
Boiling, *n* (%)	32 (84.2)	40 (87.0)	0.762
Steaming, *n* (%)	2 (5.3)	8 (17.4)	0.104
Raw, *n* (%)	30 (79.0)	41 (89.1)	0.236
Deep-frying, *n* (%)	17 (44.7)	13 (28.3)	0.170
Frying, *n* (%)	20 (52.6)	33 (71.7)	0.111
Use of soup stock, *n* (%)	35 (92.1)	44 (95.7)	0.654
Use of fermented seasoning, *n* (%)	38 (100.0)	44 (95.7)	0.499

Data are represented as numbers of patients (%). Wilcoxon rank-sum test and χ^2 tests were used.

Supplementary Table 5
Comparison of dietary composition between participants with high plasma lipopolysaccharides (LPS) concentrations and those with low plasma LPS concentrations in participants without dementia

	High LPS ($n=28$)	Low LPS ($n=41$)	p
Food			
Rice, *n* (%)	28 (100.0)	39 (95.1)	0.511
Bread, *n* (%)	19 (67.9)	28 (68.3)	1.000
Noodles, *n* (%)	14 (50.0)	14 (34.2)	0.219
Miso, *n* (%)	17 (60.7)	29 (70.7)	0.442
Fish and shellfish, *n* (%)	13 (46.4)	30 (73.2)	0.042
Vegetables, *n* (%)	26 (92.9)	40 (97.6)	0.562
Seaweed, *n* (%)	13 (46.4)	22 (53.7)	0.628
Pickles, *n* (%)	19 (67.9)	27 (65.9)	1.000
Mushrooms, *n* (%)	17 (60.7)	25 (61.0)	1.000
Fruits, *n* (%)	22 (78.6)	37 (90.2)	0.296
Beef and pork, *n* (%)	19 (67.9)	30 (73.2)	0.788
Chicken, *n* (%)	16 (57.1)	27 (65.9)	0.614
Soybeans and soybean-derived foods, *n* (%)	26 (92.9)	35 (85.4)	0.458
Milk and dairy products, *n* (%)	24 (85.7)	34 (82.9)	1.000
Green tea, *n* (%)	22 (78.6)	26 (63.4)	0.197
Coffee, *n* (%)	20 (71.4)	33 (80.5)	0.400
Cooking method			
Boiling, *n* (%)	24 (85.7)	36 (87.8)	1.000
Steaming, *n* (%)	1 (3.6)	7 (17.1)	0.130
Raw, *n* (%)	24 (85.7)	36 (87.8)	1.000
Deep-frying, *n* (%)	13 (46.4)	11 (26.8)	0.124
Frying, *n* (%)	16 (57.1)	30 (73.2)	0.199
Use of soup stock, *n* (%)	27 (96.4)	39 (95.1)	1.000
Use of fermented seasoning, *n* (%)	27 (96.4)	40 (97.6)	1.000

Data are represented as numbers of patients (%). Wilcoxon rank-sum test and χ^2 tests were used.

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Supplementary Table 6
Comparison of the gut microbiota and their metabolites between participants with high plasma lipopolysaccharides (LPS) concentrations and those with low plasma LPS concentrations in participants without dementia

	High LPS ($n = 52$)	Low LPS ($n = 56$)	p
Gut microbiome	0.608		
Enterotype I, *n* (%)	20 (38.5)	26 (46.4)	
Enterotype II, *n* (%)	2 (3.9)	3 (5.4)	
Enterotype III, *n* (%)	30 (57.7)	27 (48.2)	
F/B ratio	1.54, 0.98–2.30	1.28, 0.73–2.01	0.285
Metabolite			
Ammonia, mg/g	0.62, 0.27–0.96	0.63, 0.40–0.95	0.464
Succinic acid, mg/g	0.03, 0.03–0.14	0.03, 0.03–0.39	0.682
Lactic acid, mg/g	0.03, 0.03–0.03	0.03, 0.03–8.97	0.002
Formic acid, mg/g	0.05, 0.05–0.05	0.05, 0.05–0.05	0.113
Acetic acid, mg/g	2.26, 0.03–4.96	3.58, 0.83–9.24	0.071
Propionic acid, mg/g	0.14, 0.03–1.25	0.71, 0.03–1.83	0.182
Iso-butyric acid, mg/g	0.04, 0.03–0.18	0.07, 0.03–0.19	0.613
n-butyric acid, mg/g	0.14, 0.03–0.57	0.14, 0.03–0.84	0.541
Iso-valeric acid, mg/g	0.03, 0.03–0.31	0.03, 0.03–0.26	0.649
n-valeric acid, mg/g	0.19, 0.03–2.13	0.39, 0.03–2.59	0.379
Phenol, μg/g	0.92, 0.0003–1.97	1.02, 0.0003–1.78	0.841
P-cresol, μg/g	0.43, 0.0003–118.71	0.20, 0.0003–63.03	0.298
4-Ethylphenol, μg/g	0.23, 0.0003–0.91	0.07, 0.0003–0.98	0.785
Indoles, μg/g	0.78, 0.0003–28.63	1.30, 0.13–24.32	0.806
Skatole, μg/g	0.0003, 0.0003–5.61	0.0003, 0.0003–0.24	0.418

Data are represented as medians, interquartile ranges, or numbers of patients (%). Wilcoxon rank-sum test and the χ^2 tests were used. Enterotype I: *Bacteroides* >30%, enterotype II: *Prevotella* >15%, enterotype III: others. F/B ratio, Firmicutes/Bacteroidetes ratio.

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Section 4

Oral Microbiome and Periodontal Function in Alzheimer's Disease

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Periodontal Inflammation in Relation to Cognitive Function in an Older Adult Danish Population

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Abstract. Inflammation plays a significant role in Alzheimer's disease (AD) pathogenesis. Studies have shown that systemic, peripheral infections affect AD patients. Cognitive dysfunction is a consistent finding in AD and periodontal disease is a chronic, peripheral infection often resulting in tooth loss. We hypothesized that older adults with periodontal inflammation (PI) or many missing teeth would show impaired cognition compared to subjects without PI or with few missing teeth, and among subjects with PI, those with many missing teeth would show impaired cognition compared to those with few missing teeth. The effect of PI/tooth loss on cognitive function [measured by Digit Symbol (DST) and Block Design (BDT) tests] was assessed in 70-year old Danish subjects. We found: 1) subjects with PI obtained lower mean DST scores compared to subjects without PI ($p < 0.05$); 2) subjects with many missing teeth had lower mean DST and BDT scores compared to subjects with few missing teeth ($p < 0.05$); 3) the association of PI with DST and BDT scores was dependant on the number of missing teeth (interaction: $p = 0.03$ and $p = 0.06$); and 4) education and previous cognitive scores (age 50) were important covariates. Subjects with PI had significantly lower adjusted mean DST scores compared to subjects without PI. However for adjusted BDT, the significance held only for subjects with few missing teeth. No difference in the adjusted DST and BDT scores was seen between subjects with many missing teeth compared to those with few missing teeth. These results support the hypothesis that PI may affect cognition.

Keywords: Alzheimer's disease, BDT, cognition, DST, inflammation, periodontal disease, tooth loss

INTRODUCTION

Worldwide, more than 25 million persons suffer from dementia, with 50–60% of these individuals having Alzheimer's disease (AD). As the world population ages, the number of persons with AD is expected to

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increase. Cognitive dysfunction is a consistent finding in AD and therefore, investigating factors that increase the risk of cognitive dysfunction will likely shed additional light on disease mechanisms for AD and provide strategies to reduce this risk.

The specific factors involved in the etiology and pathogenesis of AD are not well-characterized [1]; however, inflammation is thought to play a significant role [2]. Brain inflammation is characterized by elevated levels of inflammatory molecules, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP), that participate in activating and perpetuating pathways leading to neurodegeneration. The significance of the inflammatory hypothesis is that factors capable of altering levels of these molecules, such as peripheral inflammatory/infectious conditions, may alter the expression and progression of AD [3]. In support of this hypothesis, several clinical studies investigated the value of systemic inflammatory markers in predicting the onset of AD. For example, elevated CRP increased the risk of both developing AD [4] and of cognitive decline in various populations [5], while other studies have failed to demonstrate these associations [6, 7]. Studies examining proinflammatory cytokines such as IL-6 and TNF-α are also contradictory [6, 8–10]. The variation in results may be a function of differences in the timing, duration, and nature of the inflammatory processes, or because the inflammatory molecules studied may not represent the complete inflammatory phenotype.

Peripheral infections may also be linked to the "inflammatory hypothesis" [11]. Studies showed that in persons with AD, peripheral infections increased the risk of delirium [12, 13] and impaired cognitive function [13–15]. In a study of twins, peripheral infections were found to hasten AD onset [16]. Several bacterial agents have been proposed to be implicated in the pathogenesis of AD including *Chlamydophila pneumoniae (C. pneumoniae), Helicobacter pylori,* and several types of spirochetes including various types of periodontal spirochetes [17–22]. With respect to the involvement of *C. pneumoniae* and various types of spirochetes in AD, recent reviews critically evaluated all the positive and negative data available in the literature [22, 23].

Periodontal disease (PD) is a peripheral infectious chronic disease caused by specific bacteria [24]. Approximately 85% of these bacteria are Gram negative [25], with *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Tannerella forsythensis (T. forsythensis), Porphymonas gingivalis (P. gingivalis),* and *Treponema denticola (T. denticola)* considered prime periodontal pathogens [25, 26]. The later three are all Gram negative anaerobic species and *P. gingivalis, A. actinomycetemcomitans,* and *T. denticola* are capable of tissue invasion. Facilitated by the presence of inflamed and ulcerated pockets that are features of PD, periodontal bacteria and their products can gain access to the circulation resulting in bacteremia and systemic dissemination of bacterial products [27]. Studies showed that *P. gingivalis* invaded human aortic endothelial cells and induced production of proinflammatory molecules, and *A. actinomycetemcomitans, P. gingivalis,* and *T. denticola* were recovered in atherosclerotic plaques [28, 29] and brain tissue [20]. One study showed that *Treponema* species, including *T. denticola,* were detected with higher frequency in postmortem AD specimens compared to age-matched non-AD specimens, and specimens from AD subjects had a greater number of *Treponema* species than controls [20, 21]. Although not replicated, these results raise important questions as to the role of peripheral infections including PD in AD pathogenesis. PD may also result in significant systemic inflammation characterized by high CRP [30] and inflammatory molecules such as IL-1β, IL-6, and TNF-α [31]. Therefore, periodontal bacteria and systemic inflammatory molecules may contribute to brain inflammation that characterizes AD and affects its expression [3].

Clinical data also link PD to AD. Both cross-sectional and longitudinal studies have shown that gingival bleeding, loss of periodontal attachment, periodontal probing depth, alveolar bone loss (indexes of PD), and antibodies against periodontal pathogens (an index of infection and host response to infection with periodontal pathogens) were significantly associated with lower cognitive function and decline even after extensive adjustment for covariates [32–34]. In addition, patients with AD had higher numbers of positive tests for antibodies against periodontal bacteria compared to normal controls [35].

Tooth loss may be the ultimate outcome for a tooth with PD. Therefore, tooth loss may be a good index of exposure [36] for cognitive impairment/AD. Indeed, studies have reported that tooth loss is associated with cognitive impairment in females [37], cognitive decline in men [32], and an increased likelihood of being diagnosed with AD or dementia later in life [38, 39], while findings from other studies are not in concurrence [37, 40]. PD may be a risk factor for cognitive dysfunction and AD, but alternatively poor cognition may also lead to poorer oral health [41–43]. Therefore,

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it is important to evaluate whether previous cognition affects the relationship between PD and current cognition.

PD has been linked to other systemic inflammatory conditions such as cardiovascular disease [44–47]. In these studies, several measures of periodontal infection have been used including clinical PD, bacterial burden, systemic antibody levels against periodontal pathogens, and tooth loss. When tooth loss is used as an exposure for PD, several factors should be considered. On one hand, tooth loss may represent past severe periodontal infection, and studies have shown that subjects with a large number of missing teeth had severe PD [48]. On the other hand, tooth loss may represent a removal of periodontal infection. Consequently, it is possible that tooth loss can be either positively or negatively associated with systemic inflammation [48]. To further complicate the relationship between systemic and oral health, tooth loss is also dependent upon on a multitude of other factors including dental caries history, general health, socio-economic factors, education, specific population, culture, and previous cognition [40].

In the current study, we tested the hypothesis that subjects with periodontal inflammation (PI) obtain lower scores on cognitive tests compared to subjects without PI. Since tooth loss may represent long-term severe, periodontal infection and inflammation, we also hypothesized that subjects with many missing teeth obtain lower scores on cognitive tests compared to subjects with few missing teeth and that among subjects with PI, those with many missing teeth have lower cognition compared to those with few missing teeth.

MATERIALS AND METHODS

Study population

Participants. In 1964, the Copenhagen County Hospital and the County Mental Hospital in Glostrup, Copenhagen, Denmark began their cooperation on a medical-psychiatric prevalence study of people born in 1914 who lived in municipalities close to the two hospitals [49]. In total 976 individuals were invited and 802 participated in the medical component of the study, and 698 completed the Wechsler Adult Intelligence Scale (WAIS), which measures several domains of cognitive function. In 1984 when the participants were 70 years old, a follow-up study was conducted that included 4 subtests of the WAIS administered to 341 subjects. The oral/periodontal examinations were performed only at the follow-up examination in 1984 on 375 dentate subjects. The present analyses were performed on the 152 individuals with data on both WAIS assessments and oral/periodontal examination.

Cognitive assessment. Due to lack of resources, a substantial proportion of the cohort completed only four subtests [digit span, digit symbol (DST), picture completion, and block design (BDT)] of the WAIS at the 70-year (1984) follow-up. The current analysis was based on the DST and BDT because anomalous results indicated that the digit span and picture completion subtests had not been administered correctly at the 70-year assessment [50].

Of note, the same Danish translation of the WAIS was used in all studies of the 1914 cohort, and an effort was made to use the original testing procedures and scoring criteria [51]. Importantly, for the included subsample, the cognitive tests at ages 50 and 70 years were administered by different testers.

Outcome variables and the exposure of interest

The DST, a measure of psychomotor speed, involves multiple cognitive processes, including sustained attention, visuospacial perception, working memory, and visuomotor function [52]. For this test, subjects were presented with pairs of digit-symbols and then asked to match the corresponding symbols to the digits presented to them. The number of correct symbols paired in 90 seconds is the test score (range 0–90). The BDT tests visuospatial abilities and also involves multiple processes such as perceptual analysis, visuospacial and visuoconstructive abilities. For this test, the subject is asked to replicate a shown design with red-white blocks within a time limit (score range 0–48). Both the DST and BDT require efficient working memory, and studies have shown that persons with preclinical and early AD have impaired DST and BDT scores [53].

Assessment of the periodontal condition was conducted using clinical measures. Each tooth was assessed by inspection, probing, and scored using a Modified Community Periodontal Index (MCPI) as described previously [54]. Briefly, the MCPI was recorded for each tooth present and clinically scored as follows:

MCPI score 0: no sign of inflammation or periodontal pockets around the tooth;
MCPI score 1: signs of inflammation partially circumscribing the tooth, no periodontal pockets;
MCPI score 2: signs of inflammation circumscribing the tooth, no periodontal pockets;

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MCPI score 3: signs of inflammation circumscribing the tooth, periodontal pockets are 4–6 mm;
MCPI score 6: signs of inflammation circumscribing the tooth, periodontal pockets >6 mm;
MCPI score 8: signs of inflammation circumscribing the tooth, advanced periodontal destruction.

PI, the primary exposure of interest, was defined as "positive" if the MCPI score was ≥3 for at least 10% of the remaining teeth and "negative" in all other instances [48, 55].

Tooth loss (secondary exposure) was assessed by counting the number of missing teeth. Two groups of tooth loss (TL) were defined: 0–10 and 11+ missing teeth.

Covariates

Several social, oral, and general health covariates were used in the analysis. The measure of education was collected from the subjects when they were 50 year olds in 1964 and included data on school education (scored on a 1- to 3-point scale) and vocational training (scored on 1- to 5-point scale) [50]. The scores were combined to "low" (scores 1–2); "medium" (scores 3–5); and "high" (scores ≥6). Smoking was defined as never (for never smokers), past (ever smokers who had quit smoking), current light smoker (current smoking, 1–13 cigarettes/day), and current heavy smoker (current smoking, ≥14 cigarettes/day). Alcohol consumption was expressed as the number of drinks/day (continuous). The variable decayed and filled teeth (DFT) was used as an index of caries experience and defined as the total number of carious and filled teeth. Hypertension was defined by using a combination of systolic (SBP) and diastolic (DBP) blood pressures (both in mmHg), and history of hypertension at age 70 was classified as "no hypertension" (SBP <145 and DBP ≤90 and no history of hypertension), "mild" (SBP ≥145 but <155 and DBP <90 and no history of hypertension), and "moderate/severe" hypertension (SBP ≥145 or DBP ≥90 and a history of treated or untreated hypertension). Physician-diagnosed diabetes was coded as yes or no. Signs of cardiac ischemia (yes versus no) were derived from electromyography (ECG) data (age 70). Serum/plasma levels of triglyceride and total cholesterol were measured in mmole/l. We created a cardiovascular disease risk (CVDR) score, modeled after the Framingham risk score [56], by using individual scores for systolic and diastolic hypertension, diabetes, smoking, cholesterol, and triglycerides.

Statistical methods

Statistical analyses were performed using SPSS version 17 (Chicago, IL). High versus low DST and BDT scores at age 70 were defined by dichotomizing the DST and BDT scores and using the upper tertile versus the lower tertiles and the upper tertiles versus the lower tertile, respectively. The cut-point was 29 (29+ versus <29) for the DST and 32 for the BDT (32+ versus <32). Seventy percent of the subjects were classified as having PI.

Following bivariate descriptive analyses, we performed our subsequent analysis in 3 steps. First, we used two-way ANOVA to test whether DST or BDT mean scores are different: 1) between subjects with and without PI; 2) between subjects with 11+ missing teeth and those with 0–10 missing teeth; and 3) whether there was an interaction between PI and tooth loss groups (i.e., whether the effects of PI on cognitive tests depend on tooth loss). In these analyses, DST and BDT scores at age 70 years are the dependent variables, and PI and tooth loss are the independent variables.

Education, previous cognition score, gender, cardiovascular disease factors, behavior (smoking, alcohol), and other oral health measures (DFT scores) may affect cognitive performance [51]. Therefore, our second analyses (ANCOVA) extended the primary ones by adding these covariates sequentially to the two-way ANOVA models, and thus several adjusted means (AdjMean) were computed. In each analysis, the main effects and interactions between independent variables on cognitive scores were evaluated.

Third, we used multivariable logistic regression to model the association between PI (y/n) and cognitive performance (low versus high cognitive test scores). The dichotomized DST and BDT scores were the dependent variables. Education, cognitive test scores at age 50, gender, cardiovascular (ECG signs of cardiac ischemia, CVDR score), behavior (smoking, alcohol consumption), and oral health (tooth loss, DFT scores) measures were used as covariates to construct several alternative models determined *a priori*. These covariates were selected based on their potential to confound the primary associations of interest. ANCOVA modeling of BDT revealed an interaction between PI and tooth loss. Therefore we conducted our logistic regression analyses after stratifying by tooth loss category (0–10 and 11+ missing teeth). The total number of significance tests is relatively large; however, given the exploratory nature of the study, an unadjusted 5% level of significance was used.

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Table 1
Cognitive scores in relation to study subject characteristics ($n = 152$)

	n%	DST score mean (SD)	p-value	BDT score mean (SD)	p-value
Oral health					
PI					
No	30	41.0 (10.7)		33.0 (6.8)	
Yes	70	33.4 (10.5)	0.00 (T)	31.2 (7.9)	0.03 (T)
# missing teeth					
0–10	51	38.8 (11.5)		32.9 (7.4)	
11+	49	32.3 (9.6)	0.00 (T)	29.1 (6.7) T	0.00 (T)
Demographic					
Gender					
Female	48	36.7 (11.3)		29.1 (6.2)	
Male	52	34.8 (11.0)	0.29 (T)	32.8 (7.9)	0.00 (MW)
Education					
Low	26	30.4 (10.3)		27.3 (5.4)	
Medium	60	36.2 (9.9)		31.7 (6.0)	
High	14	43.9 (12.0)	0.00 (A)	35.5 (8.5)	0.00 (KW)
Behavior					
Smoking					
Never	24	35.2 (10.3)		31.3 (7.5)	
Past	42	36.3 (11.4)		31.3 (6.9)	
Current <14	24	35.3 (12.6)		30.4 (8.1)	
Current ≥14	11	35.7 (8.2)	0.96 (A)	31.1 (6.3)	0.93 (A)
Systemic health					
Hypertension					
None	49	35.3 (10.2)		31.4 (7.2)	
Mild	23	38.6 (9.6)		30.6 (7.0)	
Mod/Sev	14	34.0 (14.3)	0.17 (A)	31.2 (7.6)	0.81 (A)
Cardiac ischemia (ECG)					
No	28	36.7 (11.0)		30.8 (7.1)	
Yes	72	33.3 (10.0)	0.09 (T)	31.2 (7.4)	0.80 (T)

DST = Digit Symbol Test; BDT = Block Design Test; ECG = Electrocardiography (cardiac signs of ischemia). Mean (SD) = unadjusted means and standard deviations. Group-differences for PI, missing teeth groups, ECG were assessed using the t-test (T), for gender Mann-Whitney U test (MW), and for education, smoking, and hypertension ANOVA (A) or Kruskal-Wallis (KW).

RESULTS

The population used in this study was homogeneous in terms of race/ethnicity and age, with all subjects aged 70 years in 1984. Females and males were approximately equally distributed (Table 1). When we compared subjects who were included in the analysis versus those who were not, we found that included subjects tended to have more teeth and higher mean DFT scores. However, if edentate subjects (those without teeth) were excluded, the differences disappeared. Included subjects tended to be more educated and had higher DST and BDT scores at 50 and 70 years of age.

DST scores ranged from 13–77, with a mean of 35.7 (sd = 11.0), while BDT scores ranged from 4–48, with a mean of 29.9 (sd = 7.5). As shown in Table 1, individuals with PI had, on average, lower DST and BDT scores than persons without PI ($p < 0.05$), and subjects with 11+ missing teeth had statistically significantly lower mean DST and BDT scores than those with fewer lost teeth ($p < 0.01$). Further, mean BDT scores were statistically significantly higher for men than women and for persons with higher education ($p = 0.00$). Higher DST scores were significantly related to having higher education, mild hypertension ($p = 0.03$), and lack of signs of cardiac ischemia, based on ECG ($p = 0.09$).

Table 2 and Fig. 1 show PI and tooth loss in relation to DST and BDT scores. Before adjusting for other covariates, the main effect of PI was statistically significant as regards mean DST score (means 38.8 versus 33.6; $p = 0.01$), but this was not the case for BDT (means 31.8 versus 30.3; $p = 0.25$). The main effect of tooth loss was significant for both DST and BDT cognitive tests (means 39.7 versus 32.7 and 33.2 versus 28.8; $p < 0.01$ for both), while the interaction between tooth loss and PI was statistically significant for DST ($p = 0.03$) and approached significance for BDT ($p = 0.06$). After stratifying the analysis on tooth

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Table 2
Cognitive test scores in relation to PI and tooth loss

	No PI		PI				
	Missing teeth		Missing teeth		*P* values		
	0–10	11+	0–10	11+	$PI_{(M)}$	$TL_{(M)}$	$PI*TL_{(I)}$
N	32	15	46	59			
WAIS subsets							
[Means (SEM)]							
DST							
Crude	44.3 (1.8)	33.3 (2.6)*	35.0 (1.5)*	32.1 (1.3)*	***0.01***	***0.00***	***0.03***
AdjMean1	43.0 (1.7)	34.1 (2.5)*	34.9 (1.5)*	32.7 (1.3)*	***0.01***	***0.00***	0.06
AdjMean2	38.8 (1.3)	36.4 (1.8)	34.9 (1.1)*	34.5 (0.9)*	***0.02***	0.33	0.45
AdjMean3	38.5 (1.3)	36.2 (1.8)	35.0 (1.0)*	34.6 (0.9)*	***0.04***	0.31	0.50
AdjMean4	38.5 (1.3)	37.2 (1.9)	34.9 (1.0)*	34.4 (1.0)*	***0.02***	0.54	0.80
AdjMean5	39.8 (1.8)	36.1 (1.2)	36.0 (1.2)*	33.5 (1.4)*	***0.02***	0.16	0.71
BDT							
Crude	35.2 (1.2)	28.3 (1.8)*	31.3 (1.0)*	29.3 (0.9)*	0.25	***0.00***	0.06
AdjMean1	34.3 (1.1)	28.8 (1.7)*	31.2 (1.0)*	29.7 (0.9)*	0.37	***0.01***	0.10
AdjMean2	33.2 (0.8)	29.4 (1.2)*	30.4 (0.7)*	30.8 (0.6)*	0.40	***0.05***	***0.01***
AdjMean3	33.1 (0.8)	29.4 (1.2)*	30.4 (0.7)*	30.9 (0.6)*	0.44	0.10	***0.02***
AdjMean4	33.4 (0.8)	30.0 (1.2)	30.5 (0.7)*	31.3 (0.6)*	0.33	0.15	***0.02***
AdjMean5	33.1 (1.1)	30.2 (1.3)	30.4 (0.8)*	31.5 (0.9)	0.44	0.55	***0.02***

* $p < 0.05$ when compared to subjects with NoPI and 0–10 missing teeth (LSD).
- Crude means: For DST scores, there was a significant difference between subjects with PI versus subjects without PI, but not for BDT scores ($p = 0.01$ and $p = 0.25$). For both cognitive tests there was a significant difference between subjects who had lost 11+ versus 0–10 teeth (for all $p = 0.00$); the PI* tooth loss interaction term was $p = 0.03$ for DST and 0.06 for BDT (2-way ANOVA). Adjusted means: the adjusted means computed were: AdjMean1 (covariate: education); AdjMean2 (covariate: cognitive test at age 50); AdjMean3 (covariates: education, cognitive test at age 50 and gender); AdjMean4 (covariates: education, cognitive test at age 50, gender and ECG signs of cardiac ischemia, CVDR score; and AdjMean5 (covariates: education, cognitive test at age 50, gender and ECG signs of cardiac ischemia, CVDR score, smoking, alcohol, and DFT scores). For DST there was a significant difference in fully adjusted means (AdjMeans5) between subjects with PI versus those without PI ($p = 0.02$) but not for BDT ($p = 0.44$). For all cognitive tests the significant difference between subjects who had lost 11+ versus 0–10 teeth was no longer present ($p = 0.55$; $p = 0.16$). The interaction term for PI and tooth loss was lost for the DST ($p = 0.71$) but acquired statistical significance for the BDT ($p = 0.02$). PI = Periodontal inflammation; WAIS = Wechsler Adult Intelligent Scale; DST = Digit Symbol Test; BDT = Block Design Test; (M) = Main effect; (I) = Interaction; LSD = least significant difference.

loss, significant mean differences in DST were seen between subjects with and without PI among participants with 0–10 missing teeth (means 35.0 and 44.3, $p < 0.05$), but no significant difference was observed between subjects with and without PI among subjects with 11+ missing teeth (means 32.1 and 33.3, $p > 0.05$).

In the fully adjusted model (Table 2 and Fig. 1), the main effect of PI remained significant for DST (means 38.0 versus 34.3; $p = 0.02$), but this was not the case for BDT (31.9 versus 30.8; $p = 0.44$). The main effect of tooth loss was not statistically significant for either of the two tests (means 37.7 versus 34.6 and 31.4 versus 30.4; $p > 0.05$ for both tests). The interaction between PI and tooth loss was not statistically significant for DST ($p = 0.71$), but significant for BDT ($p = 0.02$). Stratified analysis showed a significant effect of PI on BDT means among subjects with 0–10 missing teeth ($p = 0.02$), but no effect among subjects with 11+ missing teeth ($p = >0.05$). These results showed that together, education and previous cognitive scores had a significant impact on subsequent cognitive scores and affected the relationship between PI/tooth loss and cognition at age 70.

Table 3 shows the association between cognitive scores (low versus high) in subjects with versus those without PI using logistic regression. In our crude analysis the presence of PI was strongly associated with having a low DST score (OR = 5.38). This was only modestly attenuated (OR = 4.79) when we further adjusted for DST score at age 50 years, education and gender (Table 3, Model 2). While the addition of cardiovascular factors slightly increased the adjusted OR to 5.89 (Table 3, Model 3), the addition of oral health measures increased the OR to 6.80 (Table 3, Model 4), and when all the variables were entered simultaneously (Table 3, Model 5), the adjusted OR for PI and DST score remained strong ($OR_{adj} = 7.00$). These results showed that subjects with PI are more likely to score low on the DST even after adjusting for previous cognition and other potential confounders.

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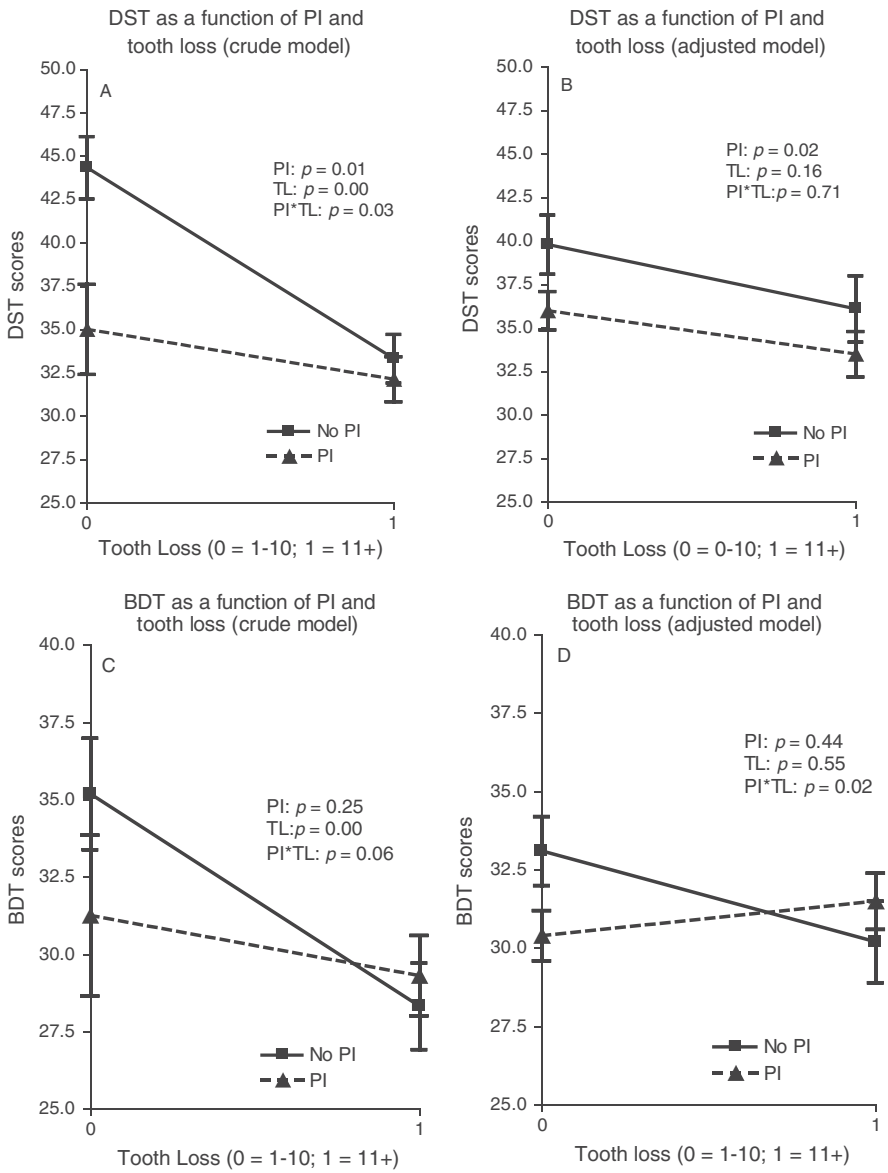


Fig. 1. The effect of periodontal inflammation (PI) and tooth loss on cognitive scores. A) ANOVA showed significant interaction [F (Interaction) = 4.8, $p = 0.03$] between PI and tooth loss on the mean DST scores. B) The interaction shown in A lost its significance in fully adjusted model [F (Interaction) = 0.34, $p = 0.71$]. The difference in DST scores between ± PI was maintained. C) For BDT scores, ANOVA showed a trend for interaction [F (Interaction) = 4.78, $p = 0.06$] between the PI and tooth loss. D) The interaction between PI and tooth loss became significant in fully adjusted model [F (Interaction) = 6.0, $p = 0.02$]. DST = Digit Symbol Test; BDT = Block Design Test; PI = periodontal inflammation. Fully adjusted model = covariance: Education, cognitive test at age 50, gender, and ECG signs of cardiac ischemia, CVDR score, smoking, alcohol, and DFT scores.

In our analysis using ANOVA, we found an interaction between PI and tooth loss on BDT scores after adjustment for previous BDT score and education (Fig. 1). Therefore, we examined the odds of testing low on the BDT separately for subjects with 0–10 versus 11+ missing teeth (Table 4). For subjects with 0–10 missing teeth, our crude analysis with the dichotomized BDT score as the outcome variable revealed that the presence of PI was associated with having a low BDT score (OR = 3.26). The adjusted ORs tended to increase with the addition of additional covariates, and when all covariates were included in Model 5, the adjusted OR was 7.62 (Table 4, Model 5). For those subjects with 11+ lost teeth, there was

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Table 3
Odds ratios for a low versus high Digit Symbol Test score at age 70 by periodontal inflammation status.

WAIS subset		OR	95% CI	p-value
PI				
No		1.00	Ref	
Yes				
	Crude	5.38	1.97–14.73	0.001
	Model1	4.70	1.53–14.99	0.007
	Model2	4.79	1.47–15.63	0.009
	Model3	5.89	1.63–21.31	0.007
	Model4	6.80	1.83–25.31	0.004
	Model5	7.00	1.74–28.16	0.006

Crude, Model 1 variables: PI and DST scores age 50, Model 2 variables: Model 1+ education, gender, Model 3 variables: Model 2+ ECG signs of cardiac ischemia, CVDR score, Model 4 variables: Model 3+ tooth loss and DFT, Model 5 variables: Model 4+ smoking and alcohol. WAIS = Wechsler Adult Intelligence Scale.

an inverse association between having PI and low BDT scores (Table 4, 11+ missing teeth, all models); however, none of the OR point estimates were statistically significant. These results showed that among subjects with 11+ missing teeth, persons with PI were less likely to score low on the BDT than persons without PI.

DISCUSSION

In this study involving an older Danish population, we showed that the effect of PI on DST and BDT scores was dependent, at least in part, on the number of missing teeth (PI by tooth loss interaction). Among subjects with 0–10 missing teeth, we found that persons with PI had lower mean DST and BDT scores compared to those without PI.

When we adjusted for previous cognitive test score, the combined effect of PI and tooth loss was different for DST and BDT scores. Subjects with PI had significantly lower DST scores compared to those without PI. Subjects with PI also had lower mean BDT scores compared to those without PI but only among subjects with fewer than 11 missing teeth. These patterns persisted when further adjustments were made for the relevant covariates. The results demonstrate that cognition at the age of 50 has an important impact on cognition at age 70 and that adjusting for the former was an essential element in our analysis. Cognition scores at age 70 can reflect both the initial development of cognition and possibly age-related changes. Changes in cognition from 50 to 70 years are analyzed when the model includes previous cognitive test scores.

In line with the above results, the strength of association between PI and DST scores was strong even after extensive adjustment for potential confounders. The positive association between PI and BDT scores was also strong, but existed only in subjects who had lost fewer than 11 teeth. There was an inverse relationship between PI and BDT scores among subjects who had lost 11+ teeth, but the association was not statistically significant.

The finding of an association between PI and decreased cognitive function is consistent with other studies [33–35]. The ORs for the association between PI and DST and BDT were stronger in our study compared to other studies [33, 34] possibly due to inherent differences in the populations studied, definition of PD, tests used to measure cognitive function, and statistical management of potential confounders.

Another important finding of our study was that there was a statistically significant difference in unadjusted DST and BDT scores between subjects with 0–10 versus 11+ missing teeth as we hypothesized. However, when we adjusted for all covariates,

Table 4
Odds ratio for a low versus high Block Design score at age 70 by periodontal inflammation among participants with 0–10 and 11+ missing teeth

WAIS subsets		0–10 Missing teeth			11+ Missing teeth	
		OR	95% CI	*p*	OR	95% CI
Block Design						
No PI		1	Ref		1	Ref
Yes						
PI	Crude	3.26	1.26–8.38	0.01	0.41	0.84–2.04
	Model1	4.32	1.16–16.12	0.03	0.23	0.04–1.50
	Model2	5.74	1.37–24.17	0.02	0.25	0.03–2.03
	Model3	5.18	1.19–22.54	0.03	0.54	0.05–5.30
	Model4	7.88	1.39–44.69	0.02	0.61	0.06–6.32
	Model5	7.62	1.29–44.89	0.03	0.35	0.01–17.39

WAIS = Wechsler Adult Intelligent Scale. Crude, Model 1 variables: PI and BDT at age 50, Model 2 variables: Model 1+ education and gender, Model 3 variables: Model 2+ ECG signs of cardiac ischemia, CVDR score, Model 4 variables: Model 3+ tooth loss and DFT, Model 5 variables: Model 4+ smoking and alcohol.

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including education and previous cognitive scores, these differences were no longer statistically significant for either of the cognitive tests. These results showed that although subjects with many missing teeth tended to have lower DST and BDT scores compared to those with few missing teeth, these differences could be explained by other variables, such as previous cognition and education. It is possible that previous cognition or cognitive changes and education played a significant role in a given subject's oral health and their treatment decisions over the life course [41, 57–59]. Our results are also consistent with those of Starr et al. who reported that lower original intelligence explained the difference in cognition between dentate and edentate subjects [40].

The interaction between PI and tooth loss for BDT scores appeared to be due mainly to the significant decrement in BDT scores in subjects without PI but with 11+ missing teeth compared to the ones with 0–10 missing teeth. This decrement could be due to the fact that subjects with many missing teeth may have had a history of previous PD, had the teeth removed, but the impact of the tooth-related inflammation/infection persisted [36, 60, 61] and thus affected the BDT scores. Once the periodontally-involved teeth were removed, the remaining teeth were less likely to have serious PD.

Assuming that tooth loss largely represented past severe PD, we also hypothesized that among subjects with many missing teeth, those with periodontal inflammation would have lower cognitive scores compared to those without periodontal inflammation; however, our results showed no difference in cognitive scores between these 2 groups. It is possible that the combination of PI and many missing teeth did not result in significant systemic exposure compared to the combination of many missing teeth and no PI. This can be due to less severe PI or removal of periodontal teeth early when the damage was minimal. In addition, tooth loss may also be due to other causes. Poor oral health may lead to poor diet that associates with lower cognition [62]. However, to further elucidate the role of PI and tooth loss on cognition, larger prospective studies are needed. Evaluation of cognition, PI and tooth loss longitudinally paralleled by measures of inflammation/infections and host defense are desirable.

Education and previous cognitive scores were found to be important covariates although the interaction between PI and tooth loss was significant for BDT scores but not for DST scores. These different patterns may reflect the nature of the cognitive test itself. There are commonalities as well as differences between these tests. Classically, DST [or its revised Digit Symbol Substitution (DSST)] and BDT tests are used to assess visuospatial abilities. A recent study showed a strong relationship between DSST and performance on episodic memory [52], tests that are also dependant on executive [63] and the speed-processing functions [64, 65]. The authors argued that DSST may be a reliable predictor of memory decline as the relationship between memory performance and DSST was stronger than between memory and speed processing or executive function. In our Glostrup data, DST and BDT were among the WAIS subtests showing the strongest association with aging [50]. Both BDT and DST are sensitive to brain damage and correlate with the general g-factor of intelligence [66] known to impact general and oral health [40]. DST and BDT may also reflect different anatomical and physiological processes. For example, functional studies showed that BDT activated predominantly parietal lobe [67, 68] while DST activated in addition to parietal lobe several areas of the frontal lobe [69]. Importantly, these two tests may be administered differently by different examiners and this may have played a role in our study as the cognitive tests at age 50 and 70 were not administered by the same testers.

Our results support the hypothesis that PI may affect cognition and perhaps AD pathogenesis [70]. Inflammation is one of the key features of AD and provides the foundation for the hypothesis that inflammatory and infectious conditions may pose a risk for AD. PI appears to have significant systemic effects in our population as our previous study also found an association of PI with mortality at 21-years follow up [54]. It is also possible that both conditions (AD and PD) stem from a hyperinflammatory response to a specific injurious agent. Still another mechanism may be related to the PI contribution to cardiovascular disease [71].

As in most cross-sectional studies, reverse causation should be considered. An observed association between cognition at age 70 and PI may reflect both effects on cognition and effects of cognition on periodontal health. It is possible that patients with poor cognition or cognitive decline have poorer oral health measures such as tooth loss, caries level, and plaque control [43, 72, 73]. Although studies assessing the presence of PD in individuals with lower cognitive function or dementia are few and contradictory [43, 74], the potential effect of cognition on periodontal condition cannot be ignored [32]. In order to mitigate the concern about the directionality of the observed relationships cognitive test scores from subjects at the age 50 were included in our adjusted models. When interpreting our results, it should be considered that

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our study sample had higher education and higher cognitive scores compared to the subjects not included in the analysis.

There are several strengths of the current analysis. Our investigation centered on a homogeneous population; therefore variation in that regard was virtually eliminated, and we were able to detect statistically significant differences despite a limited number of subjects. Further, the availability of oral and systemic health variables, as well as previous cognitive scores, allowed for the inclusion of these factors as covariates in our models.

In addition to the cross-sectional design of our study, the limited battery of cognitive tests is also a limitation. One of the earliest cognitive impairments in AD is thought to be in episodic memory [75] consistent with early AD specific pathology [76]. However, recent studies have shown that in cognitively normal subjects but with amyloid pathology, cognitive decline was not limited to episodic memory but also included visuospatial abilities (assessed also by DST and BDT) [77]. Consistent with these findings, a meta analysis [53] showed that several other cognitive deficits exist early in the disease process including executive functioning, perceptual speed, verbal ability, visuospatial skill, and attention [78] as multiple brain structures and functions may be affected before the AD diagnosis [78].

The sample size was relatively small given the hypotheses being tested; nevertheless, we observed statistically significant main effects and interactions even after controlling for a number of covariates. Moreover, effect sizes were strong. That said, statistical power to detect significant differences in some analyses was likely compromised by the particularly small number of subjects (PI and 11+ missing teeth, $n = 15$). The MCPI index cannot differentiate between gingivitis, a common less severe form of PD and periodontitis, nevertheless, the index has been used previously in associations between PD and cardiovascular disease, and in our population was associated with other systemic processes [54]. At least some measurement misclassification of both outcome and exposure variables is possible; however, because measures for the independent variables included in the analysis were originally obtained by examiners who were blinded to both DST and BDT scores, it is most likely that such misclassification would have biased our estimated effect sizes toward the null. Finally, although homogeneity of the study population was generally strength of the project, it may limit the generalizability of our findings.

In conclusion, based upon data from an older Danish population, our data support the hypothesis that periodontal inflammation can affect cognition.

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Oral Inflammation, Tooth Loss, Risk Factors, and Association with Progression of Alzheimer's Disease

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Abstract. Periodontitis is a polymicrobial chronic inflammatory disease of tooth-supporting tissues with bacterial etiology affecting all age groups, becoming chronic in a subgroup of older individuals. Periodontal pathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are implicated in the development of a number of inflammatory pathologies at remote organ sites, including Alzheimer's disease (AD). The initial inflammatory hypothesis proposed that AD hallmark proteins were the main contributors of central nervous system (CNS) inflammation. This hypothesis is expanding to include the role of infections, lifestyle, and genetic and environmental factors in the pathogenesis of AD. Periodontal disease (PD) typifies a condition that encompasses all of the above factors including pathogenic bacteria. These bacteria not only are the source of low-grade, chronic infection and inflammation that follow daily episodes of bacteremia arising from everyday tasks such as brushing, flossing teeth, chewing food, and during dental procedures, but they also disseminate into the brain from closely related anatomical pathways. The long-term effect of inflammatory mediators, pathogens, and/or their virulence factors, reaching the brain systemically or otherwise would, over time, prime the brain's own microglia in individuals who have inherent susceptibility traits. Such susceptibilities contribute to inadequate neutralization of invading agents, upon reaching the brain. This has the capacity to create a vicious cycle of sustained local inflammatory milieu resulting in the loss of cytoarchitectural integrity and vital neurons with subsequent loss of function (deterioration in memory). The possible pathways between PD and AD development are considered here, as well as environmental factors that may modulate/exacerbate AD symptoms.

Keywords: Alzheimer's disease, inflammation, oral health, periodontal disease

INTRODUCTION

Alzheimer's disease (AD), a form of dementia, is the most common neurodegenerative disease worldwide [1]. The prevalence of AD increases exponentially with age, rising from 3% among the 65 to 74 year age group and to almost 50% among those around 85 years and older [2, 3]. Mental deterioration is slow

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but progressive, contributing to poor memory, disorientation, confusion, and eventually profound dementia. Susceptible individuals can take decades before clinically presenting with the disease. This implies that the etiology of AD is heterogeneous and that the importance of finding new risk factors for development in the case of late-onset AD remains a priority.

Although partial efficacy of non-steroidal anti-inflammatory drugs in some AD patients [4, 5] gave rise to the inflammatory hypothesis that accounts for the intrinsic elements of CNS inflammation [6], support also exists for the extrinsic inflammatory mediators. The elderly, for example, having suffered multiple episodes of recurrent infections, can present with dementia like clinical symptoms in likely late-onset AD cases [7] as well as those subjects with confirmed clinical diagnoses of AD [8, 9].

The innate immune responses suggest extrinsic inflammatory cytokines are involved in exacerbating neurocognition [9] and cytokine-related genes are being implicated in the susceptibility to inflammation in late-onset AD [10–12]. Furthermore, human brain tissue specimens from postmortem AD patients demonstrate evidence for neuroinflammation via the activated complement system as C1q, C3b, and reactive oxygen species are all involved in the amyloid fibril formation [11, 13, 14]. These observations are strengthened by genome-wide studies supporting the role of innate immune components such as complement receptor 1 (CR1) and a fluid-phase regulatory protein clusterin [15, 16].

PATHOLOGICAL HALLMARKS OF ALZHEIMER'S DISEASE

In AD, marked neuronal loss is observed in the hippocampus where high densities of the classical hallmark lesions are initially observed [17]. An accumulation of intraneuronal neurofibrillary tangles (NFTs) and extracellular amyloid-β (Aβ) plaques are the two easily demonstrated histological features of AD brains [18]. Synaptic dysfunction is considered as one of the earliest structural defects [19]. Specifically, NFTs indicate the severity of disease with Aβ plaques depicting disease progression [17]. NFTs in neurons constitute hyperphosphorylated tau protein that alters the polymerization and stability of microtubules [20]. The loss of synapses between neurons correlates well with cognitive decline [19]. The cumulative knock-on effect of these cytoarchitectural changes is compromised protein-protein interactions with other cytoskeletal elements eventually leading to further synaptic loss and the demise of the NFT-bearing neurons [17, 18, 20, 21].

Amyloid plaques are largely made up of fibrillary Aβ peptides $A\beta_{40/42}$ amino-acid residues and are the result of α-, β-, and γ-secretase enzymes cleaving the transmembrane amyloid-β protein precursor expressed by all CNS cells. The proteolytic fragment consisting of the last 28 residues of the amyloid-β protein precursor ectodomain prior to the membrane and including the first 12 to −14 residues of the transmembrane region generate $A\beta_{40/42}$ amino-acid residues that lead to deposition of extracellular, insoluble fibrillar Aβ. Clearing insoluble Aβ peptides from the brain involves phagocytosis by microglial cells [22, 23], an activity that invariably fails with the consequences of further accumulation of fibrillary Aβ.

Aβ peptides and insulin are known substrates of an insulin-degrading enzyme [24, 25]. Both of these proteins are important in the pathogenesis of AD and type II diabetes mellitus, respectively. Literature supports the risk-factor relationship between type II diabetes with increased risk of cognitive impairment and dementia via its potential pro-inflammatory toxicity from perturbed glucose metabolism [26]. However, it is not clear if Aβ itself promotes insulin resistance in AD with the generation of subsequent oxidative stress, reactive oxygen species, and related pro-inflammatory cascades [27]. Streptozotocin used for generating experimental diabetic rats as a model for investigating late-onset AD in relation to insulin resistance is a toxin derived from *Streptomyces* species of bacteria [28]. It is, therefore, plausible to suggest that an initial microbial trigger may be responsible for insulin resistance and the subsequent deposition of Aβ in late-onset AD.

Aβ plaques can be observed in the brains of cognitively intact individuals, but they are fewer and are generally of the diffuse ($A\beta_{40}$) type, which so far appear to have little pathological significance. Of the two forms of amyloid, fibrillary Aβ is regarded as being neurotoxic [29] and *in vitro* studies have shown it to lyse all types of cells by apoptosis [30]. Evidence from neuroradiological and neuropathological investigations link this hallmark ($A\beta_{40/42}$) to the initiation of intracerebral inflammatory response in the inherited forms of AD as well as late-onset AD [14, 31, 32].

The inflammatory element of the disease may be a significant risk factor for late-onset AD, specifically as innate immune components such as C1q and C3b are involved with plaque maturation [11, 13]. The relevance of detecting C1q and C3b within $A\beta_{40/42}$ also draws attention to the cellular mechanisms involved

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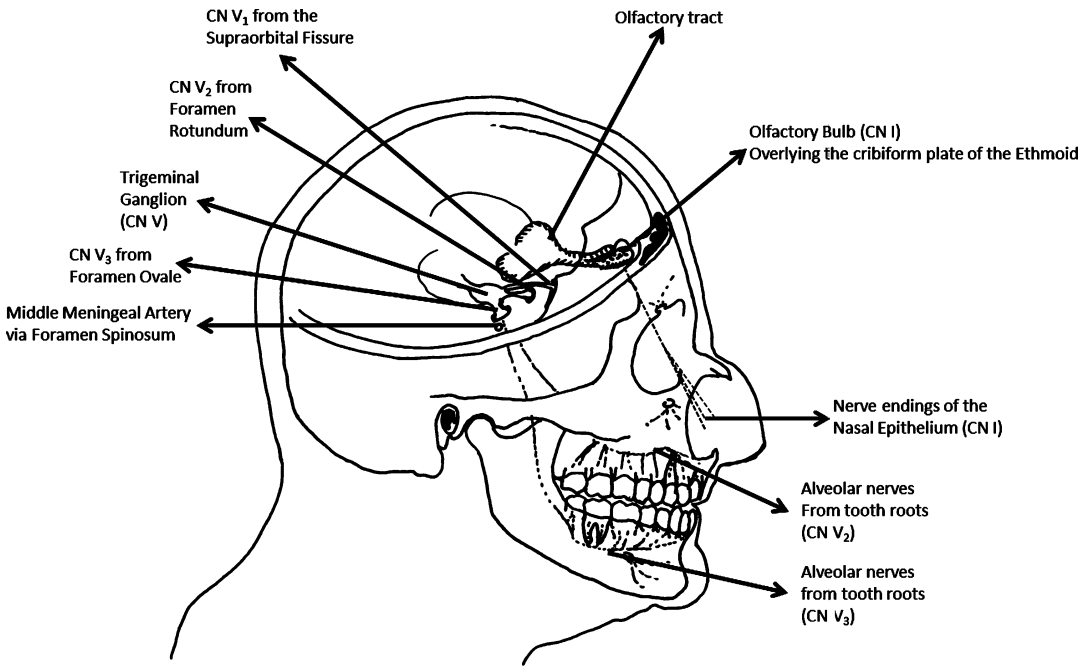


Fig. 1. Nerve pathways from the oral and nasal cavity to the brain, showing the 2nd and 3rd branches of the Trigeminal (CN V) and the Olfactory nerve (CN I). The middle meningeal artery enters the brain at the foramen spinosum in the lateral portion of the greater wing of the sphenoid, and then follows the cranial base and lateral potions of the vault, supplying the dura and bones of the calvarium.

with regulating inflammation in neurodegeneration and in clearing $A\beta_{40/42}$. The inflammatory components corroborate data from genetic studies proposing a relationship between AD and cytokine polymorphisms [12, 33], as well as the complement proteins such as clusterin and CR1 in its pathogenesis [15, 16]. With respect to the complement activation cascade, CR1 is a membrane-associated complement inhibitory protein that binds C4b and C3b acting at the C3/C5 convertase stage of the alternative and classical complement pathway, and clusterin is a fluid-phase protein that interferes with the assembly of the lytic membrane attack complex. Decrease in clearance of the lesion by the innate immune system contributes to bystander damage that promotes a vicious cycle of chronic intra-cerebral inflammatory (high endogenous levels of inflammatory mediators) response [14, 34].

WHAT IS INTRA-CEREBRAL INFLAMMATION?

In the past the brain was considered as an "immunoprivileged organ," as elements of the immune system [such as neutrophils, naive T cells (adaptive immune system), plasma proteins, and extra-cerebral-toxins to prevent inappropriate glial cell activation] cannot cross the blood-brain barrier (BBB). However, it is now understood that the circumventricular organs are not subject to the BBB [35], a region of the brain where infections (both systemic and direct via the trigeminal and olfactory nerve pathways (see Fig. 1) and inflammatory mediators can access the brain [36–38].

The brain deals with inappropriate toxins derived locally (Aβ deposits) or from extracerebral sources (infectious agents) using its own innate immune system consisting of ependymal cells, microglia, astrocytes, and oligodendrocytes [39]. Normally these cells regulate the production and uptake of endotoxins and secrete trophic factors that nurture the CNS cells and protect their functions [40]. However, following physical damage and/or invasion by foreign agents (lipopolysaccharide, LPS), glial cells (specifically microglia) bearing the LPS receptor (CD14) and the highly conserved toll-like receptors 2 and 4 (TLR 2 and 4) undergo a number of phenotypic (resting to activated state) and functional changes. Key morphological changes include thickening and shortening of branching processes attached to a hypertrophic glial cell body [41]. Once activated, microglia upregulate the expression of MHC class II molecules along with the secretion of pro-inflammatory cytokines (TNF-α

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and IL-β), complement proteins, quinolinic acid, arachidonic acid and its metabolites, nitric oxide, platelet activating factor, α and β chemokines excitatory amino acids, and free radicals [14]. When these innate factors are secreted by microglia, a local CNS inflammatory response is mounted.

In AD brains, Aβ is recognized as a nidus for intracerebral inflammation placing chronic neuroinflammation downstream of this primary hallmark [13, 14]. However, this view is changing, especially with late-onset AD cases as the importance of the innate immune molecules is being uncovered by genome-wide studies [11]. If nerve cell death and chronic CNS inflammation are common precursors of the development of dementia, explaining equivalent numbers of Aβ deposits and NFTs in clinical and subclinical AD in the very elderly who bypass dementia is important [42–45] and adds further complications to the etiological nature of AD.

Even Aβ in subclinical AD subjects, which initiates intracerebral inflammation [11, 46, 47], appears not to lead to clinical decline in cognition. One possible explanation for the lack of cognitive decline could lay in the inflammatory genetic traits of these and the non-impaired individuals with vital neuronal cells being rescued from death and so providing a "cognitive reserve." The inflammatory signals that initiate phagocytosis by microglia are driven by Aβ that involve the CD14 and TLR 2 and 4 signaling [22, 23, 48, 49], a pathway also used by microglia for bacterial LPS recognition [50]. Spontaneous loss-of-function mutation in the TLR 4 gene has been demonstrated to have an inhibitory effect on microglial cell activation. For example, presence of Aβ in the microglia with mutated gene expression [49] demonstrated reduced secretion of inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and nitric oxide. These findings further strengthen the cognitive reserve hypothesis in very old subjects in the presence of classical AD hallmark lesions.

Using an *ex-vivo* experimental model to examine the expression of pro-inflammatory cytokine profiles in whole blood from the healthy middle-aged offspring of patients affected by late-onset AD, van Exel et al. [10] reported higher levels of specific cytokines than were found in the siblings from non-AD parents. This finding also demonstrates that inflammation-related risk factors are present in currently healthy subjects who may have a genetic 'susceptibility profile' a phrase, coined by McGeer and McGeer [33] to late-onset AD. Despite the inheritance factors, the brain's response to inflammation is slow as supported by animal models of AD [51]. Currently, the greater accumulation of Aβ deposits in late-onset AD is considered to be the result of defects in the clearance system [52]. Therefore, the pertinent question would be, "what initially triggers $A\beta_{40/42}$ release in patients with late-onset AD?"

Infections are common in elderly individuals and are the main cause of death in a majority of neurodegenerative conditions. One hypothesis is that the elderly, *per se*, are immunocompromised and this correlates with their susceptibility to an increased incidence of infection [53, 54]. Recurrent bacteremia from common infections in the elderly, due to, for example, chronic periodontitis [55, 56], intra-abdominal [57], and urinary tract infections [58], will contribute to systemic circulation infections. In addition, chronic periodontitis demonstrates a promising link that encompasses environmental influences, susceptibility profiles, infectious agents, and a multitude of host's factors that affect its episodic re-occurrence [59]. Numerous studies show that tooth loss due to PD to correlate with cognitive impairment in AD [60–63]. Further studies have demonstrated systemically derived immune components such as antibodies to the periodontal pathogens circulating in the blood plasma of AD subjects [64]. In addition, AD patients with antibodies to the periodontal pathogens in their blood have displayed inflammatory mediators (cytokines) in the systemic circulation [64]. As Holmes et al. [9] suggest, these cytokines and those from alternative sources in the peripheral circulation have the potential to reach the brain parenchyma, and subsequently prime microglia to mount a local immune response with appropriate stimuli, and impair memory.

An alternative hypothesis for the role of Aβ in subclinical and/or clinical AD individuals is that Aβ is acting as an antimicrobial peptide [65] to counteract infections by functioning as part of the early innate immune defense mechanisms that mediate innate and adaptive immune responses [66]. Traditionally, antimicrobial peptides act as look-outs for invading micro-organisms to maintain the balance between commensals. The main target for antimicrobial peptides is the pathogen cell membrane, as most antimicrobial peptides are cationic [67]. Antimicrobial peptides undergo electrostatic interactions with negatively charged molecules to penetrate bacterial cell walls, including anionic lipids and LPS [67]. They then invade the lipid bilayer, creating trans-membrane pores through which leakage of ions and metabolites, cytoplasmic components, dissipation of electrical potentials, and cell death of microbes takes place [68]. This hypothesis suggests the involvement of a

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pathogenic precursor in the initiation of Aβ release before inflammation becomes detectable in the presence of this hallmark. We support this hypothesis and propose a suite of susceptibility traits and immunosuppressive (stressed or rundown) episodes during life that give way to chronic bacterial infections. These bacterial elements in the individuals with susceptibility profiles may trigger release of Aβ to neutralize their effect. Over time Aβ will accumulate in the brains of healthy but susceptible individuals and initiate neuroinflammation that may cross the threshold from subclinical to late-onset AD.

The etiological hypothesis suggests that viruses and bacteria and/or their virulence factors access the brain and thereby contribute to AD pathogenesis. A review by Holmes and Cotterell [69] provides a range of infective agents consistently being linked to AD. These include viruses such as Herpes simplex virus type I [70], *Chlamydophila pneumoniae* [71], *Treponema* spp., [72] *Borrelia burgdorferi* [73], and more recently LPS from *Porphyromonas gingivalis* (*P. gingivalis*) [74], one of the key bacteria causing PD in humans.

AGE-RELATED PERSONAL HYGIENE CHANGES AS RISK FOR INFECTIONS

Advancing age is the greatest risk factor for all forms of AD. Some consequences of advancing age are a compromised immune system [53, 54] and a neglect of general and oral personal hygiene [61, 75, 76], and such conditions are associated with recurrent, chronic infections. Recurrent, chronic infections enhance systemic hyperinflammatory profile that may lead to confusion and other dementia-like clinical features [7–9] in which the exact structural/cellular changes taking place at the time remain unknown.

Several studies support deterioration in oral health with increasing age [77–80]. The exact reasons are poorly understood, but advancing age is likely to compromise the manual dexterity of senior citizens and this may make cleaning their teeth more difficult, or perhaps it is because as general health concerns and conditions increase with age, maintenance of oral health becomes a lower priority. The elderly are more likely to be on multiple medications, many of which, as a side effect, cause xerostomia and this will inevitably be a factor in deteriorating oral health [81]. Furthermore, if the elderly suffer from physical impairments, accessing the dentist may become more difficult. Elderly people resident in care institutions are, to a certain extent, dependent on the level of care within the establishment for the level of oral hygiene and dental health they receive. These factors were supported in a large-scale survey carried out in the US by Griffin et al. [79], which found that older age groups were more likely to be edentulous or have untreated dental disease and root caries. Those who were either residents in institutions or homebound had higher levels of untreated cavities, gingivitis (a marker of poor oral hygiene), and poorer overall oral health than the elderly living independently. The study shows that cost, lack of transportation, and limited mobility were key barriers to accessing dental care for nursing home residents [79]. Other groups of elderly that show higher untreated dental disease and lower levels of oral health are those from ethnic minorities and low-income families [79].

PERIODONTAL DISEASE

PD is a polymicrobial inflammatory disease that has been estimated to affect 10–15% of the developed world population and is a major cause of tooth loss. The prevalence of PD increases with age, affecting around 50% of people over the age of 55 years [82]. The disease affects tooth supporting tissues wherein the interaction of specific bacteria and the host's immune responses play a pivotal role [59]. The host's response to bacteria and their products is an important factor in determining the extent and severity of PD [83]. The acute bacterial challenges stimulate junctional transformed pocket epithelium to produce a broad range of inflammatory mediators to guard against gingival tissue damage. The inefficient clearance of subgingival pathogens by the innate immune system compromises the integrity of periodontal tissues and eventually results in the formation of periodontal pockets.

Periodontitis, involving specific bacteria coupled with the host's response, initiates an acute phase receptor-mediated cytokine production by epithelial cells and simultaneous neuropeptide release, resulting in vasodilation of local vessels. Chemokines mobilize neutrophils from blood vessels for migration to the area of bacterial invasion. Gingival bleeding, swelling, and redness together with the presence of neutrophils/macrophages within the inflamed gingivae indicate clinical signs of inflammation. The infection is both confined to, and subsequently cleared by, neutrophils and macrophages, or expands to include other cells and structures [84]. In the high susceptibility profile group of individuals, the acute phase responses fail to clear the infection, and chronic inflam-

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matory lesions develop within a matter of weeks. The pathogenic consortium consisting of *P. gingivalis*, *Tannerella forsythia* (*T. forsythia*), and *Treponema denticola* (*T. denticola*) appear to be the main organisms involved in the development of chronic PD [85, 86]. The subgingival sulcus serves as a niche enabling a cyclic chronic inflammatory process which in turn facilitates recurrent bacteremia following routine oral health regimes [55, 56, 87]. A number of inflammatory pathologies are said to develop in this way, including cardiovascular diseases [88, 89], rheumatoid arthritis [90–92], diabetes mellitus [93], and others as well as AD [60, 61, 64, 72, 74].

ANATOMICAL RELATIONSHIP OF FACIAL NERVES AND THE BLOOD SUPPLY TO THE BRAIN

The position of the oral cavity, serving the need for speech and food consumption, connects with the brain via series of nerves. Cranial nerve 1 (CN1) is the special sensory nerve for olfaction and contributes not only to our sense of smell but also to that of taste. CN1 has complex pathways that trigger visceral responses (salivation and nausea or accelerated peristalsis in the intestinal tract and increased gastric secretion) to various odors. Although CN1 is recognized and named as the olfactory nerve, the majority of the olfactory tract comprises of secondary, rather than primary sensory axons; thus it is really not a "nerve" but rather a bulb and tract. There is a physical connection between the oral and nasal cavity, extending onto the superior nasal conchae and nasal septum and contains neurosensory cells and olfactory glands, which keep the mucosa moist and in which the dissolution of inhaled scents (aromatic molecules) occurs. The peripheral processes of the primary sensory neurons in the epithelium perform as sensory receptors and transmit sensation centrally, which congregate into around 20 bundles, which, in turn, pass through foramina of the cribiform plate of the ethmoid bone. The cribiform plate of the ethmoid bone is the porous barrier between the nasal passages and the brain itself. Once they have passed through the cribiform plate, the central processes synapse on the secondary sensory neurons in the olfactory bulb itself, which houses the nerve cell bodies. Behind this area is the olfactory tract and trigone; the nerve cell bodies travel to the three olfactory areas, located in the anterior part of the entorhinal cortex area, encompassing the hippocampal gyrus and all ultimately lead to the hippocampus [94].

Cranial nerve V (CN V) or the trigeminal nerve, arising from the mid-lateral surface for the pons, is primarily a general sensory nerve with smaller motor component. There are three divisions of the CN V which are ophthalmic (V I), maxillary (V_2) and mandibular (V_3) where, the motor root of CN V travels with the mandibular branch. The ophthalmic division (V I) exits the neurocranium through the supraorbital fissure, the maxillary division (V_2) through the foramen rotundum in the sphenoid bone and the mandibular (V_3) branch through the sphenoid's foramen ovale. CN V is a general sensory nerve to the scalp, face, nasal and oral cavities (including the teeth and tongue), and brachial motor nerve to the muscles of mastication (temporalis, masseter, medial pterygoid, and lateral pterygoid), tensor tympani, tensor (veli) palitini, mylohyoid, and the anterior belly of the digastric. When dental or periodontal therapy is performed using local anesthesia (e.g., novocaine, xylocaine), the drug is injected into the oral mucosa covering the bony foramina where the sensory branches of the CN V exit into the oral cavity. For the maxillary dental arcade, the injection is aimed toward the pterygopalatine ganglion; for the mandibular teeth, this is directed toward the mandibular foramen. In the case of the pterygopalatine ganglion, this supplies sensation via branches of V_2 from the nasal cavity, plate, nasopharynx, and maxillary teeth. The lingual and inferior alveolar nerve branches carry sensation from the entirety of the lower jaw, mandibular teeth, gums, and anterior two thirds of the tongue as shown in Fig. 1. As with most nerves, the branches of the trigeminal nerve are accompanied by veins and arteries along the peripheries of their pathways [94].

The olfactory and the trigeminal nerve(s) pathways are also exploited by periodontal pathogens as a means of bypassing the BBB for direct entry into the CNS [72, 95, 96], an observation supported by studies in immunosuppressed animal models using *T. denticola* [97]. The animal model study allows some insight into the virulence of the organism and the host's immune defenses as being important for this occurrence.

The systemic route as an alternative mode of bacterial entry into the brain is favored due to bacteremia as mentioned earlier, association of periodontal pathogens with atherosclerotic lesions and in particular *P. gingivalis* having the ability to adhere to erythrocytes for innate immune evasion [87, 98, 99] as well as gaining advantage for transportation to remote body organs [99].

The brain is supplied by three paired blood vessels: the right and left internal carotid arteries, arising from

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the common carotid artery at the base of the neck. It has three divisions that enter the cranium, anteriorly through the carotid canal of the temporal bone and through foramen lacerum in the middle cranial fossa. The vertebral arteries arise from the subclavian arteries, bilaterally and both enter the cranium via the foramen magnum. The vertebral and internal carotid arteries unite on the base of the brain at the Circle of Willis, via a series of interconnecting smaller arteries. The basilar artery is created when the vertebral arteries join. The Circle of Willis itself is composed of the posterior cerebral, posterior communicating, internal carotid, anterior cerebral and anterior communicating arteries; all these arteries branch to supply the brain itself [94] including the circumventricular organ regions where bacteria and bacterial products access the brain.

THE ASSOCIATION BETWEEN PERIODONTAL DISEASE AND ALZHEIMER'S DISEASE

Longitudinal studies have shown that people with PD who progressed to the development of AD had poorer oral health [61, 76, 79, 100–102]. Does poor oral health always mean that the pathogens will disseminate to the brain even in AD patients? Both our unpublished data from controlled experiments using animal models, and that of Foschi et al. [97], indicate that the presence and motility of the low virulence strains of periodontal pathogens may not be sufficient for them to access the brain. However, animal models of oral diseases (periodontitis and endodontic) may require an adjustment for the optimization of dosage and/or duration of infection to allow for bacteria to translocate to the brain. Our unpublished data demonstrates that *P. gingivalis* (FDC381) accessed the brain of $ApoE^{null}$ mice following an oral infection while *P. gingivalis* (ATCC 33277) failed, even in SCID mice mono and poly infections [97]. It therefore appears that the greater virulence of *P. gingivalis* (FDC 381) due to having acquired fimbriae, likely allowed its adherence to erythrocytes for innate immune evasion, a process that has gained the bacterium an advantage for dissemination [99], to the brain (Poole et al. unpublished results). When the duration of active infection supersedes the virulence of the bacteria, there remains a high possibility that the host harboring the periodontal pathogens will demonstrate these bacteria disseminating to the brain. Immunocompromised status seen in AD patients will also enhance the infection process. This begs the question as to what causes some individuals to harbor periodontal bacteria rather than other species? Could there be as yet unknown genetic factors/inflammatory traits, lifestyle driven environmental stressors?

However, it should be noted that patients suffering from AD cognitive impairment are poor at managing their personal oral health. In addition, a caregiver or dentist may face a marked decrease in cooperation from the AD patient, making management of oral health more challenging. With patients having increased cognitive impairment, such as reduced adaptation to change, dentists often choose not to carry out the dental treatment that would give optimal oral health. For example, patients with AD may well be unable to cope with extensive, potentially unpleasant dental procedures, leaving them with fewer teeth, which could have a detrimental impact on their eating ability.

Nutritional deficiencies are documented in the elderly as well as in the dementia subjects, especially with regard to lessened intake of B-vitamins and folic acid in the diet. The marker that indicates these deficiencies also correlates with cognitive decline, but as consequence of disease rather than a cause [103]. The mechanism of cognitive decline is suggested via synaptic dysfunction, which is one of the earliest structural defects associated with decline in memory [19]. Diet provides the essential B-vitamins, phospholipids, and other micronutrients, which are required for the formation of new synapses [104].

EPIDEMIOLOGICAL EVIDENCE FOR THE ASSOCIATION BETWEEN CHRONIC PERIODONTITIS AND ALZHEIMER'S DISEASE

Clinical/epidemiological studies so far, all agree on loss of teeth leading to poor memory [60–63]. Further studies have examined possible inflammatory biomarkers in an attempt to link and/or to find new diagnostic makers of AD. Others have, however, used more specific measures including IgG levels to *P. gingivalis* and other specific periodontal pathogens [64, 105]. A study by Sparks Stein et al. [105] used cohort methodology analyzing levels of serum antibodies to periodontal disease. At the start of the study period, all participants were cognitively intact, but higher levels of serum antibodies to periodontal pathogens at baseline led to some individuals developing AD [105]. As baseline measures were taken years before diagnosis of AD, the elevation in serum antibod-

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ies cannot be attributed to secondary effects of AD (for example, poor oral hygiene). Although clinical measurements of oral health were not taken in the Sparks Stein et al. [105] investigation, periodontal bacterial species are generally accepted as being specific enough to PD and assessing serum antibody levels to these pathogens may prove to be a true indicator of PD in AD patients.

POSSIBLE CONFOUNDERS

Several environmental, epidemiological, and risk factors show similar trends and patterns in both PD and AD. Whether or not this is coincidental or arises through shared developmental pathways remains unclear. The impact of these associations is the potential for these factors to act as confounders, influencing the true relationship between PD and AD.

The incidence of both PD and AD increases with age; this has already been mentioned in previous sections. Gender related trends exist between AD and periodontal disease. The incidence of AD has been shown to be higher in women after 85 years of age. This is thought to be due to the protective effect of pre-menopause estrogen [106]. Men, however, have been shown to have a greater incidence of PD (up to 50%) than women overall [107]. Interestingly, this study also showed that men with PD also had increased incidence of coronary artery disease, suggesting fewer men than women survive to old age. Physical activity has been shown to have a positive effect on cognitive function and those aged 70–79 years with high levels of activity show lower levels of inflammatory markers such as IL-6 and C-reactive protein [106]. Likewise obesity has shown similar trends, in that those who are obese suffer a greater incidence of AD. Although physical activity and the incidence of PD have not been investigated directly, many studies have shown an indirect relationship, highlighting a greater incidence of PD with obesity and diabetes [107, 108]. It has been suggested that obesity may be the second highest risk factor for PD after smoking [109]. The underlying mechanism for this association is thought to be related to proinflammatory cytokines, including IL-6 and C-reactive protein that are released by adipose tissue, along with hormones adipokines or adipocytokines. With the increasing levels of evidence supporting inflammatory processes in AD development, it is possible that the relationship between AD and obesity may follow similar mechanisms [109]. Poorer general health may be associated with PD due to a compromising immune system and therefore, the ability of the host to defend against periodontal bacterial infections.

It is generally accepted that smoking is the major risk factor in periodontal disease. Smokers are 2–7 times more likely to present with PD than non-smokers and this is unrelated to oral hygiene. Disease progression is more rapid and response to treatment is poorer [109]. Smoking is thought to affect neutrophil function, reducing the host's ability to eliminate periodontal pathogens. Smokers with PD have distinct patterns of pathogenic microbial profile than non-smokers with PD [110–112]. Smoking is also thought to lead to release of reactive oxidative species and oxidative stress mediated tissue damage. Inflammatory cytokine and chemokine expression in smokers compared to non-smokers show many differences reflecting the immunosuppressant effect of smoking, which may contribute to an enhanced susceptibility to periodontitis [113].

The relationship between smoking and AD is less clear; with some studies suggesting smoking has a beneficial effect due to nicotine treatment improving cognitive performance in age associated memory impairment [114], while others suggesting that smoking increase the risk of AD. In the proposed theory, an increased risk of AD is similarly related to the factors causing periodontal disease, whereby smoking increases free radical generation leading to high oxidative stress, or affects the inflammatory immune system leading to phagocyte activation and further oxidative damage.

PD would appear to have an increased prevalence in both ethnic minority groups and lower socioeconomic status. This has been attributed to a complex combination of social, psychological, and structural factors including nutrition, oral hygiene, healthcare utilization, and access to care. It is thought that having lack of resources to pay for care, not having a regular source of care, or availability of transportation to healthcare centers contribute to these trends [115–117].

The relationship between race and socioeconomic status and AD appears more complex. Meta-analysis assessing the relationship between education level and AD showed that overall those with low or no education were more likely to develop AD, but this was not shown in all studies. This relationship was stronger in developed, compared to developing countries possibly due to life-expectancy being shorter in developing countries. Individuals with less education appear to have lower cognitive function compared to those with higher education levels. Education-dementia relation-

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ship appears to vary according to age, gender, and race/ethnicity and the suggestion is that the relationship ties in with a person's life events beginning prior to and carrying on beyond years of formal education [118]. These epidemiological trends associated with both PD and AD demonstrates the importance for excluding the impact of confounders when investigating a true relationship between PD and AD.

GENETIC RISK FACTORS FOR LATE-ONSET ALZHEIMER'S DISEASE AND PERIODONTITIS

The apolipoprotein E (ApoE) gene is a known genetic risk factor associated with late-onset AD, and more recent investigations suggest some further genetic risk factor associations with innate immune molecules and inflammatory traits in late-onset AD [10, 15, 16]. In particular, cytokine-related genes appear to be involved in the susceptibility to inflammation in late-onset AD [10, 12, 33] as well as in PD [119–121].

As the immune system plays an important role in PD pathogenesis [59], it is thought that PD itself may have genetic associations. Polymorphisms in IL-α, IL-1β, IL-6, and TNF-α genotype are reported for periodontitis [119–121] and similarly IL-1α, IL-1β, IL-6, TNF-α, α2-macroglobulin, and α1-antichymotrypsin are all upregulated in AD [12, 33] suggesting commonalities between susceptibility profiles in these two disease conditions. As mentioned earlier, offspring of parents with AD have higher inflammatory cytokines in their blood than those who are descendants of non-AD parents [10]. Similarly, parents with poor oral health tend to have children with poor oral health; however, it is difficult to conclude that the poor oral health trait is a result of the genetic makeup of the individual and not simply an environmental influence [122].

STRESS ENVIRONMENTAL AND GENETIC FACTORS

Stress: In health

Acute stressors in the environment, such as facing a dangerous situation, activate physiological systems designed to enhance self-preservation [123]. These include the sympathetic-adrenal-medullary and the more slowly responding hypothalamus pituitary adrenal (HPA) axis. This response prepares the body to cope with threat [124]. The two response systems work by increasing certain hormones, such as adrenaline and cortisol. These, in turn, act on the biological functioning of the individual, such as increased heart rate, inhibition of the digestive system, or increased glucose supply to the muscles. Although the physiological changes enhance our ability to mount a physical response to threat, the associated neurochemical changes can lead to cognitive failures, which may present as dementia-like even within a cognitively normal population. For example, elevations in cortisol as a result of activation of the HPA axis can lead to impairments in attention [125], working memory and inhibitory control [126], and declarative memory [127]. Cognitive impairments due to acute stressors are reversible. Further acute stress responses may actually have some benefits for health with enhancements in immune function being reported [128]. However, when short-term beneficial adaptations designed to maintain homeostasis during acutely stressful events become excessive or prolonged, problems can occur [129].

Stress: Periodontal disease

Psychological stress may affect periodontal disease. Such stressors can lead to a change in health behaviors, which in turn may lead to associated increased risk of PD. For example, poorer oral hygiene coupled with increased smoking [130] and alcohol intake [131]; visiting the dentist less regularly, and eating less healthily with higher fat and sugar diets [132] encourage bacterial growth and worsen periodontal status.

Stress impairs the balance between pro- and anti-inflammatory responses. Alterations in inflammatory polymorphic gene function, in particular of IL-1 and IL-6 (119–121), will affect polymorphonuclear leukocyte chemotaxis. The net effect will be reduced lymphocyte proliferation and this may increase the vulnerability of periodontal tissue to microorganisms leading to further tissue destruction [133]. Inhibition of T cell responses by glucocorticoids appears to explain, in part, susceptibility to PD and pro-inflammatory cytokines are potent activators of the HPA axis [134, 135]. Patients with PD who are stressed show increased IL-6 and IL-1β levels in gingival crevicular fluid [133]. Results of several studies were reviewed and demonstrated a correlation between psychological stress and salivary and blood stress markers relating to inflammatory response and progression of PD [134]. However, a cause and effect relationship has not, so far been found, therefore stress is considered a risk factor for PD rather than a cause.

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Stress: Alzheimer's disease

High levels of environmental stressors could lead to impairments in cognitive processes, which are important for maintaining oral hygiene. For example, acute and chronic activation of the HPA axis can lead to elevated basal cortisol levels. High levels of circulating cortisol causes hippocampal damage and so impair hippocampus-dependent memory processes [125]. In a cognitively intact elderly population, higher cortisol levels were indicative of impairments of declarative memory and executive functioning [135], both of which are needed to maintain good oral hygiene.

The hippocampus is the area of pathology in AD and is vulnerable to effects of stress and trauma [136]. Chronic stress can impair immune responses and so compromise the body's ability to resist disease [137]. The brain attempts to compensate cellular stress, by regulatory mechanisms, involving upregulation of heat shock proteins [138]. Loss of heat shock proteins, *in vitro,* was shown to contribute to accumulation of hyperphosphorylated tau, a component of NFTs, and a hallmark of AD pathology [139]. In addition, stress activated protein kinases, for example, mitogen-activated protein kinase 38 and the c-jun N-terminal kinases are activated in AD [140, 141]. These two stress pathways can also be activated by oxidative stress [142, 143], a common denominator of environmental, pathological, and habitual factors.

CRITICAL REMARKS ABOUT THE PRESENT STATE OF THE RESEARCH TO RELATIONSHIP BETWEEN ALZHEIMER'S DISEASE AND CHRONIC PERIODONTITIS

Clinical observational studies thus far all correlate with loss of teeth leading to poor memory. However, direct evidence is lacking to support a causal association between periodontal bacteria and progression of AD. The clinical studies have been performed on elderly cohorts where overlapping features of the aging process, such as deposition of Aβ in the brain is likely to have already begun. When assessing periodontal status clinically, levels of caries may not have been accounted for. Oral hygiene studies were based on retrospective questionnaire surveys, which may introduce selection bias depending on the response rate. People who are motivated to complete questionnaires may be more likely to visit the dentist than those who do not. Genetic factors have not given importance in some of the studies. Both the Riviere et al. [72] and the Poole et al. [74] studies lacked information concerning the periodontal status of the cases analyzed.

FUTURE RESEARCH

How PD contributes to impaired memory remains intriguing and future studies should be directed towards addressing these mechanisms. There is paucity of information relating to the exact risk factor(s) for the development of deteriorating memory from missing teeth in the prodromal phase of AD. Are these factors downstream of co-morbidities such as diabetes affecting periodontal status of the individual? Or common susceptibility profiles play a key role in loss or gain of function in future generations of AD parents. Further evidence to support an association between PD and AD, research would need to demonstrate that the inflammatory and immunological responses that the bacteria and their virulence factors induce may subsequently lead to the onset of AD. Perhaps associations between parents with AD and their children at a much younger age should be monitored for oral health as that should eliminate any overlapping confounders that may be masking the true links in these two conditions.

This review clearly indicates significant gaps in our current understanding of the causal association of pathogens (spirochetes, bacteria, viruses) in a slowly progressive and debilitating disease such as AD. Although various types of neurotropic spirochetes including oral (*Treponema* spp) and non-oral spirochetes (*B. burgdorferi*) have been detected directly in association with the pathological hallmarks of AD [73] but, a causal relationship has not been tested in animal models. To the best of our knowledge, there is no single *in vivo* animal model study that has examined the role of periodontal bacteria during chronic infection (9–12 months of exposure times) to study the sequence of neuropathological events associated with the development of AD pathology. Even though infectious agents have been implicated in relation to AD hallmark pathology for over a century ago, several clinical and molecular studies strongly support an association between PD and AD. However, to date, there is a paucity of reports supporting a causative relationship between periodontal pathogens and AD cases and *in vivo* transgenic mice.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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Oral Infections and Cytokine Levels in Patients with Alzheimer's Disease and Mild Cognitive Impairment Compared with Controls

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Abstract.
Background: Oral infections are prevalent in the adult population. Their impact includes the implication as a risk factor for Alzheimer's disease (AD), altering its progression. One of the potential mechanisms involves immune mediators such as circulating cytokines.
Objective: The goal of the present study was to investigate the prevalence of oral infections and blood levels of IL-1β, TNF-α, and IL-6 in patients with AD, mild cognitive impairment (MCI), and controls.
Methods: Sixty-five elderly were evaluated (25 AD, 19 MCI, and 21 controls) by the following methods: Mini Mental State Exam, Questionnaire of Functional Activities, periodontal and oral evaluation, and blood concentrations of IL-1β, TNF-α and IL-6.
Results: Patients with AD had high serum IL-6 levels ($p = 0.029$), and patients with periodontitis had high serum TNF-α levels ($p = 0.005$). There was an association between IL-6 and TNF-α in patients with AD/MCI and periodontitis ($p = 0.023$).
Conclusion: The increased levels of TNF-α and IL-6 in this study suggests their implication in the overlapping mechanisms between oral infections and AD. Longitudinal studies are necessary for further investigation.

Keywords: Alzheimer's disease, IL-6, inflammation, mild cognitive impairment, oral infection, periodontitis, TNF-α

INTRODUCTION

Alzheimer's disease (AD) is the most common acquired cerebral degeneration and the main cause of dementia in Western countries (50%–66%). It interferes in personal, social, and work activities. Its pathophysiology is based on molecular and cellular neuronal abnormalities that include senile plaques and neurofibrillary tangles with a chronic inflammatory background [1]. One of the main factors involved in the development and progression of AD is a cerebral inflammatory process. Some inflammatory biochemical markers seem to be elevated in patients

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with AD when compared with healthy subjects of the same age. The plasmatic levels of interleukin 6 (IL-6) and the cerebral levels of IL-6, interleukin 1 Beta (IL-1β) and tumor necrosis factor alpha (TNF-α), were increased in these patients [2].

Dental infections, especially periodontitis, are associated with dementia and are risk factors for the onset or the aggravation of AD [3–6]. Chronic periodontitis is an infectious-inflammatory disease caused by bacteria that destroy the connective and bone tissue of the periodontium [7]. This chronic infection generates chronic inflammation with the release of local and systemic inflammatory and immune mediators such as cytokines, which are elevated in the serum of patients [8]. Besides, oral infections at the mucosa, such as candidiasis, are more frequent in AD. They are also able to increase serum IL-6 and TNF-α levels [9]. Thus, the objective of this study was to investigate the prevalence of oral infections and serum levels of IL-6 and TNF-α in patients with AD, mild cognitive impairment (MCI), and non-demented elderly.

MATERIALS AND METHODS

Subjects

Twenty-five patients with AD and 19 patients with MCI diagnosed according to the criteria of the National Institute for Communicative Disorders and Stroke (Alzheimer's Disease and Related Disorders Association) (NINCDS-ADRDA) [10] were evaluated. The diagnosis was performed by an experienced neurologist of the Group of Behavior and Cognitive Neurology of the Hospital das Clinicas (Medical School of the University of Sao Paulo). The patients were compared with 21 healthy subjects. They were non-demented elderly that had been followed at the Group of Multidisciplinary Assistance of Elderly of the Geriatric Clinic at the same hospital. All patients, controls, relatives and/or guardians were informed about the purposes of the study and all signed the informed consent. The local Ethics Committee approved the protocol.

Patients with AD, MCI, and controls were matched according to educational status, age, gender and the prevalence of hypertension and diabetes ($p > 0.05$; SPSS 17.0; SPSS Inc., Illinois, USA). A single trained neurologist who was blinded to the dentist evaluation performed the neurological evaluation of all subjects.

Exclusion criteria were other neurodegenerative or neurological conditions (e.g., Parkinson's disease, multiple sclerosis, other causes of dementia, neural infections such as Hansen's disease, stroke) and cerebral, facial, or cervical tumors.

Protocol of evaluation

A single trained dentist performed the complete oral exam and clinical questionnaire to identify absences of teeth and aspects of the oral mucosa, tongue, and orofacial pain [11]. The decayed, missing, and filled tooth index was determined as well as the O'Leary plaque index (PI). The periodontal probing was performed to determine the gingival bleeding index (BI), probing pocket depth (PPD), the clinical attachment level (CAL), and cement-enamel junction distance (CEJ) [12, 13].

The serum level of cytokines (IL-6, IL-1β, and TNF-α) were investigated by venous blood samples of the subjects, acquired concomitant to clinical exam. The blood samples were obtained in dry tubes of 9 mL (Vacuette®) and immediately centrifuged, and the serum was immediately stored in a –80°C freezer. After the storage of all samples, a multiplex panel (MILLIPLEX map High Sensitivity Human Cytokine Panel®) was used to perform the cytokine analysis.

Statistical analysis

Data were tabulated and analyzed according to means, standard deviations, frequencies, and normality tests for the distribution of quantitative variables (Shapiro-Wilk test and Q-Q plot). Variables with normal distribution were analyzed with one-way ANOVA, MANOVA and Pearson's correlation coefficient. Non-parametric chi square test was also performed. The level of significance was 5%.

RESULTS

The demographic characteristics and the results of cognitive and functional evaluation are illustrated in Table 1. Patients with AD had higher impairment of cognition and function than the other groups ($p < 0.001$).

AD patients showed higher prevalence of candidiasis as well as coated tongue (Table 2). There were no differences in periodontal indexes. Patients with AD and MCI had more absent teeth ($p = 0.032$) and a longer duration of edentulism ($p = 0.032$)

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Table 1
Demographic characteristics, cognitive (Mini-Mental State Exam) and functional evaluation (Pfeffer questionnaire) ($N = 65$)

	Study Group		Control Group	
	AD ($N = 25$)	MCI ($N = 19$)	Non demented ($N = 21$)	p^*
Female gender	15 (16.0%)	13 (68.4%)	14 (66.7%)	0.822
Age (years)	77.68 ± 6.03 (63–92)	73.11 ± 6.79 (56–86)	75.33 ± 5.75 (62–87)	0.244
Marital status				
Married	12 (48.0%)	9 (47.4%)	8 (38.0%)	
Divorced	2 (8.0%)	0 (0%)	1 (4.8%)	0.748
Widowed	11 (44.0%)	10 (52.6%)	12 (57.1%)	
Color (Race)				
White	21 (84.0%)	16 (84.2%)	19 (90.5%)	
Black	2 (8.0%)	1 (5.3%)	1 (4.8%)	0.620
Yellow	2 (8.0%)	2 (10.5%)	1 (4.8%)	
Educational status				
4 years or less	10 (40.0%)	8 (42.1%)	12 (57.1%)	
4–8 years	11 (44.0%)	6 (31.6%)	7 (33.3%)	0.547
8 years or more	4 (16.0%)	5 (26.3%)	2 (9.5%)	
Economic status				
Minimum salary or less per month	0 (0%)	1 (5.3%)	3 (14.3%)	
2–4 minimum salaries per month	20 (80.0%)	15 (78.9%)	15 (71.4%)	0.425
4–8 minimum salaries per month	4 (16.0%)	1 (5.3%)	2 (9.5%)	
More than 8 minimum salaries per month	1 (4.0%)	1 (10.5%)	1 (4.8%)	
Functional score	12.16 ± 6.42 (4–23)	1.21 ± 1.03 (0–4)	0.19 ± 0.51 (0–2)	<0.001*
Cognitive score	21.3246 (17–27)	27.63 ± 1.06 (25–29)	27.76 ± 1.54 (25–30)	<0.001*

*Statistical difference: chi-square and one-way ANOVA; Frequencies (%) and means ± standard deviations (range). AD, Alzheimer's disease; MCI, mild cognitive impairment.

than healthy elderly (Table 3). The main cause for tooth loss in AD patients was periodontal disease ($P < 0.001$); in opposition, decay was the main cause of tooth loss of the MCI group and the control group (Table 3).

The cytokine levels (IL-1β, TNF-α, and IL-6) can be observed in Fig. 1. Patients with AD had significantly higher IL-6 levels than controls ($p = 0.029$), and patients with periodontal disease had significantly higher TNF-α levels than patients with healthy periodontal tissues ($p = 0.005$). The multivariate analysis showed an association between IL-6 and TNF-α in patients with AD or MCI and periodontal disease ($p = 0.023$).

The concentration of IL-6 was negatively correlated with the MMSE score ($p = 0.018$; Pearson) and positively correlated with the Pfeffer's Functional Assessment Questionnaire (FAQ) ($p < 0.001$; Pearson), which means that the high serum IL-6 level was observed in subjects with low performance in cognitive tests. The concentration of TNF-α was positively correlated with the periodontal indexes PPD, CAL, BI ($p < 0.001$; Pearson), and PI ($p = 0.008$; Pearson), which means that patients with worse periodontal condition had higher serum TNF-α level. Finally, IL-6 and TNF-α were positively correlated in all studied groups ($p < 0.001$; Pearson).

DISCUSSION

This study shows that the elevated concentration of IL-6 in patients with AD was associated with high serum TNF-α level of subjects with periodontal disease. This finding suggests that immune-inflammatory mechanisms of periodontitis may underlie its role in the onset, progression or aggravation of AD. IL-6 is a biomarker for cognitive impairment [14], and TNF-α might be a biomarker of the association of periodontitis with AD by these results.

The etiology of AD is not yet completely elucidated, and the involvement of chronic inflammation in its pathophysiology is still controversial. The cerebral inflammatory process could be a consequence of the deposits of senile plaques and neurofibrillary tangles, and its immune-inflammatory mediators might accelerate the neurodegenerative process [15, 16]. This hypothesis is supported by the evidence of IL-6 and C reactive protein as biomarkers of cognitive impairment [14, 17, 18].

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Table 2
Dental characteristics and periodontal indexes ($N = 65$)

	Study group		Control Group	
	AD ($N = 25$)	MCI ($N = 19$)	Non demented ($N = 21$)	p^*
DMFT	27.16 ± 5.83 (15–32)	25.84 ± 5.65 (17–32)	24.42 ± 5.07 (15–32)	0.258
Teeth				
Healthy	9 (36.0%)	9 (47.4%)	13 (61.9%)	
Decayed	5 (20.0%)	3 (15.8%)	4 (19.0%)	0.610
Fractured	2 (8.0%)	1 (5.3%)	0 (0%)	
All missing	9 (36.0%)	6 (31.6%)	4 (19.0%)	
Periodontal tissues				
Healthy	6 (24.0%)	6 (31.6%)	8 (38.2%)	
Periodontitis	10 (40.0%)	5 (26.3%)	9 (42.8%)	0.184
Gingivitis	0 (0%)	2 (10.5%)	0 (0%)	
Edentulous	9 (36.0%)	6 (31.6%)	4 (19.0%)	
Oral mucosa				
Healthy	15 (60.0%)	17 (89.5%)	17 (81.0%)	
Candidosis	8 (32.0%)	0 (0%)	2 (9.5%)	0.050*
Benign lesion	2 (8.0%)	2 (10.5%)	2 (9.5%)	
Malign lesion	0 (0%)	0 (0%)	0 (0%)	
Coated tongue	18 (72.0%)	3 (15.8%)	4 (19.0%)	<0.001*
Orofacial pain				
No pain	18 (72.0%)	12 (63.2%)	17 (81.0%)	
At chewing	6 (24.0%)	4 (21.0%)	4 (19.0%)	0.356
Diffuse	1 (4.8%)	2 (25.0%)	0 (0%)	
Bruxism	1 (4.0%)	0 (0%)	1 (4.8%)	0.436
Oral infection	10 (40.0%)	7 (36.8)%	9 (42.8%)	0.977
Mean PPD (mm)	2.82 ± 1.68 (1.00–5.78)	3.05 ± 1.61 (1.16–5.89)	2.63 ± 3.25 (1.00–5.31)	0.766
Maximum PPD (mm)	4.68 ± 2.15 (2.00–8.00)	4.30 ± 1.88 (2.00–8.00)	4.52 ± 2.32 (1.00–9.00)	0.847
Mean CAL (mm)	4.15 ± 3.90 (1.12–7.23)	4.32 ± 3.12 (0.00–7.90)	3.92 ± 1.44 (1.57–5.95)	0.851
Maximum CAL (mm)	5.10 ± 4.45 (0.00–12.00)	5.66 ± 3.84 (0.00–11.00)	6.61 ± 3.22 (0.00–11.00)	0.476
O'Leary plaque index(%)	71.87 ± 26.58 (20–100)	67.69 ± 28.41 (15–100)	58.47 ± 26.52 (10–90)	0.357
BI*** (%)	46.00 ± 33.32 (0–80)	44.61 ± 34.26 (0–87)	29.17 ± 26.58 (0–80)	0.247

*Statistical difference: chi-square and one-way ANOVA; Frequencies (%) and means ± standard deviations (range); AD, Alzheimer's disease; MCI, mild cognitive impairment; DMFT, decayed, missing, fulfilled teeth; PPD, probing pocket depth; CAL, clinical attachment level; BI, bleeding index.

Table 3
Prevalence of missing teeth, edentulism time, and main reason of tooth loss according to group ($N = 65$)

		Study Group		Control Group	
		AD** ($N = 25$)	MCI** ($N = 19$)	Non demented ($N = 21$)	p^*
Missing teeth	Third molars	0 (0%)	0 (0%)	1 (4.8%)	
	<7 missing teeth	2 (8.0%)	0 (0%)	1 (4.8%)	
	7–12 missing teeth	3 (12.0%)	4 (21.1%)	1 (4.8%)	0.032*
	13–20 missing teeth	2 (8.0%)	6 (31.6%)	10 (47.6%)	
	>20 missing teeth	18 (72.0%)	9 (47.4%)	8 (38.1%)	
Edentulism duration (years)		33.80 ± 13.48 (10–60)	31.40 ± 13.93 (10–50)	23.52 ± 12.26 (0–50)	0.033
Main reason for tooth loss	Periodontal disease	20 (80.0%)	8 (42.1%)	6 (28.6%)	<0.001
	Decay	5 (20.0%)	11 (57.9%)	15 (71.4%)	

*chi-square; Frequencies (%); one-way ANOVA; means ± standard deviations (range). **AD, Alzheimer's disease; MCI, mild cognitive impairment.

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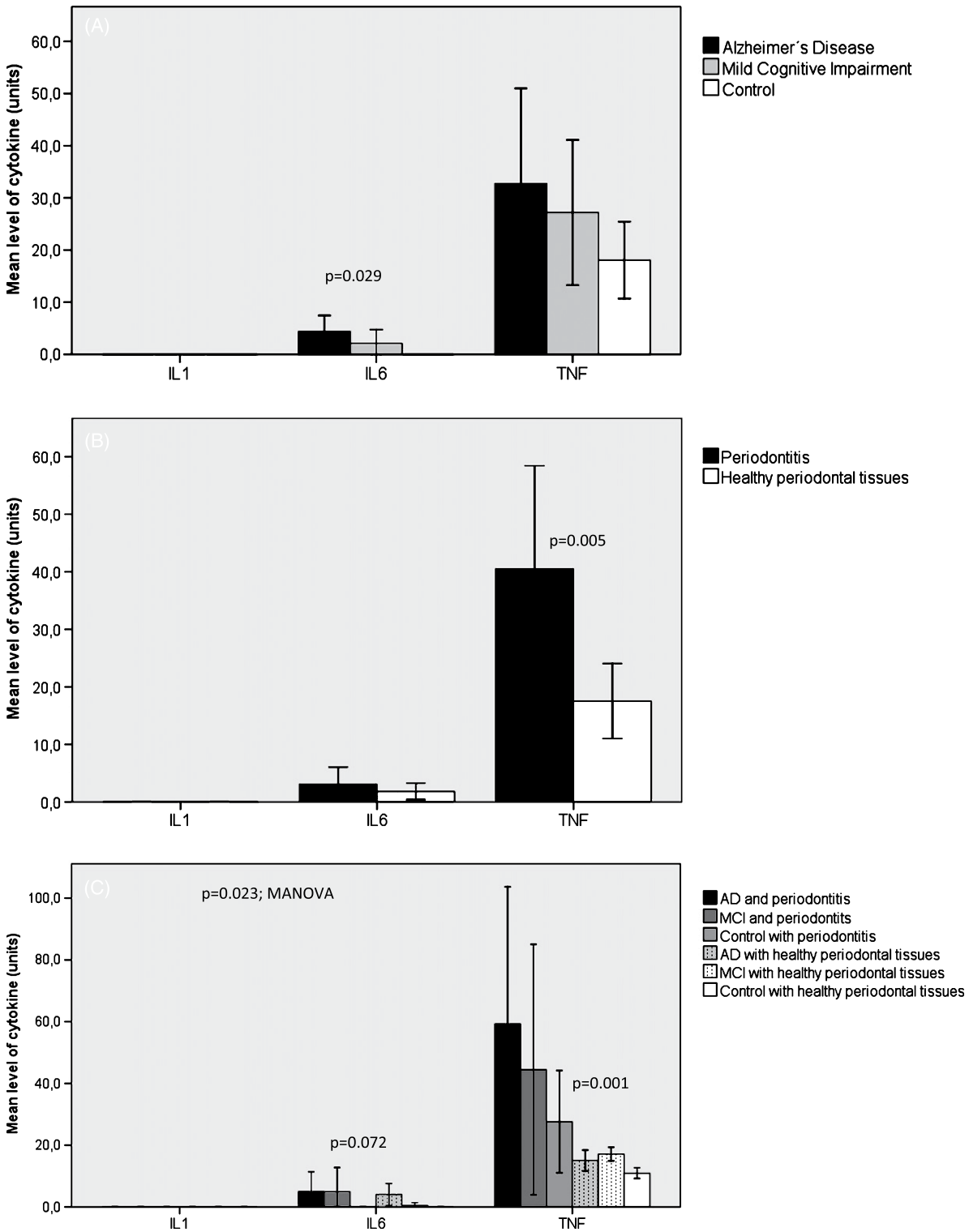


Fig. 1. A) Cytokine levels (IL1β, TNFα, and IL6) in Alzheimer's disease, mild cognitive impairment, and in healthy subjects. B) Cytokine levels (IL1β, TNFα, and IL6) in periodontitis and in healthy periodontal tissues. C) Cytokine levels (IL1β, TNFα, and IL6) in Alzheimer's disease, mild cognitive impairment, healthy subjects, and in periodontitis compared to healthy subjects.

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Common immune-inflammatory backgrounds of neurodegeneration in AD and bone destruction in periodontitis supports their overlapping. Oral infections are frequent in the adult population and their systemic impact is important, supporting prevention and early treatment to avoid complications, such as AD, at old age [3, 19–21]. Systemic inflammation of periodontitis is implicated in the progression and aggravation of several chronic illnesses [5], and it includes dementia.

The present results are in agreement with previous observations showing the association of early tooth loss due to periodontal disease with dementia [22]. The main cause of tooth loss was periodontitis, which corroborates to the association of cytokine expression and AD. The impact of tooth loss on nutrition, especially when occurring under the age of 35 is another risk factor of AD [23]. Oral rehabilitation with prostheses is compulsory to improve nutritional status that have an implication in general health and in prevention of cognitive deficit. Thus, oral diseases may play a role in AD by immune-inflammatory and indirect nutritional consequences [24]. Besides systemic inflammatory mechanisms, oral bacteria can reach the cerebral tissue and induce chronic local infection and inflammation in the brain [25].

In the present population studied, there were no statistical differences in the prevalence of dental diseases and periodontal parameters, except infections of the oral mucosa, a finding similar to earlier observations, which was the subject of a previous publication [11]. In that previous publication, the severity of periodontitis was not different but its prevalence was higher in patients with AD. Although the current evaluation showed similar prevalence of periodontitis between studied groups, the previous history of causal factors for dental extractions showed periodontitis as the reason for teeth loss only in AD patients. However, candidiasis can also increase the evaluated cytokines in blood serum [9]. We suggest monitoring blood serum levels of IL-6 and TNF-α and evaluating the clinical signs of periodontitis and other oral infections in AD is essential.

TNF-α, IL-1β, and IL-6 are capable of stimulating the synthesis of Aβ-amyloid 1-42 peptide ($A\beta_{42}$) and the phosphorylation of tau protein (P-tau), and $A\beta_{42}$ and P-Tau can stimulate the production of TNFα, IL-1β, and IL-6 by glial cells [26]. Thus, periodontitis and oral mucosa infections, as potential causes of TNF-α increase in the blood serum, is a candidate for a modifying factor for the development of AD [19, 21].

The world's population got older in the last century, and as dementia is becoming more prevalent, it is becoming a public health problem. Co-morbidities are frequent and may play a role in the aggravation of symptoms. Non-modifiable risk factors include age, presence of APOE ε4, and family history [27]. Oral infections can be assessed, treated, and prevented [11, 28, 29], and their blood serum biomarkers (IL-6 and TNF-α) may help in the analysis of their risks in dementia.

In conclusion, this case-control transversal study shows evidence of the association between cytokines expression in the blood serum with oral infections and AD. The next step is to investigate the role of periodontitis and oral mucosa infections in the incidence of dementia in longitudinal studies, to elucidate the mechanisms underlying the overlap between TNF-α and IL-6 in neurodegeneration. Examining clinical implications is necessary to prevention, and the treatment of oral infections in adults and the elderly will improve the quality of aging because these infections potentially have a role as risk factors for AD.

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Can Better Management of Periodontal Disease Delay the Onset and Progression of Alzheimer's Disease?

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Abstract. A risk factor relationship exists between periodontal disease and Alzheimer's disease (AD) via tooth loss, and improved memory following dental intervention. This links the microbial contribution from indigenous oral periodontal pathogens to the manifestation of chronic conditions, such as AD. Here, we use *Porphyromonas gingivalis* infection to illustrate its effect on mental health. *P. gingivalis* infection, in its primary sub-gingival niche, can cause polymicrobial synergy and dysbiosis. Dysbiosis describes the residency of select commensals from the oral cavity following co-aggregation around the dominant keystone pathogen, such as *P. gingivalis*, to gain greater virulence. The initial process involves *P. gingivalis* disturbing neutrophil mediated innate immune responses in the healthy gingivae and then downregulating adaptive immune cell differentiation and development to invade, and subsequently, establish new dysbiotic bacterial communities. Immune responses affect the host in general and functionally via dietary adjustments caused by tooth loss. Studies from animals orally infected with *P. gingivalis* confirm this bacterium can transmigrate to distant organ sites (the brain) and contribute toward peripheral and intracerebral inflammation, and compromise vascular and microvascular integrity. In another study, *P. gingivalis* infection caused sleep pattern disturbances by altering glial cell light/dark molecular clock activity, and this, in turn, can affect the clearance of danger associated molecular patterns, such as amyloid-β, via the glymphatic system. Since *P. gingivalis* can transmigrate to the brain and modulate organ-specific inflammatory innate and adaptive immune responses, this paper explores whether better management of indigenous periodontal bacteria could delay/prevent the onset and/or progression of dementia.

Keywords: Alzheimer's disease, manageable risk factor, memory, *P. gingivalis*, periodontitis, sleep

MAKING A CASE FOR PERIODONTITIS AS A RISK FACTOR FOR ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common example of dementia, constituting 60–80% of all dementias, and the healthcare costs associated with the management of individuals with this condition keeps rising [1]. Unfortunately, the pathogenesis of the disease remains an enigma. There is no adequate treatment or prophylaxis for the disease at present. The solution rests on prevention, or finding an effective treatment for AD.

To this end, Baumgart and colleagues [2] reported on the mission of the newly formed World Dementia Council and outlined the key challenges of tackling dementia. One key priority is to examine the

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modifiable risk factors that influence the development of dementia [2]. A risk factor relationship between periodontal pathogens/periodontal disease and AD exists, as previously discussed by Olsen and Singhrao [3]. Salient points are that periodontal disease is co-morbid with clinically demented subjects [4–7]. Tooth loss in early and mid-life has consequences for poor memory in later life [4, 7–12]. A prospective longitudinal study reported by Ide et al. [7] upholds the view that periodontitis is associated with a marked increase in cognitive decline, which is independent of baseline cognition, in men with a mild to moderate clinical diagnosis of dementia. A study following the impact of a dental intervention on patients with a clinical diagnosis of mild AD found over 50% of participants (who also suffered from gingivitis/periodontitis) showed improvements in their memory [6]. Oral bacteria and their virulence factors affect the central nervous system pathology [13, 14], and indiscriminate release of inflammatory mediators cause neuronal injury [5, 15–17]. The host's humoral responses to periodontal pathogen infections are ineffective, but if combined with other tests, they may prove useful as predictive biomarkers of AD [18–20]. The failure of Baumgart et al. [2] to recognize these facts prompted a response in the form of a letter to the Editor by Kamer et al. [21]. We also lend our support to recognize periodontitis as a risk factor in the context for AD pathogenesis [21] and provide further laboratory based research evidence from rodent brains infected with *Porphyromonas gingivalis* (*P. gingivalis*).

While the aging process is associated with an increased likelihood of periodontitis [22] and dementia, their onset is by no means inevitable. Just as some individuals fail to progress beyond gingivitis [23], others retain cognitive functioning throughout their life [24, 25]. Individuals who retain cognitive functioning in later life also show a low lifetime risk for cancer, diabetes, cardiovascular disease(s) [26, 27], and possibly periodontitis. Oral bacteria are associated with diverse pathologies, which are often co-morbid with AD (Fig. 1) as described elsewhere [28]. As periodontitis is an easily manageable risk factor for a number of conditions, we argue that reducing the incidence of periodontal disease is a preventative measure for numerous health benefits in later life.

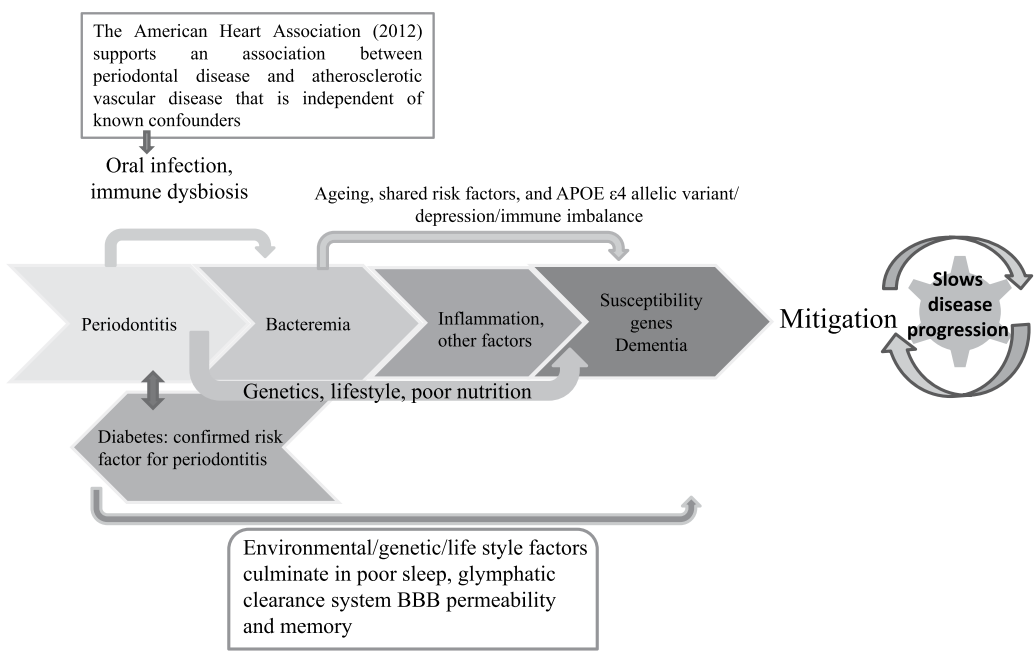


Fig. 1. Schematic to show knock-on effect from an oral condition such as periodontitis to the development of mixed pathologies in the order of periodontal disease, cardiovascular, and AD with input from metabolic disorders like type 2 diabetes. The arrows point to major risk factors with plausible effect on each condition. Mitigation of poor lifestyles are modifiable with dental intervention potentially slowing down/preventing these related conditions.

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PERIODONTAL DISEASE

Periodontitis describes a chronic polymicrobial infection in which *P. gingivalis* represents a keystone pathogen [29] and initiates dysbiosis [30] for nutritional dependency and imparting greater immune armamentarium to sustain the newly developing microbial communities in the sub-gingival niche. Until recently, it was acceptable to relate *P. gingivalis* interactions with specific species of bacteria, for example, those belonging to the red complex (*Treponema denticola* and *Tannerella forsythia*) [31]. However, to explain periodontal disease pathology, Hajishengalis and Lamont [30] have revised this view into a polymicrobial synergy and dysbiosis model. Hajishengalis and Lamont [30] propose that several hundreds of bacteria identified from the oral cavity (the oral microbiome, http://www.homd.org) all have the potential to subvert immune defenses, and overcome protective host barriers by gaining greater virulence from the keystone pathogen in the susceptible host. This process is like a form of bacterial *radicalization* where seemingly inert species co-aggregate, while dwelling in the oral cavity. Co-aggregation of *P. gingivalis* with *Streptococcus gordonii* is a typical example of how synergy between local commensals and a keystone pathogen may be possible [32]. Taken together these newly armed, select communities of bacteria with greater virulence can impair the host's immune surveillance, and tip the balance from homeostasis to dysbiosis [30].

Periodontitis consistently shows progressive destruction of gingival connective tissue and adjacent alveolar bone [3, 29]. The initial gingival epithelial cell breach is the result of *P. gingivalis* being able to infect and survive within neutrophils that normally survey the gingival health [33]. The transient disablement of inflammatory signaling pathways attenuate neutrophil recruiting chemokine, interleukin 8 (IL-8), and cause imbalance of T cell IL-17 levels [34]. These changes promote local dysbiosis and therefore increase local inflammation, which is beneficial for selection and survival of the most resilient inflammophilic dysbiotic species within that oral niche [33, 35]. The oral bacterial/virulence factor will then transmigrate to the AD brain, with neuroinflammation as an obvious outcome [14]. Already disturbed adaptive immune responses, as is apparent from the host suffering from periodontal disease [34], will give rise to oral pathology. Marsh et al. [36] have verified the role of adaptive immune cells in the immune-deficient transgenic AD mouse model (Rag-5xfAD). This report confirms that adaptive immune cells in an immune-sufficient host (control animals) help to restrain both neuroinflammation and accumulation of amyloid-β (Aβ) protein [36], suggesting organ specific hallmark pathology can develop in the immunosuppressed vulnerable host.

The suggested association between periodontal disease and AD relies on several key observations. First, there are significant correlations between the rate of cognitive decline and measures of periodontal disease progression, such as pocket depth, alveolar bone loss, and tooth loss [37]. Currently, the relevance of newly identified risk factor, cognitive deficit for periodontitis [8], is more difficult to measure and pinpoint. We have suggested an explanation following our studies performed in the *P. ginigivalis* mono-infection model of periodontal disease in apolipoprotein E knockout ($ApoE^{-/-}$) mice (see section on blood-brain barrier below). Future research will undoubtedly highlight a better understanding of the pathophysiology of poor memory development in relation to the human form of periodontitis.

ALZHEIMER'S DISEASE

AD is a neurodegenerative disease characterized clinically by generalized loss of numerous markers of social values. Of major interest here are irreversible memory loss and sleep pattern disturbances [38–41]. Pathologically, the presence of significant numbers of two specific microscopic lesions (extracellular Aβ deposits, and intracellular hyperphosphorylated tau protein coated neurofibrillary tangles) is critical to AD diagnosis. Major risk factors for AD include aging, and genetic susceptibilities [42]. Co-morbid states include cerebrovascular strokes [43–45] with and without diabetes [46, 47]. The salient environmental factors, which can increase AD onset, include physical injury and peripheral infections [48] (Fig. 2).

A diverse list of candidate microbes that appear to be associated with, and survive the highly inflammophilic environment of the AD brain, is given by Olsen and Singhrao [3]. However, there is a paucity of information as to whether there is synergy among multiple-species identified from AD brains, as is suggested, for example, in the oral biofilm bacteria [30]. Miklossy implicates oral and non-oral spirochaetal infections [13, 49] as dominant AD pathogens. However, microbes, such as *Chlamydophila pneumoniae* [50], *Helicobacter pylori* [51], Herpes simplex virus

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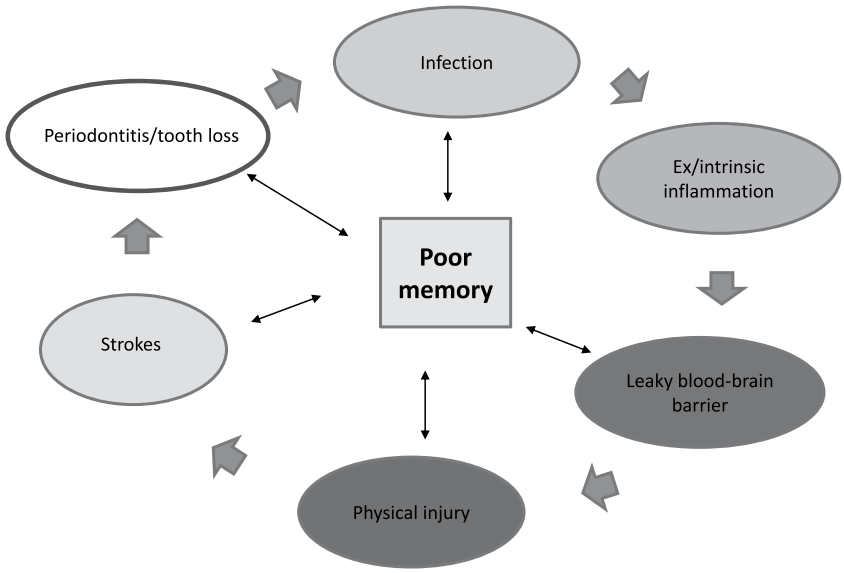


Fig. 2. Schematic shows the relationship of various pathologies affecting memory. The single head arrows suggest a possible direction of disease progression with periodontitis as the initial condition. The double arrow heads point to known examples of diseases that negatively affect memory.

type 1 [52], and LPS from Gram-negative bacteria [14, 53], are documented as being identified in AD postmortem brain specimens. This hypothesis implies the existence of an AD specific, but yet incomplete microbiome, consisting of a consortium of inflammophilic pathogens indigenous to oral and remote body organs. Intensive research is underway with newer hypotheses incorporating Aβ senile plaques as by-products of bacterial biofilm matrices [54, 55], and determining the existence of synergy among multiple-microbial species. If this is the case, oral bacterial infections will reveal their potential to cause/modulate the late-onset AD with possible synergy with other spirochetes as suggested elsewhere [49, 56].

THE BLOOD-BRAIN BARRIER

Finding oral bacteria in postmortem AD brains is intriguing. To this end, our research in the *P. gingivlais* infected ApoE$^{-/-}$ periodontal disease mouse model [57] continues to search for scientifically relevant clues. Our research findings in chronic *P. gingivalis* infection of ApoE$^{-/-}$ mice suggest the integrity of the hippocampal blood-brain barrier (BBB) gradually erodes via an inflammation mediated physical injury of the cerebral parenchyma [58]. For the first time, *P. gingivalis* infection in mice has been associated with the loss of BBB integrity [58]. This is interesting, as the same pathological feature of the BBB exists during human aging [59] and in AD subjects with the APOE4 genetic phenotype [60]. AD subjects with the APOE4 susceptibility gene demonstrate a high proinflammatory cytokine (tumor necrosis factor-alpha or TNF-α, IL-6, and IL-1β) expression [61]. Since microbial infections promote interleukin class of cytokines (IL-6 and IL-1β) and TNF-α secretion via the inflammasome formation [62], it is plausible to suggest that individuals with APOE4 genotype are prone to harboring chronic infections and the cytokine burden is secondary to infections. For example, the genetically modified phenotype of ApoE$^{-/-}$ mice is predisposed to intrinsic stress in the form of higher burden of vascular inflammation, due to the constitutive expression of the pro-inflammatory cytokine TNF-α [63, 64]. Consequently, they are more sensitive to local and peripheral innate immune mediators (cytokines) released following daily bacteremia from experimental periodontitis [57]. This correlates with daily bacteremias that occur in humans due to periodontitis [65, 66]. A defective BBB [59, 60] would allow entry of pathogens and their immunogenic virulence factors and non-infection related molecules into the brain. Entry of bacteria and bacterial products will trigger oxidative stress followed by continually stimulating microglia to express inflammatory cytokines locally and generate neurodegeneration by mitigating protection of neurons from extrinsic insults.

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There is little doubt that recurrent infections of the elderly increase the odds for developing AD [67]. The deteriorating BBB following *P. gingivalis* infection in mice [58] helps to explain the possible delirium post infections observed in elderly awaiting clinical AD diagnosis [67]. With regard specifically to periodontal disease, one longitudinal study has suggested a link between higher Aβ load in the hippocampus of elderly humans with mild forms of periodontitis [37]. Singhrao et al. [58] are suggesting the role of *P. gingivalis* in inflammation mediated injury with progressive deterioration of the hippocampal microvascular integrity in the very regions of the brain that demonstrate a high burden of neuropathological hallmarks of AD. These observations are similar to the effect of physical insults, such as traumatic brain injury and stroke, which also lead to cognitive deficit (Fig. 2), aggregated hallmark protein accumulation and eventually AD [45, 68]. Both infections and physical brain trauma show common inflammatory mediators in their eventual manifestation of dementia [17, 69, 70].

Nutritional deficiency also correlates with cognitive decline [71], and fewer teeth may lead an individual reducing their intake of fresh foods, which in turn may decrease intake of B-vitamins and folic acid and other forms of nutrition [28]. Taken together, factors causing deterioration in cognition are multifactorial and complex with likely contribution from primary infection and nutritional deficiency due to loss of teeth. For example, depression can manifest from the generalized feeling of being unwell and can suppress appetite. Furthermore, AD is a depressive condition that can also lead to sufferers having reduced appetite.

Bacterial infections, specifically those that are involved in the onset of stroke, can also lead to AD in survivors [43–45]. Systemic hypoxia from co-morbid vascular states (atherosclerosis/stroke) [72, 73] and paucity of peripheral adaptive immune cells in the AD brain, and from Rag-5xfAD mice, demonstrates enhanced neuroinflammation and Aβ accumulation compared to control animals [36]. This research helps to explain Aβ deposition and abnormal tau phosphorylation post stroke. The chronic *P. gingivalis* infection of $ApoE^{-/-}$ mice show inflammation mediated physical damage that appears to be similar to that seen in traumatic brain injury or concussion cases with shared inflammatory biomarkers [58, 69]. These facts demonstrate how chronic infection of the brain with a keystone pathogen, such as *P. gingivalis*, can cause potentially serious consequences for the BBB and subsequent poor mental health. Future research should include AD transgenic mice with oral infections to support the preliminary findings of Singhrao et al. [58].

SLEEP

Sleep is a physiological process, which reduces responsiveness and induces immobility on a temporary basis [74] in order to rest the body and support several brain functions including mood, memory, and consciousness [75]. Two main biological drivers govern sleep. The first is the homeostatic pressure to sleep, which rises as the amount of time increases since the last sleep period [76]. The second driver is the body's endogenous circadian timing mechanism, which controls the timing and organization of sleep and is located in the hypothalamic suprachiasmatic nuclei (SCN) [40, 77]. During sleep, changes in the brain's electrical activity observed via cortical electroencephalogram (EEG) patterns define two different behavioral states. The first observable state is non-rapid eye movement sleep characterized by the presence of "theta waves" and "delta waves". Second is rapid eye movement sleep characterized by EEG patterns similar to people who are awake but distinguished by the presence of phasic events (twitching limbs), and tonic phenomena (loss of muscle tone) [78].

Research has pointed to a clear link between sleep duration and quality and physical health [79]. Short sleep durations (less than 6 h) and poor sleep quality can increase the likelihood of mortality [80, 81] and specifically increase susceptibility to illnesses, such as coronary heart disease [82] and Type 2 diabetes [83]. Indeed, lack of sleep and poor sleep quality appear to impact negatively on the body's metabolic [84], endocrine, and immune functioning [85]. Furthermore, recent research has suggested that although healthy younger cohorts can compensate for the impact of sleep-deprivation or poor sleep quality, older adults and those with AD appear to suffer more markedly from disrupted sleep-wake cycles [86]. In addition, the process of aging can affect negatively on the mechanisms controlling sleep. In the elderly, the aging of the SCN can lead to a decrease in neuronal activity, which in turn can cause changes in the circadian regulation and secretion of the sleep hormone melatonin [76]. Furthermore, an older population may have more psychological [87] and physiological [88] risk factors for poor sleep.

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Specifically, within the AD population, there are marked changes in sleep with increases in disturbed night sleep and increased daytime napping. It is therefore plausible to suggest that poor sleep (both duration and quality) could increase the risk of developing AD [89] in the elderly. However, once AD is established, deterioration in the circadian system that controls sleep-wake cycles [90] may be the direct or indirect result of disease processes that lead to increased sleep disturbances. Patients with AD who have been institutionalized (hospital setting), for example, show the marked disturbances in circadian patterns perhaps due to limited exercise and daylight exposure [91]. However, as AD progresses, the damage to the neuronal pathways that initiate and maintain sleep become more pronounced and lead to increased problems with sleep [89, 92, 93]. For example, AD patients show increased theta activity during non-rapid eye movement sleep [94]. Such changes in sleep can destabilize both physiological processes, such as immune function, and impact on cognitive processes, such as memory (Fig. 3). As the impact of sleep appears to be bidirectional, it may be that stabilization of sleep patterns reduce the symptoms [95].

It is clear that sleep can affect the development [89] and progression [96] of AD. However, the precise way in which deregulated sleep may influence AD is not clear. An interesting link between sleep and the host's immune system is suggested [97], which links infections, inflammation, and disturbed sleep-wake cycles. However, until the recent discovery of the glymphatic system, it was not clear what processes could be mediating the link between poor sleep and AD. The glymphatic system was discovered using two-photon imaging of live mice via a closed cranial window [98]. The glymphatic system uses the glial water channels to perform a clearance function within the brain similar to that of the peripheral lymphatic system [99, 100]. Therefore, the glymphatic system is important for the removal of danger associated molecular patterns such as microbial debris, interstitial solutes and Aβ plaques from the brain [101]. Interestingly, the glymphatic system appears to be "turned on" during normal sleep and reduces in function during the awake state [101]. Therefore, if individuals are suffering from sleep abnormalities then the reduced functioning of the glymphatic system could increase levels of Aβ plaque formation and hyperphosphorylated tau neurofibrillary tangles. This may explain why poor sleep duration and quality can lead to the development of, and accelerate the progression of AD [102, 103]. Therefore, the down regulation of the glymphatic system because of aging and mixed pathological factors and intrinsic stress due to infections, which affect sleep quality and duration, may interact to influence AD development and progression (Fig. 3).

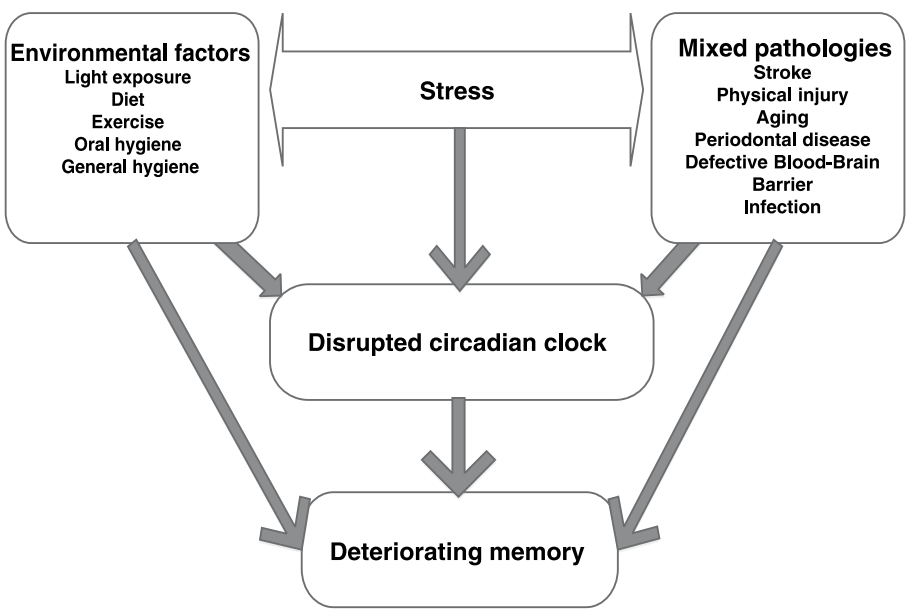


Fig. 3. Schematic to show stress and the main risk factors influencing the circadian clock. All these factors, over time, lead to deterioration in memory. The arrows suggest a simplistic path for functional loss.

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Impairments to the glymphatic system may specifically reduce the brains ability to clear microbes such as *P. gingivalis*. Direct infiltration of the brain by microbes such as *P. gingivalis* can directly lead to neuroinflammation through activation of cortical microglia and contribute to local immune responses [104]. Paolicelli and colleagues [105] reported that microglia play a key role in scavenging and pruning normal neuronal network connections for brain development, and this activity is greater when the animal is asleep. Takayama et al. [106] noted that *P. gingivalis* infection of mice brains caused sleep pattern disturbances by altering glial cell light/dark molecular clock activity. *P. gingivalis*, therefore, can subvert the host's microglial cell phagocytic activity by disrupting the circadian system that controls sleep-wake cycles. Consequently, the glymphatic system appears less efficient. This important and original finding by Takayama et al. [106] links the keystone periodontal pathogen, *P. gingivalis*, to sleep disturbances and subversion of microglial cell function, and paves the way for aggregated protein build-up. This report has crucial relevance to sleep pattern disturbances in AD and supports how poor oral hygiene and the associated rising levels of intrinsic and extrinsic sources of cytokines may act as crucial early modifiers of neurodegeneration and disease severity leading to deteriorating memory, sleep and ultimately the development of pathology.

WAYS TO MITIGATE RISK FACTORS

Oral hygiene

From an oral hygiene aspect, even having teeth (being dentate) but not maintaining daily oral hygiene appears to increase the risk (22–65%) of dementia compared to those who brush their teeth daily [107]. A healthy oral cavity has a normal microbiome, consisting largely of non-pathogenic commensal bacteria. These adhere to oral surfaces in the form of a biofilm. Any disruption of their environmental equilibrium can lead to polymicrobial synergistic dysbiosis resulting in periodontitis. For good gingival health and protection against periodontal disease, effective tooth brushing techniques (twice a day) for 2 minutes using toothpaste with fluoride (containing a minimum of 1350 parts per million fluoride) is recommended. Combining brushing with interdental cleansing (e.g., interdental brushing once before bedtime) will aid physical removal of plaque from in between teeth and help to control host's inflammatory response to plaque. Individuals should be encouraged to visit the dentist regularly to improve their oral health no matter what their predisposition in health. For example, patients with clinical AD are less able to perform a recommended daily oral hygiene routine mainly due to ongoing cognitive impairment, and their dental check-ups are reliant on the freedom of their caregiver. As periodontitis is treatable, it offers the potential to reduce AD progression, if managed correctly (Fig. 1). Therefore, the dental intervention for AD patients should be high on the priority scale. Ultimately, the caregivers will need to take on-board the advice from dental professionals and change personal lifestyle and habits to minimize the severity of disease.

Diet

Poor diet combined with a non-active lifestyle leads to nutritional deficiencies and development of metabolic abnormalities. These include increases in the following: insulin resistance and chronic inflammation, oxidative stress-induced proteins, lipids, DNA damage, and alterations in neuroendocrine systems [108–113]. Calorie reduction, resulting in a body mass index of a healthy range of 19–23 is beneficial for bone density [114]. Moderate exercise leads to improved glucose tolerance and insulin action, and reduction in cardiovascular disease risk factors including plasma low-density lipoproteins-cholesterol concentration [115, 116]. Excessive exercise may increase the subjects' metabolic rate, which in turn can act as a disease-based risk factor. Individuals prone to recurrent microbial infections show increased TNF-α level despite consuming a healthy diet suggesting the oxidative stress from infections is of a higher magnitude than the host can manage. Alternatively, aging and AD processes also lead to a gradual accumulation of oxidative stress and molecular damage suggesting existence of incomplete efficiency in anti-oxidative and repair mechanisms in the host [117].

Certain foods have a beneficial impact on health. Plant foods contain a range of phytochemicals and vitamins, which are associated with reduced oxidative stress and inflammation. These natural anti-oxidants help to prevent cardiovascular disease and cancer [118, 119]. A diet high in foods containing phytochemicals such as onion, garlic, wild blueberries, tea, cabbage, carrot, celery, and soya may reduce oxidative stress and inflammation [120], while

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some grain-based foods may destabilize blood glucose levels. In addition, oily fish high in omega-3 and omega-6 polyunsaturated fatty acids can positively affect the regulation of inflammation by increasing levels of eicosanoids [121]. Therefore, a healthy diet could reduce metabolic abnormalities, oxidative stress, and inflammation, which in turn, can control the progression of periodontitis and subsequently slow down AD progression.

CONCLUSIONS

Periodontitis constitutes a peripheral oral infection, the pathogenic components of which may result in infecting the brain and/or exposing the brain to immunogenic virulence factors and inflammatory mediators implicated in the pathogenesis of AD. A prospective interventional study has shown that effectively treating periodontitis can lead to improvements in cognition. A chronic oral infection with *P. gingivalis* in ApoE$^{-/-}$ mice demonstrated peripheral and intracerebral inflammation is capable of compromising BBB integrity. A dysfunctional BBB can lead to many neurological diseases, including stroke, traumatic brain injury and AD. This demonstrates some relevance of a chronic infection of the brain with a keystone pathogen such as *P. gingivalis*, causing potentially serious consequences for the BBB and subsequent mental health. Others have shown that *P. gingivalis* infection can alter the microglial cell light/dark molecular clock activity, resulting in defective phagocytosis. This increases the prevalence of danger associated molecular patterns, which prevent normal functioning of neuronal networks as does a periodontal dysbiotic microbial community in subverting hosts innate and adaptive immune responses. Thus, risk factors for AD should also include periodontitis in addition to the more accepted factors, such as aging, genetic susceptibilities, stroke, and traumatic brain injury.

Diet

The dietary regime should include foods that offer long-term protection from developing mixed pathologies (Figs. 1–3). The diet should include high intake of green leafy vegetables and blueberries, and oily fish because they appear to have anti-oxidant and anti-inflammatory properties as well as lowering cholesterol.

Sleep

Good quality sleep appears to be critical for the glymphatic system to function. Glymphatic processes appear to have the potential to prevent and slow AD progression. Health practitioners should highlight the importance of good sleep alongside guidance on diet and physical activity. Other potential non-medical interventions include focusing on a diet high in foods that promote sleep and the use of light therapy.

All of the above everyday activities (hygiene, dietary intake, and sleep) are changeable by the host's indigenous microbiome if their balance is disturbed.

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Clinical and Bacterial Markers of Periodontitis and Their Association with Incident All-Cause and Alzheimer's Disease Dementia in a Large National Survey

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Abstract. Microbial agents including periodontal pathogens have recently appeared as important actors in Alzheimer's disease (AD) pathology. We examined associations of clinical periodontal and bacterial parameters with incident all-cause and AD dementia as well as AD mortality among US middle-aged and older adults. Clinical [Attachment Loss (AL); probing pocket depth (PPD)] and bacterial [pathogen immunoglobulin G (IgG)] periodontal markers were investigated in relation to AD and all-cause dementia incidence and to AD mortality, using data from the third National Health and Nutrition Examination Surveys (NHANES III, 1988–1994) linked longitudinally with National Death Index and Medicare data through January 1, 2014, with up to 26 years of follow-up. Sex- and age-specific multivariable-adjusted Cox proportional hazards models were conducted. Among those ≥65 years, AD incidence and mortality were consistently associated with PPD, two factors and one cluster comprised of *IgG* titers against Porphyromonas gingivalis (*P. gingivalis*), Prevotella melaninogenica (*P. melaninogenica*) and Campylobacter rectus (*C. rectus*) among others. Specifically, AD incidence was linked to a composite of *C. rectus* and *P. gingivalis* titers (per SD, aHR = 1.22; 95% CI, 1.04–1.43, $p = 0.012$), while AD mortality risk was increased with another composite (per SD, aHR = 1.46; 95% CI, 1.09–1.96, $p = 0.017$) loading highly on *IgG* for *P. gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *C. rectus*, *Streptococcus intermedius*, *Capnocylophaga Ochracea*, and *P. melaninogenica*. This study provides evidence for an association between periodontal pathogens and AD, which was stronger for older adults. Effectiveness of periodontal pathogen treatment on reducing sequelae of neurodegeneration should be tested in randomized controlled trials.

Keywords: Aging, Alzheimer's disease, dementia, periodontal pathogens, periodontitis

INTRODUCTION

Dementia, a common disorder affecting older adults, has an estimated prevalence of 4.7% (≥60 years) [1], with 4.6–7.7 million additional annual cases occurring worldwide [3.5–10.5 per 1,000 in various world regions] [1–3]. Generally, ∼60–80%

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[1]MAB had full access to the data used in this manuscript and completed all the statistical analyses.

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of dementia is ascribed to Alzheimer's disease (AD) [1], a progressive neurodegenerative disorder with a multi-factorial etiology. AD triggers progressive episodic memory deterioration followed by impairment in other domains of cognition [4]. AD is likely caused by age-dependent and progressive Aβ-amyloid brain deposition [5], with a second pathological hallmark being the neurofibrillary tangles arising from hyperphosphorylated tau proteins [6]. It constitutes the primary cause of disability among older adults [7], the leading health care burden in developed countries [8], and the sixth leading cause of death in the US [9]. The number of AD-affected Americans is expected to rise from currently 5.4 million to 13.8 million by 2050 [9]. In 2016, US long-term and hospice care cost for all-cause dementia (including AD, vascular dementia and other rare forms) was estimated at $236 billion [9].

Despite no effective treatment, epidemiologic research has uncovered genetic markers for late-onset AD (e.g., ApoE ε4) and several modifiable risk factors. The combined effects of low education, smoking, physical inactivity, depression, mid-life obesity, hypertension, and type 2 diabetes explains ∼54% of AD risk [10], leaving much variation unaccounted for. Identifying novel mid-life modifiable risk factors is essential for planning cost-effective interventions. Microbial agents have recently appeared as important actors of AD's etiology [8], notably periodontal pathogens [11–15], many of which can cause periodontitis (Pd), a condition shown to increase risk of diabetes, atherosclerosis, cardiovascular events [16], and adverse cognitive outcomes [11–15].

Pd affects 20–50% of older adults and is initiated by periodontal bacteria, the most well-known being *Porphyromonas gingivalis (P. gingivais), Tannerella forsythia (T. forsythia)*, *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans),* and *Treponema denticola (T. denticola)*, triggering gingival inflammation, connective tissue destruction, periodontal pocket formation, alveolar bone loss, and edentulation [17]. Given its increased prevalence with age, Pd may be highly predictive of AD. In fact, several hypothesized pathways link Pd to AD, including brain tissue invasion by periodontal gram-negative bacteria found in the dental biofilm, release of bacterial byproducts into the brain via bloodstream invasion and direct impacts of peripheral nerves [18]. Periodontal pathogens can affect brain cytokines through systemic or neural pathways [19]. A recent comprehensive study suggests that *P. gingivalis* and its associated gingipains in the brain play a central role in AD pathogenesis and suggests that $A\beta_{1\text{-}42}$ is produced in the brain partly as a response to this infection [20]. However, the epidemiological evidence as to the relationship between various Pd-related pathogens, including *P. gingivalis*, and AD remains scarce.

We examined age and sex-specific associations of serum immunoglobulin G (IgG) humoral immune response against periodontal pathogens and Pd markers with incident all-cause and AD dementia as well as AD mortality among U.S. middle-aged and older adults (45+years at baseline) using the National Health and Nutrition Examination Survey (NHANES) III linked with Center for Medicare & Medicaid Services (CMS) data.

MATERIALS AND METHODS

Database: NHANES-CMS

The NHANES, sponsored by the National Center for Health Statistics (NCHS), consists of cross-sectional surveys providing nationally representative data on U.S. population health and nutritional status. Sampling follows a stratified, multistage probability cluster design. It includes in-home interviews for basic health and demographic information [21]. This was a retrospective cohort study whereby publicly available data was linked to restricted medical and death records and analyzed at the Research Data Center (RDC). CMS-Medicare and NDI linkage methodology are provided in Supplementary Material 1.

The present study was approved for ethical treatment of participants by the Institutional Review Board of the National Institute on Aging, Intramural Research Program.

Study sample

A participant flowchart is presented in Fig. 1, including the sample at risk and number of events. First, we included NHANES III participants aged 45+years with complete data on at least one of 19 periodontal pathogens Immunoglobulin G (*IgG)* humoral immune response (1988–1994, surplus serum, SPSDEPPX), mortality status and CMS-linkage data. Among 33,199 participants (aged 1–90 years) recruited in NHANES III (1988–1994) with complete socio-demographics (i.e., age and

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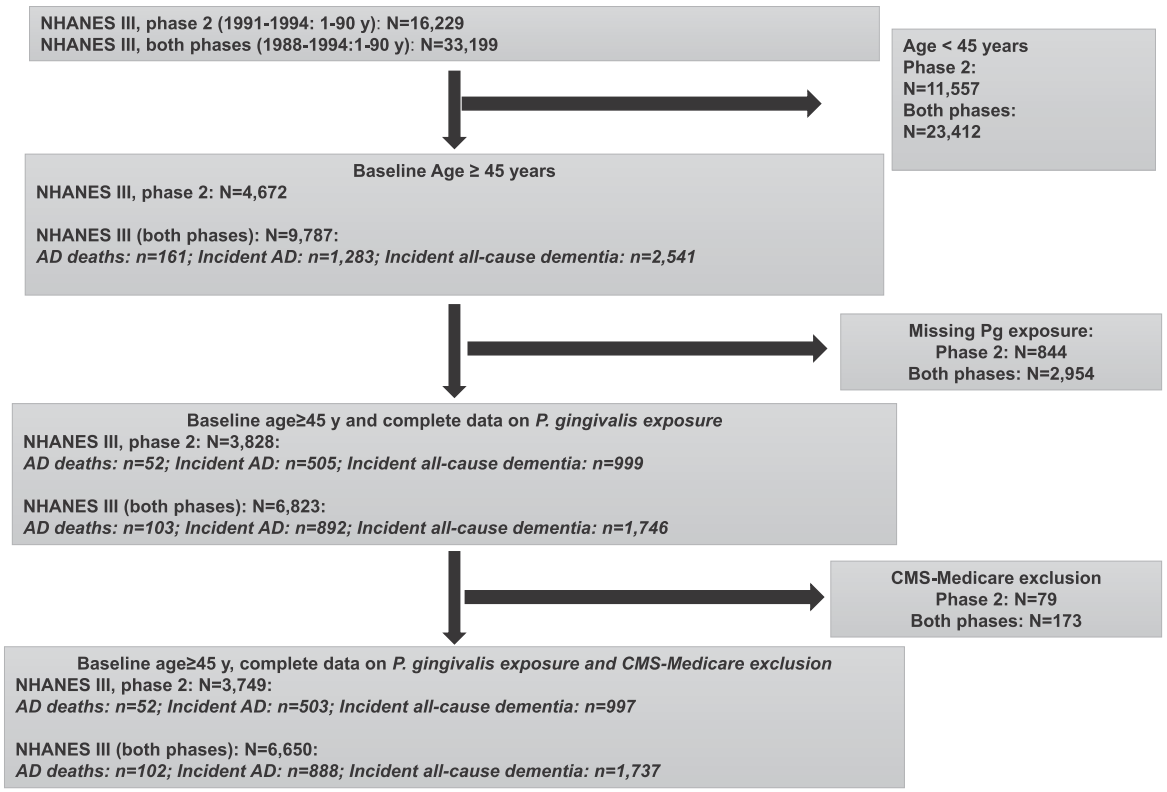


Fig. 1. Participant Flowchart. Both phases: 1988–1994; Phase 2:1991–1994. AD, Alzheimer's disease; CMS, Centers for Medicare and Medicaid Services; NHANES III, Third National Health and Nutrition Examination Surveys.

sex), 9,787 were aged 45+years, of whom 6,823 had complete surplus serum periodontal pathogen data.

Second, 4,672 participants aged 45+years had data on humoral immune response (*IgG*) against two periodontal pathogens only for phase 2 (1991–1994) of NHANES III (DEPP file), namely *Pg* and *Aa*. Of this sub-sample, 3,828 had complete data in the DEPP file (specifically on *Pg*). Sample sizes varied for both samples, depending on exposure of interest.

Participants without CMS-Medicare data were assumed to have no event of interest until end of 2013 or censored upon death. Thus, unweighted samples consisted of 6,823 participants with *Pg* measured at both phases and 3,828 with *P. gingivalis* measured at phase 2. After exclusion due to Health Maintenance Organization (HMO) utilization, those samples were reduced to 6,650 and 3,749, respectively. Third, for PPD/AL exposures, the sample size was reduced to 5,088 (45+, both phases combined, CMS-Medicare exclusion) and PPD/AL exposures with complete both phase *P. gingivalis* and CMS-Medicare exclusion amounted to N = 4,465.

Dementia and AD onset

The CMS Chronic Condition Data Warehouse Categories included a summary file with 21 chronic conditions and varying reference time periods, numbers and types of claims to qualify, exclusions and a set of International Classification of Diseases, version 9 (ICD-9)/CPT4/HCPCS codes. AD was diagnosed using ICD-9 code 331.0 (any diagnosis on the claim) from inpatient, Skilled Nursing Patient [SNP], Home Health Agency [HHA], Health Options Program [HOP] or Carrier claims during a 3-year period. All-cause dementia was assessed using similar criteria with the following diagnostic codes: 331.0, 331.1, 331.11, 331.19, 331.2, 331.7, 290.0, 290.10, 290.11, 290.12, 290.13, 290.20, 290.21, 290.3, 290.40, 290.41, 290.42, 290.43, 294.0, 294.1, 294.10, 294.11, 294.8, and 797. We computed time-to-event starting from Medical Examination Center (MEC) examination date, using the earliest occurrence date. The summary file was available for 1999–2013 follow-up period. Using the same algorithm, we estimated AD/dementia's earliest diagnosis

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date during 1991–1998 [22]. Follow-up time was truncated to January 1, 2014 and was expressed in months.

Mortality from AD

AD mortality was a primary outcome and was determined using underlying cause of death ICD-10 code G30 [23]. Additional AD cases were included when earliest AD diagnosis date was unavailable but AD-related death was assigned. Follow-up was censored at death or, if participants were alive by end of follow-up, January 1, 2014.

Dental examination and clinical periodontal markers

Attachment loss (AL) and probing pocket depth (PPD) defined Pd in our study [24]. Briefly, dental examiners assessed oral health during both phases of MEC examinations in NHANES III. AL and PPD were measured at mid-buccal and mesio-buccal sites on every tooth in two randomly selected quadrants-the maxilla and the mandible (Range: 14–28 teeth) [25]. AL was defined as distance between cemento-enamel junction and base of periodontal pocket, while PPD was distance from base of periodontal pocket to free gingival margin [26]. Mean AL and PPD were calculated for 28 sites. Two non-missing sites per tooth were required for AL/PPD measure and at least one tooth measurement was required to estimate the mean [26].

Periodontal pathogens

Serum immunoglobulin G (IgG) titers were measured for humoral immune response against 19 periodontal bacteria using a series of 1:1,000 serum dilutions and checkerboard immunoblotting as previously described [27]. Those pathogens are: 1) *A. Actinomycetemcomitans* (American Type Culture Collection [ATCC] strains 43718, 29523, and 33384); 2) *P. gingivalis* (ATCC strains 3327 and 53978); 3) *T. forsythia* (ATCC strain 43037); 4) *T. denticola* (Oral Microbiology, Gothenburg, Sweden [OMGS] strain 3271); 5) *Campylobacter rectus* (*C. rectus*, ATCC strain 33238); 6) *Eubacterium nodatum* (*E. nodatum*, ATCC strain 33099); 7) *Prevotella intermedia* (*P. intermedia*, ATCC strain 25611); 8) *Prevotella nigrescens* (*P. nigrescens*, ATCC strain 33563); 9) *Prevotella melaninogenica* (*P. melaninogenica*, ATCC strain 25845); 10) *Fusobacterium nucleatum* (*F. nucleatum*, ATCC strain 33563); 11) *Parvimonas micra* aka *Micromonas micros* (*M. micros*, ATCC strain 10953); 12) *Selenomonas noxia* (*S. noxia*, ATCC strain 43541); 13) *Eikenella corrodens* (*E. corrodens*, ATCC strain 23834); 14) *Capnocylophaga ochracea* (*C. ochracea*, ATCC strain 33624); 15) *Streptococcus intermedius* (*S. intermedius*, ATCC strain 35037); 16) *Streptococcus oralis* (*S. oralis*, ATCC strain 35037); 17) *Streptococcus mutans* (*S. mutans*, ATCC strain 25175); 18) *Vellonella Parvula* (*V. parvula*, ATCC strain 10790); 19) *Actinomyces naeslundii* (*A. naeslundii*, ATCC strain 49340) [28]. IgG titers were quantified using chemiluminescent signal-measuring instrument and compared to human IgG standard curve [27]. Specifically, 8,153 stored serums among NHANES III participants aged 40 years or older were analyzed at Columbia University College of Dental Medicine, New York, NY between 2003 and 2006 (Phase 2 then Phase 1 samples). A factor analysis was conducted among individuals >45 years of age, to extract independent common factors, using eigenvalue and scree plot criteria and varimax rotation (Supplementary Material 2). These factors were entered simultaneously into models examining associations between periodontal pathogens and AD mortality, AD incidence and all-cause dementia incidence. Finally, we created modified mutually-exclusive color-coded clusters that were determined using cluster analysis in a previous analysis of all available periodontal pathogen data (k = 19, 40+years, NHANES III, both phases) [28], and analysis also used by others [29]. Those clusters were defined based on correlated Log_e transformed pathogen IgG titers as shown in Supplementary Figure 1 [28]. In our present study, we first summed Log_e transformed IgG to form the clusters of correlated pathogen titers. Those summation clusters were then z-scored to allow for better interpretation in our main models.

In phase 2 of NHANES III, 9,371 surplus sera on two periodontal pathogens were analyzed (*P. gingivalis* and *A. actinomycetemcomitans*) among participants aged 12 years or older of whom 3,828 were 45+years. Antibody concentrations were measured in ELISA units of IgG (EU) and were examined in both untransformed and Log_e transformed metrics, for comparative purposes.

Covariates

Models were stratified by baseline age group (45+, 55+, and 65+) and sex. Demographic,

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socio-economic, social support, lifestyle and health-related factors, dietary quality and nutritional biomarkers were potential confounders included in all models (See Supplementary Material 2 for details).

Statistical analysis

We performed analyses using Stata 15.0 (StataCorp, College Station, TX) [30]. We accounted for survey design complexity by incorporating 6-year (NHANES III, 1988–1994) and 3-year (NHANES III, 1991–1994) primary sampling units and strata. Standard errors were estimated using Taylor series linearization (i.e., *svy*: commands) [30]. Multivariate imputed data (m = 5 imputations, 10 iterations) with chained equations [31] estimated means and proportions across age groups and measures of association, after adjusting for sampling design complexity.

The main exposures of interest were 19 periodontal pathogens added simultaneously (Model 1) and separately (Model 2) for the entire NHANES III sample (1988–1994). Factors extracted from those 19 periodontal pathogens, as well as pre-determined color-coded clusters, were also considered as predictors of interest. For phase 2 NHANES III, *A. Actinomycetemcomitans* and *P. gingivalis* were main predictors, analyzed as standardized z-scores (as is and Log_e transformed). These periodontal pathogens were then entered as predictors (both phases and phase 2, separately) into linear regression models with periodontitis measured with AL and PPD as outcomes while adjusting for all covariates. Finally, means of AL/PPD were considered as separate predictors in main causal models, specifically incident AD and all-cause dementia due to smaller sample size for AD mortality outcome. In those models, defining time-to-event from any age ≥45 years since baseline visit (i.e., delayed entry) until death or censoring or outcome of interest (AD death, AD incidence, all-cause dementia incidence), we conducted Cox proportional hazards models for three outcomes stratifying separately by sex and baseline age to ≥45, ≥55, and ≥65 years. We present fully adjusted models accounting for demographic, socio-economic, lifestyle/social support factors, nutritional biomarkers, and health-related factors (Supplementary Material 2). Weighted mean times of follow-up are estimated from weighted person-months of follow-up and the weighted sample in each model ([Person-months$_{(weighted)}$]/[Persons$_{(weighted)}$]). A type I error of 0.05 was considered for statistical significance and 0.10 for borderline (or marginal) significance. Multiple testing adjustment was done using a familywise Bonferroni approach while accounting for outcome multiplicity (e.g., AL/PPD: 2 outcomes; incident AD/all-cause dementia/AD mortality: 3 outcomes) and assuming that each exposure, model and strata was a distinctive hypothesis [32].

RESULTS

Participant characteristics by age group and sex

Cumulative incidence (weighted) of all three outcomes increased linearly with baseline age, with AD dementia, all-cause dementia and AD mortality reaching 18%, 38%, and 3% in the 65+ baseline age group, respectively. Women in this sample were more likely to be older, and baseline age was also directly related to Non-Hispanic white race, smaller household size, higher proportion widowed, larger means of AL and PPD, and higher proportions completely or partially edentulous (Table 1 and Supplementary Table 1). However, most periodontal pathogen titers were either unrelated or inversely linked to age, with the clearest inverse relationship shown for Orange Blue and Yellow Orange clusters, and for *Factor 3* comprised of *E. nodatum* and *A. naeslundii IgG* titers. Moreover, socio-economic status was associated with younger age, while age was directly related to better dietary quality as measured by the 1995 Healthy Eating Index, co-morbidity and AL, reduced physical activity, smoking and drug use, lower prevalence of obesity, reduced mean of 25(OH)D coupled with increased levels of folate, vitamin A, vitamin E, total carotenoids, and ferritin.

Periodontal pathogens' association with AD mortality, AD and all-cause dementia incidence

After correction for multiple testing (Table 2), Phase 2 *P. gingivalis IgG* titers (un-transformed, z-scores) were associated with increased risk for incident AD dementia, particularly among women (1 SD = 212, HR = 1.14, 95% CI: 1.05–1.23, $p = 0.004$) and individuals above 55 (HR = 1.06) or 65 years (HR = 1.12) at baseline. Log_e transformed *P. gingivalis IgG* titers were marginally associated with increased risk for AD mortality in 65+ age group, while the reverse was true for *A. Actinomycetemcomi-*

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Table 1
Baseline characteristics of selected participants by age group, NHANES III, 1988–1994 [N = 3,749 (phase 2:1991–1994); N = 6,650 (both phases)][a]

Selected participant characteristics	Age group (y)			*p*-value* (Design-based F-test)[b]	
	45–55	55–65	65+		
Unweighted N (both phases)	(N = 1,701)	(N = 1,698)	(N = 3,251)	55–65 versus	65+ versus
	25.6%	25.5%	48.9%	45–55	45–55
Cumulative incidence of AD and all-cause dementia and of AD mortality, weighted %					
AD dementia	1.7 ± 0.3	11.2 ± 1.1	18.3 ± 0.9	<0.001	<0.001
All-cause dementia	4.3 ± 0.5	18.9 ± 1.3	37.5 ± 1.1	<0.001	<0.001
AD mortality	0.1 ± 0.1	1.1 ± 0.4	2.9 ± 0.4	0.028	0.001
Dental measures					
Periodontitis, mean ± SE	(N = 1,435)	(N = 1,225)	(N = 1,805)		
Attachment Loss	1.45 ± 0.05	1.76 ± 0.07	1.98 ± 0.07	0.03	<0.001
Probing Pocket Depth	1.50 ± 0.03	1.53 ± 0.04	1.45 ± 0.03	0.29	0.28
Factors (z-scores), mean ± SE	(N = 1,597)	(N = 1,604)	(N = 3,077)		
Factor 1	–0.05 ± 0.09	+0.00 ± 0.09	0.02 ± 0.08	0.35	0.24
Factor 2	–0.05 ± 0.04	–0.09 ± 0.04	–0.15 ± 0.04	0.46	0.063
Factor 3	+0.21 ± 0.04	+0.13 ± 0.04	–0.04 ± 0.03	0.10	<0.001
Factor 4	–0.07 ± 0.06	–0.15 ± 0.04	–0.09 ± 0.04	0.09	0.62
Factor 5	+0.05 ± 0.07	–0.04 ± 0.05	+0.03 ± 0.04	0.12	0.84
Clusters (z-scores), mean ± SE	(N = 1,655)	(N = 1,655)	(N = 3,169)		
Orange Red	–0.097 ± 0.04	–0.150 ± 0.040	–0.183 ± 0.036	0.35	0.078
Red Green	–0.031 ± 0.010	–0.071 ± 0.085	–0.046 ± 0.071	0.55	0.83
Yellow Orange	+0.02 ± 0.06	–0.02 ± 0.06	–0.10 ± 0.05	0.51	0.020
Orange Blue	+0.19 ± 0.04	+0.10 ± 0.05	–0.10 ± 0.04	0.08	<0.001
Dentate status	(N = 1,701)	(N = 1,698)	(N = 3,251)		
Completely edentulous	9.2 ± 1.11	19.0 ± 1.24	32.2 ± 1.87	--	--
Edentulous in one arch	10.5 ± 1.22	14.5 ± 1.07	14.8 ± 0.95	<0.001	<0.001
Teeth complete	80.3 ± 1.48	66.5 ± 1.54	52.9 ± 2.04	0.001	<0.001
Periodontal pathogen IgG					
Phase 2	(N = 895)	(N = 914)	N = 1,826)		
P. gingivalis					
Continuous	109.2 ± 6.34	105.2 ± 4.2	122.3 ± 7.55	0.49	0.16
Log_e transformed	4.49 ± 0.03	4.47 ± 0.02	4.51 ± 0.02	0.30	0.64
A. Actinomycetemcomitans (Aa)					
Continuous	99.3 ± 5.53	102.8 ± 5.15	94.5 ± 2.87	0.66	0.39
Log_e transformed	4.45 ± 0.03	4.46 ± 0.02	4.44 ± 0.02	0.79	0.61
Both Phases, Log_e transformed[c]	(N = 1.622–1,692)	(N = 1,617–1685)	(N = 3,116–3,227)		
P. Gingivalis (Pg) mix	5.7 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	0.44	0.34
P. Intermedia (Pi)	5.6 ± 0.1	5.5 ± 0.1	5.3 ± 0.0	0.57	0.002
P. Nigrescens (Pn)	5.3 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	0.20	0.06
T. Forsythia (Tf)	4.8 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	0.37	0.76
A. Actinomycetemcomitans (Aa) mix	6.7 ± 0.1	6.7 ± 0.1	6.6 ± 0.1	0.65	0.41
F. Nucleatum (Fn)	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	0.78	0.15
S. Oralis (So)	4.3 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	0.64	0.54
M. Micros (Mm)	5.2 ± 0.1	5.2 ± 0.1	5.1 ± 0.1	0.52	0.13
C. Rectus (Cr)	4.4 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	0.11	0.73
E. Corrodens (Ec)	5.2 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	0.63	0.15
E. Nodatum (En)	7.3 ± 0.1	7.1 ± 0.1	6.8 ± 0.1	0.09	<0.001
S. Intermedius (Si)	5.2 ± 0.1	5.1 ± 0.1	5.0 ± 0.1	0.68	0.002
C. Ochracea (Co)	5.0 ± 0.1	4.8 ± 0.1	4.7 ± 0.0	0.052	0.002
V. Parvula (Vp)	3.6 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	0.97	0.021
A. Naeslundii (An)	6.1 ± 0.1	5.9 ± 0.1	5.7 ± 0.1	0.10	<0.001
P. Melaninogenica (Pm)	5.3 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	0.99	0.35
S. Noxia (Sn)	3.7 ± 0.2	3.6 ± 0.1	3.5 ± 0.1	0.21	0.21
T. Denticola (Td)	4.9 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	0.35	0.43
S. Mutans (Sm)	4.5 ± 0.1	4.5 ± 0.1	4.4 ± 0.1	0.82	0.40
Socio-demographic characteristics	(N = 1,701)	(N = 1,698)	(N = 3,251)		
Age (y)	49.1 ± 0.11	59.3 ± 0.09	73.6 ± 0.24	<0.001	<0.001
Sex, % male	48.5 ± 1.75	44.8 ± 1.12	41.4 ± 1.12	0.07	0.002

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Race/ethnicity	(N = 1,701)	(N = 1,698)	(N = 3,251)		
Non-Hispanic White	79.7 ± 1.80	79.0 ± 2.06	86.6 ± 1.30	--	--
Non-Hispanic Black	8.8 ± 0.74	9.6 ± 0.90	7.3 ± 0.76	0.39	0.04
Mexican-American	4.3 ± 0.43	2.9 ± 0.31	1.9 ± 0.17	0.003	<0.001
Other	7.3 ± 1.46	8.5 ± 1.97	4.3 ± 0.85	0.54	0.02
Urban/rural area of residence	(N = 1,701)	(N = 1,698)	(N = 3,251)		
Urban	50.1 ± 4.69	46.1 ± 4.53	43.2 ± 5.11	0.18	0.06
Rural	49.1 ± 4.69	53.9 ± 4.53	56.8 ± 5.11	--	--
	(N = 1,701)	(N = 1,698)	(N = 3,251)		
Household size	2.9 ± 0.06	2.5 ± 0.04	1.9 ± 0.03	<0.001	<0.001
Marital status	(N = 1,701)	(N = 1,698)	(N = 3,251)		
Never married	5.4 ± 0.97	3.1 ± 0.43	4.0 ± 0.41	0.02	0.93
Married	73.7 ± 2.12	71.8 ± 1.5	54.9 ± 1.45	--	--
Divorced	13.7 ± 1.72	10.4 ± 1.06	5.4 ± 0.57	0.14	0.003
Widowed	2.3 ± 0.39	9.7 ± 0.9	33.3 ± 1.17	<0.001	<0.001
Other	4.8 ± 0.61	4.9 ± 0.85	2.5 ± 0.39	0.82	0.08

25(OH)D, 25-hydroxyvitamin D; AD, Alzheimer's disease; EU, ELISA units; HEI, Healthy Eating Index; HS, high school; IgG, Immunoglobulin G; MAR, Mean Adequacy Ratio; NHANES, National Health and Nutrition Examination Surveys. [a] Values are weighted means ± SEM or percent ± SEP, taking into account sampling design complexity (PSU and strata), across 5 imputations with 10 iterations. [b]Design-based F-test accounting for design complexity in terms of sampling weights, PSU and stratum. for categorical variables, this was the equivalent of a χ^2 test of independence restricting the sample first to 55–64/45–54, then to 65 + /45–54. For continuous variables, it was the equivalent of a Wald test in a linear regression model with the variable being the outcome predicted by age group and in which 45–54 years was the referent category to which "55–64" and "65+" were compared. [c]SD of Log_e transformed periodontal pathogens across groups ranged between 1.2–1.3 (*Co, Vp*) and 1.8–2.0 (*Pg, En, An*), with the remaining ranging between 1.4 and 1.6.

tans IgG (both transformed and untransformed, 65+). When examining all 19 periodontal pathogens (Log_e transformed, z-scored, 1988–1994) in relation to the three dementia outcomes of interest (Supplementary Tables 2 and 3), and upon multiple testing adjustment, we found that *S. oralis IgG* was linked with increased risk for all-cause dementia among men, a pattern observed among women for *E. corrodens IgG* (*Model 1*: all pathogens entered). Similarly, *C. rectus IgG* was associated with increased risk for all-cause dementia in all age groups, a pattern that was consistent between models 1 and 2 among the older group (65+). *C. rectus IgG* was also marginally and directly associated with incident AD risk in the 55+ age group (Model 1), while *S. intermedius* was marginally and inversely associated with incident AD risk among women (Model 1). For AD mortality (Table 3), most of our findings emerged when each periodontal pathogen was entered separately into the model (*Model 2*). Most notably, *P. gingivalis IgG* (Log_e transformed, z-score) was associated with increased AD mortality risk among those aged 65+ at baseline (1 SD = 2.03, HR = 1.36, 95% CI: 1.10–1.69, $p = 0.010$), as was the case for *P. melaninogenica IgG* (1 SD = 1.28, HR = 1.43, 95% CI: 1.11–1.85, $p = 0.009$). The latter finding was further strengthened by adding the remaining 18 titers into the model (HR = 1.73, $p = 0.005$). Consistent with all-cause dementia (Supplementary Table 2), Table 3 also indicates that *So* was directly related to AD mortality risk among men (Model 2). Additionally, AD mortality risk was increased with higher *S. intermedius IgG*.

Periodontal pathogen factor and clusters and their association with AD mortality, AD and all-cause dementia incidence

Using factor analysis and pre-defined clusters (Table 4), our results indicated that AD incidence was associated with *Factor 4* in the 65+ age group, which loaded highly on *C. rectus* and *P. gingivalis* titers (aHR = 1.22; 95% CI, 1.04–1.43, $p = 0.012$). In this model, the effect of 1 SD increase in *Factor 4* on AD incidence was equivalent to two years of aging on the Log_e(HR) scale. Moreover, AD mortality risk was increased with higher baseline Factor 2 in that age group (per SD, aHR = 1.46; 95% CI, 1.09–1.96, $p = 0.017$) which loaded highly on *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *F. nuclatum*, *C. rectus*, *S. intermedius*, *C. ochracea* and *P. melaninogenica* titers. In both 55+ and 65+ age group, Orange-Red cluster (*P. melaninogenica*, *P. intermedia*, *P. nigrescens*, *P. gingivalis*) was associated with increased AD mortality risk, while Red-Green cluster (*T. forsythia*, *T. denticola*, *A. actinomycetemcomitans*, *E. corrodens*, *S. noxia*, *V. parvula*, *C. rectus*) was only marginally associated with AD and all-cause dementia among women ($p < 0.033$), after correction for multiple testing.

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Table 2
P. gingivalis and *A. actinomycetemcomitans* serum IgG's association with incident all-cause and Alzheimer's disease (AD) dementia and with AD mortality in multiple Cox proportional hazards model, overall and stratified by sex and race: NHANES III, 1991–1994[a]

	z-scored periodontal pathogen IgG (Phase II)[b]			z-scored, Log_e transformed periodontal pathogen IgG (Phase II)[b]		
	Log_e (HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*
All-cause dementia[c]						
Men (N = 1,646)						
P. gingivalis	–0.19	(0.17)	0.31	–0.12	(0.10)	0.26
A. actinomycetemcomitans	+0.03	(0.06)	0.62	+0.03	(0.06)	0.66
Women (N = 1,979)						
P. gingivalis	+0.07	(0.07)	0.34	–0.04	(0.10)	0.69
A. actinomycetemcomitans	+0.05	(0.05)	0.36	+0.03	(0.07)	0.62
45+ at baseline (N = 3,625)						
P. gingivalis	–0.01	0.11	0.92	–0.08	(0.06)	0.19
A. actinomycetemcomitans	+0.04	(0.04)	0.34	+0.03	(0.06)	0.60
55+ at baseline (N = 2,731)						
P. gingivalis	+0.01	(0.09)	0.88	–0.08	(0.06)	0.21
A. actinomycetemcomitans	–0.01	(0.05)	0.91	–0.01	(0.05)	0.81
65+ at baseline (N = 1,817)						
P. gingivalis	+0.04	(0.07)	0.59	–0.06	(0.06)	0.33
A. actinomycetemcomitans	**+0.12**	**(0.06)**	**0.051**	+0.08	(0.06)	0.19
AD dementia[d]						
Men (N = 1,646)						
P. gingivalis	–0.05	(0.21)	0.81	–0.11	(0.12)	0.37
A. actinomycetemcomitans	+0.10	(0.11)	0.34	+0.11	(0.11)	0.34
Women (N = 1,979)						
P. gingivalis	+0.13	(0.04)	**0.004****	+0.04	(0.06)	0.58
A. actinomycetemcomitans	–0.03	(0.11)	0.81	–0.09	(0.11)	0.40
45+ at baseline (N = 3,625)						
P. gingivalis	**+0.06**	**(0.03)**	**0.034**	–0.04	(0.06)	0.50
A. actinomycetemcomitans	+0.01	(0.06)	0.84	–0.00	(0.07)	0.95
55+ at baseline (N = 2,731)						
P. gingivalis	**+0.06**	**(0.02)**	**0.015****	–0.03	(0.06)	0.64
A. actinomycetemcomitans	–0.04	(0.08)	0.62	–0.07	(0.07)	0.28
65+ at baseline (N = 1,817)						
P. gingivalis	**+0.11**	**(0.03)**	**0.003****	+0.03	(0.07)	0.72
A. actinomycetemcomitans	+0.07	(0.07)	0.35	+0.02	(0.07)	0.73
AD mortality[e]						
45+ at baseline (N = 3,625)						
P. gingivalis	+0.11	(0.07)	0.13	+0.14	(0.14)	0.31
A. actinomycetemcomitans	–0.03	(0.34)	0.94	–0.27	(0.30)	0.31
55+ at baseline (N = 2,731)						
P. gingivalis	+0.09	(0.06)	0.13	+0.16	(0.14)	0.27
A. actinomycetemcomitans	–0.02	(0.35)	0.95	–0.33	(0.36)	0.38
65+ at baseline (N = 1,817)						
P. gingivalis	**+0.19**	**(0.15)**	**0.21**	**+0.35**	**(0.15)**	**0.033***
A. actinomycetemcomitans	**–2.61**	**(0.73)**	**0.002****	**–1.23**	**(0.28)**	**<0.001****

25(OH)D, 25-hydroxyvitamin D; AD, Alzheimer's disease; EU, ELISA units; HEI, Healthy Eating Index; HR, hazard ratio; HS, high school; IgG, Immunoglobulin G; MAR = Mean Adequacy Ratio; NHANES = National Health and Nutrition Examination Surveys. [a]Models were adjusted for age, sex, race/ethnicity, poverty income ratio, education (years), urban-rural area of residence, household size, marital status, nutritional factors (HEI, MAR), nutritional biomarkers (25(OH)D, folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium and normalized calcium), lifestyle (smoking, drug use, alcohol, physical activity), health-related factors (self-rated health, co-morbidity index, allostatic load, weight status), dentate status and social support variables. Covariates (other than exposures) were imputed and analysis is across 5 imputations with 10 iterations. [b]Standardized into z-scores. 1 SD of untransformed Pg is 434 (45+), 148 (55+), 276 (65+), 605 (Men), 212 (Women); 1 SD of untransformed Aa is 87 (45+), 82 (55+), 72 (65+),85 (Men), 88 (Women). 1 SD of Log_e transformed Pg is ~0.85–0.90 for all groups; 1 SD of Log_e transformed Aa is ~0.85–0.90 for all groups. [c]997 unweighted incident dementia cases for 45+, weighted mean follow-up time: 185 months. [d]503 unweighted incident AD cases for 45+, weighted mean follow-up time: 189 months. [e]52 unweighted AD deaths for 45+, weighted mean follow-up time: 192 months. *$p < 0.033$, marginally significant after correction for multiple testing; **$p < 0.016$, significant after correction for multiple testing.

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Table 3
Periodontal pathogens' serum IgG association with AD mortality in multiple Cox proportional hazards model, overall and restricted by baseline age group and sex: NHANES III, 1988–1994[a,b]

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*
AD mortality															
P. Gingivalis mix															
Model 1	+0.14	(0.13)	0.88	+0.15	(0.14)	0.28	**+0.35**	**(0.17)**	**0.046**	+0.25	(0.30)	0.40	+0.02	(0.25)	0.93
Model 2	+0.15	(0.10)	0.16	+0.17	(0.11)	0.13	**+0.31**	**(0.11)**	**0.010****	+0.33	(0.20)	0.11	–0.02	(0.13)	0.87
P. Intermedia															
Model 1	–0.08	(0.25)	0.74	–0.14	(0.27)	0.61	–0.19	(0.32)	0.57	+0.11	(0.52)	0.84	–0.04	(0.38)	0.93
Model 2	+0.20	(0.14)	0.15	+0.19	(0.13)	0.16	**+0.29**	**(0.13)**	**0.026***	+0.02	(0.14)	0.86	+0.11	(0.19)	0.58
P. Nigrescens															
Model 1	+0.18	(0.20)	0.36	+0.28	(0.21)	0.20	+0.18	(0.29)	0.54	–0.10	(0.66)	0.87	+0.10	(0.26)	0.69
Model 2	**+0.22**	**(0.13)**	**0.085**	**+0.25**	**0.13**	**0.063**	**+0.33**	**(0.14)**	**0.023***	**+0.43**	**(0.21)**	**0.049**	+0.11	(0.11)	0.46
T. Forsythia															
Model 1	**+0.43**	**(0.22)**	**0.058**	**+0.41**	**(0.21)**	**0.060**	+0.39	(0.25)	0.13	+0.86	(0.57)	0.14	**+0.64**	**(0.26)**	**0.019***
Model 2	+0.23	(0.14)	0.10	+0.22	(0.14)	0.12	+0.34	(0.16)	0.036	+0.49	(0.22)	**0.031***	+0.13	(0.17)	0.43
A. Actinomycetemcomitans (Aa) mix															
Model 1	**–0.38**	**(0.26)**	**0.15**	**–0.48**	**(0.27)**	**0.078**	–0.71	(0.45)	0.12	–0.05	(0.35)	0.88	**–0.80**	**(0.38)**	**0.039**
Model 2	–0.03	(0.15)	0.82	–0.10	(0.15)	0.52	–0.02	(0.19)	0.92	+0.59	(0.28)	0.042	–0.31	(0.20)	0.12
F. Nucleatum															
Model 1	–0.08	(0.28)	0.77	–0.04	(0.28)	0.89	+0.26	(0.35)	0.46	–0.77	(0.48)	0.12	+0.17	(0.40)	0.68
Model 2	+0.03	(0.13)	0.84	+0.02	(0.13)	0.86	+0.18	(0.14)	0.18	**+0.38**	**(0.19)**	**0.053**	–0.04	(0.16)	0.78
S. Oralis															
Model 1	+0.10	(0.26)	0.69	+0.03	(0.26)	0.90	+0.14	(0.31)	0.66	**+1.14**	**(0.46)**	**0.019***	–0.29	(0.29)	0.32
Model 2	+0.05	(0.13)	0.72	+0.02	(0.13)	0.87	+0.16	(0.13)	0.22	**+0.64**	**(0.25)**	**0.014****	–0.16	(0.16)	0.31
M. Micros															
Model 1	+0.21	(0.19)	0.29	+0.17	(0.20)	0.41	–0.20	(0.24)	0.39	–0.34	(0.32)	0.29	+0.42	(0.28)	0.15
Model 2	+0.15	(0.10)	0.16	+0.10	(0.12)	0.42	+0.07	(0.14)	0.64	+0.19	(0.21)	0.38	+0.13	(0.14)	0.35
C. Rectus															
Model 1	–0.13	(0.18)	0.49	–0.03	(0.19)	0.89	–0.11	(0.26)	0.69	–0.80	(0.52)	0.13	+0.02	(0.22)	0.91
Model 2	+0.07	(0.12)	0.57	+0.01	(0.16)	0.96	+0.23	(0.14)	0.10	+0.18	(0.22)	0.42	–0.04	(0.14)	0.76
E. Corrodens															
Model 1	+0.12	(0.30)	0.68	–0.01	(0.28)	0.98	+0.27	(0.35)	0.44	–0.13	(0.39)	0.74	+0.13	(0.38)	0.74
Model 2	+0.08	(0.18)	0.67	+0.02	(0.17)	0.90	+0.19	(0.15)	0.23	+0.29	(0.24)	0.23	–0.02	(0.24)	0.92
E. Nodatum															
Model 1	–0.01	(0.19)	0.96	–0.01	(0.21)	0.98	+0.15	(0.22)	0.50	–0.51	(0.40)	0.21	–0.12	(0.31)	0.71
Model 2	+0.06	(0.17)	0.73	+0.02	(0.17)	0.90	+0.20	(0.19)	0.31	**+0.32**	**(0.19)**	**0.094**	–0.12	(0.25)	0.64

(*Continued*)

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Table 3
(*Continued*)

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*
S. Intermedius															
Model 1	–0.09	(0.24)	0.71	–0.23	(0.23)	0.33	–0.11	(0.31)	0.72	+0.93	(0.37)	0.78	–0.35	(0.32)	0.28
Model 2	+0.04	(0.12)	0.72	–0.02	(0.12)	0.88	+0.15	(0.15)	0.33	**+0.72**	**(0.21)**	**0.001****	–0.19	(0.16)	0.26
C. Ochracea															
Model 1	+0.08	(0.17)	0.64	+0.10	(0.18)	0.56	+0.07	(0.23)	0.76	+0.10	(0.34)	0.78	–0.04	(0.22)	0.86
Model 2	+0.05	(0.13)	0.72	+0.06	(0.14)	0.68	+0.09	(0.17)	0.58	+0.21	(0.16)	0.19	–0.08	(0.17)	0.61
V. Parvula															
Model 1	–0.13	(0.28)	0.65	+0.02	(0.25)	0.93	–0.10	(0.29)	0.73	+0.08	(0.41)	0.85	–0.06	(0.32)	0.85
Model 2	+0.06	(0.14)	0.68	+0.02	(0.16)	0.91	+0.17	(0.15)	0.26	**+0.46**	**(0.23)**	**0.051**	–0.10	(0.18)	0.58
A. Naeslundii															
Model 1	+0.03	(0.18)	0.89	–0.02	(0.19)	0.92	–0.04	(0.23)	0.86	+0.57	(0.51)	0.27	–0.06	(0.21)	0.77
Model 2	+0.04	(0.16)	0.80	+0.22	(0.14)	0.12	+0.11	(0.19)	0.57	**+0.48**	**(0.20)**	**0.020***	–0.18	(0.21)	0.39
P. Melaninogenica															
Model 1	+0.30	(0.21)	0.17	+0.36	(0.22)	0.11	**+0.55**	**(0.19)**	**0.005****	+0.20	(0.38)	0.59	+0.42	(0.27)	0.12
Model 2	+0.20	(0.13)	0.14	+0.22	(0.14)	0.12	**+0.36**	**(0.13)**	**0.009****	**+0.41**	(0.20)	**0.047**	+0.15	(0.18)	0.40
S. Noxia															
Model 1	–0.15	(0.22)	0.50	–0.14	(0.22)	0.53	–0.14	(0.25)	0.57	–0.04	(0.32)	0.91	–0.34	(0.31)	0.28
Model 2	+0.02	(0.12)	0.87	+0.04	(0.13)	0.77	+0.13	(0.13)	0.31	**+0.37**	**(0.21)**	**0.092**	–0.16	(0.17)	0.36
T. Denticola															
Model 1	–0.10	(0.25)	0.68	–0.06	(0.24)	0.79	–0.18	(0.25)	0.48	–0.15	(0.32)	0.64	–0.03	(0.32)	0.92
Model 2	–0.09	(0.13)	0.49	–0.09	(0.13)	0.49	–0.05	(0.13)	0.70	+0.17	(0.25)	0.50	–0.13	(0.18)	0.48
S. Mutans															
Model 1	–0.26	(0.23)	0.27	–0.26	(0.24)	0.30	–0.19	(0.27)	0.49	–0.40	(0.29)	0.18	+0.03	(0.27)	0.90
Model 2	–0.07	(0.13)	0.59	–0.08	(0.13)	0.52	+0.02	(0.14)	0.86	**+0.35**	**(0.19)**	**0.074**	–0.22	(0.16)	0.17

AD, Alzheimer's disease; BMI, body mass index; HR, hazard ratio; HS, high school; IgG, Immunoglobulin G; NHANES, National Health and Nutrition Examination Surveys. [a]Models were adjusted for age, sex, race/ethnicity, poverty income ratio, education (years), urban-rural area of residence, household size, marital status, nutritional factors (HEI, MAR), nutritional biomarkers (25(OH)D, folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium and normalized calcium), lifestyle (smoking, drug use, alcohol, physical activity), health-related factors (self-rated health, co-morbidity index, allostatic load, weight status), dentate status and social support variables, as well as Phase of NHANES III. Covariates (other than exposures) were imputed and analysis is across 5 imputations with 10 iterations. Model 1: adjusted for all other periodontal pathogens; Model 2: one periodontal pathogen at a time. [b]Periodontal pathogen exposures were Log_e transformed and then standardized into z-scores. [c]Unweighted N = 6,277–6,581, weighted mean follow-up time: 200 months; [d]Unweighted N = 4,681–4,912, weighted mean follow-up time = 177 months; [e] Unweighted N = 3,077–3,229, weighted mean follow-up time: 148 months; [f]Unweighted N = 2,924–3,088, weighted mean follow-up time: 196 months [g]Unweighted N = 3,353–3,517, weighted mean follow-up time: 205 months. *$p < 0.033$, marginally significant after correction for multiple testing; **$p < 0.016$, significant after correction for multiple testing.

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Table 4
Periodontal pathogens' serum IgG (Factor scores and pre-defined clusters) association with AD mortality, AD incidence and all-cause dementia incidence in multiple Cox proportional hazards model, overall and restricted by baseline age group and sex: NHANES III, 1988–1994[a,b]

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*
Factor scores															
AD mortality															
Factor 1: So/Sm/Ec	–0.12	(0.16)	0.48	–0.23	(0.15)	0.14	–0.08	(0.17)	0.61	+0.56	(0.40)	0.17	–0.30	(0.24)	0.24
Factor 2:Pi/Pn/Pm	**+0.26**	**(0.14)**	**0.073**	**+0.31**	**(0.15)**	**0.043**	**+0.38**	**(0.15)**	**0.017****	+0.43	(0.29)	0.15	+0.19	(0.20)	0.35
Factor 3:An/En	+0.06	(0.19)	0.75	+0.02	(0.19)	0.90	+0.13	(0.23)	0.58	**+0.44**	**(0.24)**	**0.081**	–0.14	(0.27)	0.35
Factor 4:Pg/Cr	+0.12	(0.14)	0.40	+0.16	(0.15)	0.31	+0.19	(0.16)	0.24	–0.29	(0.28)	0.30	+0.16	(0.16)	0.32
Factor 5:Co/Sn	+0.04	(0.18)	0.82	**+0.23**	**(0.13)**	**0.081**	+0.18	(0.19)	0.34	–0.44	(0.39)	0.27	+0.17	(0.24)	0.48
AD incidence															
Factor 1: So/Sm/Ec	+0.06	(0.06)	0.34	+0.00	(0.07)	0.97	+0.06	(0.08)	0.48	+0.08	(0.14)	0.57	+0.08	(0.07)	0.25
Factor 2:Pi/Pn/Pm	+0.01	(0.05)	0.92	+0.01	(0.05)	0.86	+0.02	(0.06)	0.80	–0.00	(0.13)	1.00	–0.03	(0.07)	0.25
Factor 3:An/En	–0.08	(0.06)	0.20	–0.10	(0.06)	0.14	–0.11	(0.07)	0.13	–0.06	(0.11)	0.59	–0.10	(0.08)	0.25
Factor 4:Pg/Cr	+0.10	(0.06)	0.14	+0.11	(0.07)	0.11	**+0.20**	**(0.08)**	**0.012****	+0.12	(0.15)	0.44	**+0.17**	**(0.08)**	**0.038**
Factor 5:Co/Sn	–0.06	(0.09)	0.49	–0.02	(0.10)	0.83	–0.02	(0.12)	0.89	–0.05	(0.14)	0.72	–0.06	(0.09)	0.52
All-cause dementia incidence															
Factor 1: So/Sm/Ec	**+0.10**	**(0.05)**	**0.062**	+0.06	(0.05)	0.28	**+0.09**	**(0.05)**	**0.073**	+0.11	(0.09)	0.22	**+0.10**	**(0.06)**	**0.073**
Factor 2:Pi/Pn/Pm	–0.05	(0.04)	0.23	–0.03	(0.04)	0.55	–0.02	(0.04)	0.70	+0.02	(0.08)	0.83	**–0.10**	**(0.06)**	**0.070**
Factor 3:An/En	–0.07	(0.04)	0.15	–0.06	(0.04)	0.19	–0.07	(0.05)	0.16	–0.04	(0.08)	0.66	–0.08	(0.06)	0.17
Factor 4:Pg/Cr	+0.02	(0.05)	0.67	+0.04	(0.05)	0.39	+0.09	(0.06)	0.12	–0.10	(0.11)	0.33	+0.06	(0.06)	0.32
Factor 5:Co/Sn	–0.09	(0.06)	0.13	–0.06	(0.06)	0.39	–0.05	(0.07)	0.48	–0.15	(0.09)	0.10	–0.02	(0.07)	0.72
Pre-defined clusters															
AD mortality															
Cluster 1: Orange-Red	**+0.36**	**(0.16)**	**0.031***	**+0.44**	**(0.18)**	**0.016****	**+0.56**	**(0.17)**	**0.002****	+0.46	(0.47)	0.34	+0.29	(0.27)	0.28
Cluster 2: Red-Green	–0.22	(0.30)	0.47	–0.20	(0.31)	0.54	–0.16	(0.32)	0.61	–0.41	(0.75)	0.59	–0.18	(0.35)	0.61
Cluster 3: Yellow-Orange	+0.01	(0.20)	0.97	–0.07	(0.21)	0.73	–0.09	(0.25)	0.72	+0.38	(0.42)	0.37	–0.06	(0.23)	0.81
Cluster 4: Orange-Blue	+0.05	(0.18)	0.79	+0.01	(0.18)	0.94	+0.11	(0.22)	0.63	+0.34	(0.24)	0.16	–0.15	(0.26)	0.55

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Table 4
(*Continued*)

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*	Log_e(HR)	(SE)	*p*
AD incidence															
Cluster 1: Orange-Red	+0.02	(0.07)	0.75	+0.05	(0.08)	0.52	+0.05	(0.09)	0.57	–0.04	(0.14)	0.79	–0.00	(0.09)	0.99
Cluster 2: Red-Green	+0.16	(0.10)	0.12	+0.14	(0.09)	0.12	**+0.22**	**(0.11)**	**0.052**	–0.17	(0.21)	0.40	**+0.30**	**(0.13)**	**0.028***
Cluster 3: Yellow-Orange	–0.12	(0.09)	0.21	**–0.16**	**(0.09)**	**0.093**	–0.16	(0.12)	0.16	+0.22	(0.16)	0.19	**–0.22**	**(0.13)**	**0.092**
Cluster 4: Orange-Blue	–0.08	(0.06)	0.19	–0.08	(0.06)	0.17	–0.10	(0.06)	0.11	–0.05	(0.10)	0.64	–0.10	(0.08)	0.21
All-cause dementia incidence															
Cluster 1: Orange-Red	–0.05	(0.06)	0.37	–0.03	(0.06)	0.64	–0.04	(0.06)	0.53	+0.02	(0.10)	0.86	–0.11	(0.08)	0.15
Cluster 2: Red-Green	+0.12	(0.07)	0.11	**+0.12**	**(0.07)**	**0.083**	**+0.15**	**(0.09)**	**0.092**	–0.15	(0.15)	0.33	**+0.21**	**(0.09)**	**0.018***
Cluster 3: Yellow-Orange	–0.05	(0.06)	0.44	–0.07	(0.06)	0.22	–0.04	(0.09)	0.64	+0.14	(0.13)	0.25	–0.08	(0.10)	0.43
Cluster 4: Orange-Blue	–0.06	(0.04)	0.18	–0.05	(0.04)	0.21	–0.07	(0.04)	0.64	–0.02	(0.07)	0.79	–0.08	(0.05)	0.16

See Table 1 for periodontal pathogen abbreviations. AD, Alzheimer's disease. [a]Models were adjusted for age, sex, race/ethnicity, poverty income ratio, education (years), urban-rural area of residence, household size, marital status, nutritional factors (HEI, MAR), nutritional biomarkers (25(OH)D, folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium and normalized calcium), lifestyle (smoking, drug use, alcohol, physical activity), health-related factors (self-rated health, co-morbidity index, allostatic load, weight status), dentate status and social support variables, as well as Phase of NHANES III. Covariates (other than exposures) were imputed and analysis is across 5 imputations with 10 iterations. [b]19 Periodontal pathogen exposures (both phases) were Log_e transformed and then standardized into z-scores. Factor analysis was conducted from which 5 factors were extracted each explaining >4% of total variance. After varimax rotation, factor 1 loaded highest ($\lambda > 0.40$) on *T. forsythia* (0.68), *A. actinomycetemcomitans* (0.65*), F. nucleatum* (0.53), *S. oralis* (0.81), *M. micros* (0.51), *C. rectus* (0.54), *E. corrodens* (0.68), *S. intermedius* (0.63), *V. parvula* (0.64), *S. noxia* (0.62), *T. denticola* (0.63) and *S. mutans* (0.73); factor 2 on *P. gingivalis* (0.42), *P. itermedia* (0.85), *P. nigrescens* (0.84*), F. nucleatum* (0.44), *C. rectus* (0.40), *S. intermedius* (0.42), C. *ochracea*(0.44), *P. melaninogenica* (0.70); factor 3 on *E. nodatum* (0.75), *A. naselundii* (0.76); factor 4 on *P. gingivalis* (0.44) and *C. rectus* (0.42); and factor 5 on C. *ochracea* (0.45) and *S. noxia* (0.41). Factors were labelled based on up to 3 highest loadings, using a shortcut name for each. See Supplementary Figure 1 and methods section for definition of each cluster. [c]Unweighted N = 6,277–6,278, weighted mean follow-up time: 200 months (AD mortality), 197 months (AD incidence), 192 months (all-cause dementia); [d] Unweighted N = 4,681–4,682, weighted mean follow-up time: 177 months (AD mortality), 171 months (AD incidence), 165 months (all-cause dementia); [e] Unweighted N = 3,077, weighted mean follow-up time: 148 months (AD mortality), 141 months (AD incidence), 133 months (all-cause dementia); [f]Unweighted N = 2,924, weighted mean follow-up time: 196 months (AD mortality), 193 months (AD incidence), 189 months (all-cause dementia) [g]Unweighted N = 3,353–3,354, weighted mean follow-up time: 205 months (AD mortality), 200 months (AD incidence), 194 months (all-cause dementia). *$p < 0.033$, marginally significant after correction for multiple testing; **$p < 0.016$, significant after correction for multiple testing.

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Clinical Pd markers and their association with periodontal pathogens and AD/all-cause dementia outcomes

Moreover, P. gingivalis IgG, the Orange-Red, Red-Green and Yellow-Orange clusters, Factors 2 and 4 were independently associated with clinical Pd markers (AL/PPD) (Supplementary Table 3). Nevertheless, only a marginal association between PPD and incident AD risk was detected among men and older individuals upon multiple testing adjustment (Supplementary Table 4).

DISCUSSION

To our knowledge, this is the first large retrospective cohort study to examine the association between periodontal pathogens (and measures of Pd: AL/PPD) with AD incidence and mortality and incident all-cause dementia. Our findings indicated that IgG against P. gingivalis, P. melaninogenica, and C. rectus, two empirical periodontal pathogen factors, and two empirical periodontal pathogen clusters as well as PPD were consistently linked with at least one of the 3 outcomes among older adults. Moreover, findings with all-cause dementia and not AD pertained mostly to the outcome of vascular dementia, given that it is the second most common cause of dementia.

Although there are no other studies examining the association between periodontal pathogens and incidence of dementia per se, several studies have examined the relations between periodontal pathogens and cognitive impairments that could yield dementia outcomes. Our findings of positive associations between Pd and periodontal pathogens with various dementia outcomes mirror previous findings with cognitive outcomes. Specifically, using NHANES III, a study found that the highest Pg IgG (119 ELISA Units [EU]) were more likely to exhibit poor delayed verbal recall (OR 2.89, 95% CI 1.14 to 7.29) and impaired subtraction (OR 1.95, 95% CI 1.22 to 3.11) [13]. Two other nested case-control studies of periodontal pathogens found that participants with elevated A. naeslundii IgG (0.640 ng/ml) level exhibited higher risk of AD [14], as did titers for F. nucleatum and P. intermedia [15]. Similarly, the risk of developing dementia was higher among Pd patients compared to controls (HR = 1.16, 95% CI = 1.01–1.32, $p = 0.03$) [33]. Nevertheless, recent reviews and meta-analyses examining pooled evidence on Pd and dementia came to different conclusions [12]. This discrepancy highlights the need to examine associations between periodontal pathogens with AD and other types of dementia within different sub-groups (preferably at different baseline ages), as was done in our present study. Furthermore, our study indicated that PPD, a measure of current periodontitis was associated with incident AD among older adults, though that was not the case of AL, a measure of cumulative exposure. This finding needs to be replicated in other comparable cohorts.

Suggested mechanisms linking Pd or periodontal pathogens with cognitive impairment and dementia are still speculative. First, bacterial pathogens can spread from periodontal regions to blood stream into other bodily organs. Second, toxins produced by pathogens can damage the vascular system via oxidative stress leading to atherosclerosis which may trigger dementia or stroke [12]. Third, inflammatory mediators of Pd including cytokines, chemokines, and prostaglandins can contribute to AD by triggering brain inflammation [19]. P. gingivalis and P. melaninogenica are related rod-shaped, black-pigmented, strictly anaerobic gram-negative bacteria [18]. Perhaps the most characteristic feature of P. gingivalis induced periodontitis is the production of gingipains, enzymes that can cleave proteins specifically after arginine or lysine amino acids [34], and which are secreted through a complex known as Type IX Secretion System (T9SS) protein secretion system [34]. Gingipains target host peptides with antimicrobial or anti-inflammatory activities, and by inactivating them induce edema and bleeding, in addition to allowing bacterial cells to infiltrate neutrophils [35]. Together with other virulence factors, this allows P. gingivalis to induce inflammation while evading host immune response [36], and to make use of inflammatory fluids as a source of essential nutrients (e.g., iron) [37] required for bacterial growth [38]. P. gingivalis produces proteolytic enzymes that target immunoglobulins and cell surface adhesion proteins, which could facilitate invasion and weaken host immune resp Mouse models show that the lipopolysaccharides (LPS) and the gingipains produced by Pg respectively increase accumulation of amyloid-β (Aβ) [20, 39] and enhance migration and inflammation of microglia [40], which are two hallmark pathologies of AD. Recent mouse studies have demonstrated that repeated exposure to P. gingivalis, resulting in gingipain accumulation in and around brain cells, was responsible for neurodegeneration and strongly correlated with hippocampal Aβ accumulation [20, 41] onset [42]. Importantly, Poole et al.

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confirmed in an *in vitro* study of AD brain tissue and controls that that LPS from periodontal bacteria can access the AD brain during life given that labeling in the matched controls was absent. This demonstration of a known chronic oral-pathogen-related virulence factor reaching the human brains suggests an inflammatory role in the existing AD pathology [43]. Moreover, Dominy et. al have found that small-molecule inhibitors of gingipains may be an effective treatment against P. gingivalis-induced brain inflammation and bacterial colonization and thus may slow neurodegeneration [20]. The present study adds to the epidemiological evidence suggesting that P. gingivalis eradication among others may be an effective means to delay onset of AD, pending randomized clinical trials.

Just like P. gingivalis, P. melaninogenica expresses a complete T9SS secretion system. Although P. melaninogenica does not use gingipains, T9SS is important in biofilm formation and is involved in Pd, possibly by secreting proteases [44]. Our results indicate that Pg and Pm may independently or interactively induce cognitive impairment leading to AD as an underlying cause of death among older adults.

Moreover, another study showed that P. gingivalis LPS alone was sufficient to antagonize IL-6 and IL-8, but not IL-1β stimulation by another pathogen, namely C. rectus, suggesting that mixed infections, particularly interactions between P. gingivalis and C. rectus may impair host immune responses through cytokine level reduction of direct relevance to both periodontitis and AD [45].

Our study has several notable strengths including the use of a large, nationally representative sample, inclusion of middle-aged adults (≥45 years), assessment of AD incidence and mortality and incident all-cause dementia over a long follow-up period of up to 26 years, measurement of serum antibody levels for periodontal pathogens combined with dental examination and adjustment for key potential confounders.

Limitations include observational study design, even though temporality of associations were ascertained. Underdiagnosis of AD and other dementias is a possibility despite the fact that over 90% of the US population is eligible for and uses Medicare after the age of 65 years and that the linkage was comprehensive, including all aspects of health care utilization (e.g., inpatient and outpatient) with continuous follow-up between 1991 and 2014. Nevertheless, a few cases missed by Medicare were added using NDI to assess incident AD and all-cause dementia. Moreover, the data lacked some key biochemical biomarkers of AD (such as blood or CSF markers of Aβ and tau) and neuroimaging of patients. Additionally, clinical periodontal measures were only estimated based on partial-mouth examination. This could underestimate Pd severity, thus attenuating observed associations. An in-depth study examining other alternative measures of clinically defined categories for periodontitis may be warranted. Furthermore, serum IgG humoral immune response exposures, though normalized through Log_e transformation, exhibited moderate collinearity. Finally, residual confounding bias particularly by genetic risk factors (e.g., ApoE4 status) cannot be discounted.

This study provides evidence for an association between periodontal pathogens and AD, which was stronger for older adults and calls for a line of inquiry, including randomized controlled trials, on the effectiveness of periodontal treatment against onset and progression of neurodegenerative disorders such as AD.

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SUPPLEMENTARY MATERIAL

Clinical and Bacterial Markers of Periodontitis and Their Association with Incident All- Cause and Alzheimer's Disease Dementia in a Large National Survey

SUPPLEMENTARY MATERIAL I: CMS-MEDICARE AND NDI LINKAGE

Administered by CMS, Medicare is the primary health insurance program for people ≥ 65 years, people < 65 years with specific disabilities, and all individuals with end-stage renal disease (ESRD) [1]. Medicare enrollment and fee-for-service (FFS) claims data were linked to NHANES III participants by matching on Social Security Number (SSN), date of birth (month, day, year), and sex. CMS performed linkage on NHANES participants matching them with corresponding Medicare FFS annual claims data, and with Medicare Part D prescriptions since 2006 [1]. Annual files are available for Part A (inpatient, outpatient, Skilled Nursing Facility [SNF], hospice, or Home Health Agency [HHA]) and Part B (Carrier, Durable Medical Equipment) [1]. These files were used to identify the first report of dementia and AD diagnoses for NHANES participants. Methods published elsewhere were used to estimate earliest occurrence of the two incident outcomes of interest 1991 through 1998 [1]. Restricted mortality data linked to NHANES III through the National Death Index was used to examine AD mortality risk through 2013 in all analyses [2].

SUPPLEMENTARY MATERIAL II: FACTOR ANALYSIS FOR PERIODONTAL PATHOGEN TITERS

The common factor model can be summarized as follows:

$$\mathrm{Pp_i} = \sum\nolimits_{-j} = 1^{k} \lambda_{ij} * Factorj + \phi_i \qquad (1)$$

where Pp_i is the standardized z-score for each Loge transformed periodontal pathogen titer (IgG),λ_{ij} is the factor loading for each Pp and each factor, $Factor_j$ is the standardized z-score for each $factor_j$, and φ_i is the residual error, the squared value of which is the uniqueness. The sum of squared factor loadings for each Pp_i is the communality or the common variance that is accounted for by the extracted factors.

An eigenvalue > 1 rule was used and the scree plot was observed to determine the adequate number of extracted factors that would produce the best model fit. Moreover, additional factors were extracted when the next factor explained < 2% of the total variance. The factor loadings were then rotated using varimax orthogonal rotation and the factors were interpreted and Factors labeled accordingly, with cutoff point of 0.40 or more for significant loading. The factor scores (z-scores) were predicted and used as markers of normalized periodontal pathogen IgG concentration, taking into account correlations between titers. Thus, the predicted rotated factors had weak correlation ($|r| < 0.20$) (Table II.1).

SUPPLEMENTARY MATERIAL III: COVARIATES

Socio-demographic factors and socio-economic status (SES)

Key socio-demographic covariates included age (y), sex, race [Non-Hispanic white (NHW), Non-Hispanic black (NHB), Mexican-American (MA), other ethnicity (OTHER)]; marital status (Never married, Married, Divorced, Widowed, Other), household size and urban-rural residence (Urban, Rural). Among SES factors, educational level (<High school, High school,>High school); poverty income ratio (PIR) ($\leq$100%,>100–$\leq$200%,>200%) were selected.

Lifestyle and social support factors

Lifestyle factors include "substance abuse" operationalized as "alcohol consumption (g/d)" and drug use (Ever, Never); "nutritional factors" as 1995-Healthy Eating Index (1995-HEI) ranging from 0–100 [3], and mean adequacy ratio score (MAR) [4–6] (Tables III.1 and III.2); "Physical activity" measured with 3 items: 1) "Compare activity for past month to past year (less, same, more), 2): "Active compared with men/women your age" (less, same, more), and 3): "Active now compared with self, 10 years ago" (less, same, more); "Smoking" with two items: 1): "number of cigarettes smoked per day" (0 among non-smokers); 2): "years smoked cigarettes" (0 among non-smokers), drug and alcohol use. Five items were used for social support: "In a typical week, how many times do you" ... 1) talk on the telephone with family, friends, or neighbors?", 2) "get together with friends or relatives? (# per year)", 3) "visit with

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Supplementary Table II.1
Rotated factor loading matrix for Log_e transformed periodontal pathogen titers (IgG), NHANES III, both phases, 1988–1994 (Age: 45+)

	FACTOR 1	FACTOR 2	FACTOR 3	FACTOR 4	FACTOR 5
Both Phases, Log_e transformed[3]					
P. Gingivalis (Pg) mix	+0.29	**+0.42**	+0.05	**+0.44**	+0.08
P. Intermedia (Pi)	+0.23	**+0.85**	+0.08	+0.19	+0.00
P. Nigrescens (Pn)	+0.27	**+0.84**	+0.12	+0.04	+0.09
T. Forsythia (Tf)	**+0.68**	+0.31	+0.25	+0.33	–0.04
A. Actinomycetemcomitans (Aa) mix	**+0.65**	+0.39	+0.16	+0.21	+0.30
F. Nucleatum (Fn)	**+0.53**	+0.45	+0.16	+0.03	+0.11
S. Oralis (So)	**+0.81**	+0.34	+0.17	+0.01	+0.16
M. Micros (Mm)	**+0.51**	+0.38	+0.19	+0.01	+0.20
C. Rectus (Cr)	**+0.54**	**+0.40**	+0.15	**+0.42**	–0.02
E. Corrodens (Ec)	**+0.68**	+0.18	+0.15	+0.32	–0.00
E. Nodatum (En)	+0.31	+0.08	**+0.76**	+0.13	–0.02
S. Intermedius (Si)	**+0.64**	**+0.42**	+0.20	–0.03	**+0.45**
C. Ochracea (Co)	**+0.40**	**+0.44**	+0.16	+0.11	+0.24
V. Parvula (Vp)	**+0.64**	+0.34	+0.24	+0.15	+0.12
A. Naeslundii (An)	+0.12	+0.15	+0.76	–0.02	+0.23
P. Melaninogenica (Pm)	+0.35	+0.70	+0.10	–0.02	+0.12
S. Noxia (Sn)	**+0.62**	+0.29	+0.17	+0.22	+0.41
T. Denticola (Td)	**+0.63**	+0.01	+0.17	+0.34	+0.20
S. Mutans (Sm)	**+0.72**	+0.34	+0.13	–0.01	+0.18

Factors were extracted using eigenvalue > 1 rule or a change in % variance explained of at least 2%. The scree plot was used to visualize the best number of factors to extract.

any of your other neighbors, either in their homes or in your own? (# per year)", 4) "attend church or religious services? (# per year)", 5) "attend meetings of the clubs or organizations (# per year)".

Nutritional biomarkers and health-related factors

Several nutritional biomarkers were included among potential confounders, namely serum 25-hydroxyvitamin D [25(OH)], folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium, and normalized calcium.

Serum 25-hydroxyvitamin D (25(OH)D)

The INCSTAR 25(OH)D assay consists of a two-step procedure, with the first being a rapid extraction of 25(OH)D and other hydroxylated metabolites from serum or plasma with acetonitrile, and the second being the assay of the treated sample by using an equilibrium RIA procedure [7].

Serum ferritin

Ferritin, like hemoglobin, is a major iron storage protein. Serum ferritin levels increase as a result of iron overload, aging, infection, inflammation, liver disease, juvenile rheumatoid arthritis, leukemia, and Hodgkin's disease; and decrease as a result of iron deficiency. Ferritin is measured by using the Bio-Rad Laboratories' "QuantImune Ferritin IRMA" kit, a single-incubation two-site immunoradiometric assay (IRMA) based on the general principles of assays [7, 8].

Serum folate and vitamin C

Folate is required in cellular metabolism and hematopoiesis, and prolonged deficiency leads to megaloblastic anemia. In NHANES III, serum folate is measured by using the Bio-Rad Laboratories "Quantaphase Folate" radioassay kit [7, 9].

Serum normalized calcium

To measure the total calcium concentration, protein-bound and complexed calcium must be released [7]. Within the pH range of 6.9–8.0, the NOVA 7 analyzer can predict the normalized calcium level within 2% of the theoretical value [10].

Serum selenium

Serum selenium is measured by atomic absorption spectrometry [7] in a procedure based on Lewis et al. [11] and Paschal and Kimberly [12].

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Supplementary Table III.1
1995-HEI

Components	Criteria[1] <50 y	Criteria[1] ≥50 y	Score
Grains	9.1 servings/d	7.4 servings/d	10; 1 point less for each 10% less than intake required for full score Range: 0–10
Vegetables	4.2 servings/d	3.5 servings/d	Same as above
Fruit	3.2 servings/d	2.5 servings/d	Same as above
Milk	2.0 servings/d	2.0 servings/d	Same as above
Meat	2.4 servings/d	2.2 servings/d	Same as above
Total fat	≤30% energy	≤30% energy	10
	31–44% of energy	31–44% of energy	5
	≥45% of energy	≥45% of energy	0
Saturated fat	≤10% of energy	≤10% of energy	10
	11–14% of energy	11–14% of energy	5
	≥15% of energy	≥15% of energy	0
Cholesterol	<300 mg	<300 mg	10
	301–449 mg	301–449 mg	5
	≥450 mg	≥450 mg	0
Sodium	≤2,400 mg	≤2,400 mg	10; 1 point less for each 10% less intake required for full score
Variety	Top 10% intake of sum of unique foods	Top 10% intake of sum of unique foods	Same as above

[1]Based on 2,200 kcal for the < 50 y category and 1,900 kcal for the ≥ 51 y category. *Source: [3, 15]*

Supplementary Table II.2
Recommended Dietary Allowance (RDA) Values for Nutrients accessed from USDA DRI Tables to MAR Score

Nutrient	Men	Men	Men	Women	Women	Women
	19–30 y	31–50 y	51–70 y	19–30 y	31–50 y	51–70 y
Vitamin A	900 μg/day	900 μg/day	900 μg/day	700 μg/day	700 μg/day	700 μg/day
Vitamin C	90 mg/day	90 mg/day	90 mg/day	75 mg/day	75 mg/day	75 mg/day
Vitamin C for smokers	125 mg/day	125 mg/day	125 mg/day	110 mg/day	110 mg/day	110 mg/day
Vitamin D	15 μg/day	15 μg/day	15 μg/day	15 μg/day	15 μg/day	15 μg/day
Vitamin E	15 mg/day	15 mg/day	15 mg/day	15 mg/day	15 mg/day	15 mg/day
Vitamin B6	1.3 mg/day	1.3 mg/day	1.7 mg/day	1.3 mg/day	1.3 mg/day	1.5 mg/day
Vitamin B12	2.4 μg/day	2.4 μg/day	2.4 μg/day	2.4 μg/day	2.4 μg/day	2.4 μg/day
Thiamin	1.2 mg/day	1.2 mg/day	1.2 mg/day	1.1 mg/day	1.1 mg/day	1.1 mg/day
Riboflavin	1.3 mg/ day	1.3 mg/ day	1.3 mg/day	1.1 mg/day	1.1 mg/day	1.1 mg/day
Niacin	16 mg day	16 mg day	16 mg/day	14 mg/day	14 mg/day	14 mg/day
Folate	400 μg/day	400 μg/day	400 μg/day	400 μg/day	400 μg/day	400 μg/day
Iron	8 mg/day	8 mg/day	8 mg/day	18 mg/day	18 mg/day	8 mg/day
Copper	900 μg/day	900 μg/day	900 μg/day	900 μg/day	900 μg/day	900 μg/day
Zinc	11 mg/day	11 mg/day	11 mg/day	8 mg/day	8 mg/day	8 mg/day
Calcium	1,000 mg/day	1,000 mg/day	1,000 mg/day	1,000 mg/day	1,000 mg/day	1,200 mg/day
Magnesium	400 mg/day	420 mg/day	420 mg/day	310 mg/day	320 mg/day	320 mg/day
Phosphorous	700 mg/day	700 mg/day	700 mg/day	700 mg/day	700 mg/day	700 mg/day

μg, micrograms; mg, milligrams; g, grams http://www.nal.usda.gov/sites/default/files/fnic_uploads//RDA_AI_vitamins_elements.pdf http://ods.od.nih.gov/factsheets/VitaminC-HealthProfessional/

Serum vitamin E, retinol, retinyl esters, carotenoids

Serum levels of vitamin A (retinol), vitamin E (α-tocopherol), retinyl esters, and carotenoids are measured by isocratic high-performance liquid chromatography with detection at three wavelengths, namely 300, 325, and 450 nm. Quantitation is accomplished by comparison of peak heights with a standard solution [7].

The "Health" construct was operationalized with self-rated health, co-morbidity index and an allostatic load (AL) score. Self-rated their health

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Supplementary Table III.3
Allostatic load indicator criteria [13]

	High-risk clinical
Albumin (g/dL)	<3.8 [16]
C-reactive protein (mg/dL)	≥0.3 [17]
Waist:Hip Ratio	>0.9 for men;>0.85 for women [18]
Total cholesterol (mg/dL)	≥240 [19]
HDL (mg/dL)	<40 [19]
Glycated hemoglobin (%)	≥6.4 [20, 21]
Resting heart rate (beat/min)	≥90 [22]
Systolic BP	≥140 [23]
Diastolic BP	≥90 [23]

as: "Excellent" (referent), "Very good", "Good", "Fair" or "Poor." The co-morbidity index is an unweighted sum of 14 binary self-reported conditions, including "arthritis", "congestive heart failure", "stroke", "asthma", "chronic bronchitis", "emphysema", "hay fever", "cataracts", "goiter", "thyroid disease", "lupus", "gout", "skin cancer", and "other cancer". The allostatic load (AL) total score (0–9) consists of 9 items which are described in details elsewhere [13]. AL total score sums up cardiovascular (systolic and diastolic blood pressure, pulse rate), metabolic (total cholesterol, HDL-cholesterol, glycosylated Hb, sex-specific waist-to-hip ratio) and inflammatory (albumin and C-reactive protein (CRP)) risk indicators. Clinical criteria are summarized in Table III.3. Weight status was measured using body mass index (weight/squared-height, $kg.m^{-2}$) categorized as: < 18.5 (underweight), 18.5–24.9 (normal weight), 25–29.9 (overweight), ≥ 30 as obese.

Total cholesterol (mg/dl), HDL-cholesterol (mg/dl), CRP (mg/dl), albumin (g/dl), and glycosylated hemoglobin (%) were measured by laboratories using reference analytical methods (See Laboratory Procedures for NHANES III) [14]. Using standard protocols, waist-to-hip ratio, radial pulse (beats/min), and systolic and diastolic blood pressure (mmHg) were measured by trained examiners. Blood pressure was determined using a mercury sphygmomanometer [14]. The arithmetic mean of three systolic and diastolic pressures was used in the analysis. Finally, dentate status was as follows: "fully dentate", "partially edentulous", "fully edentulous".

MAR scores

RDAs of 16 vitamins and minerals were used to determine the nutrient adequacy ratio (NAR), using the following formula: NAR = [Subject's daily intake of nutrient] / [RDA of nutrient]. An adjustment of an additional 35 mg Vitamin C must be applied to the RDA for participants who were current smokers.

The NAR of each nutrient is converted to a percent, and percentages greater than 100 are truncated to 100. The total quality of the diet is then calculated from the NARs to form a mean adequacy ratio (MAR) using the following formula: MAR = [Sum of all 16 nutrient NARs]/16. The NAR and MAR for each day were calculated and then averaged over the two days.

Allostatic load (AL)

A total AL score was computed using a method described in a previous study [13]. AL total score sums up cardiovascular (systolic and diastolic blood pressure, pulse rate), metabolic (total cholesterol, HDLcholesterol, glycosylated Hb, sex-specific waist-to-hip ratio) and inflammatory (albumin and C-reactive protein (CRP)) risk indicators. Clinical criteria summarized in Table III.3 were used to obtain risk indicators which were summed with equal weighting to compute total AL score (range: 0–9).

Total cholesterol (mg/dl), HDL-cholesterol (mg/dl), CRP (mg/dl), albumin (g/dl), and glycosylated hemoglobin (%) were measured by contract laboratories using reference analytical methods (See Laboratory Procedures for NHANES III) [14]. Using standard protocols, waist-to- hip ratio, radial pulse (beats/min), and systolic and diastolic blood pressure (mmHg) were measured by trained examiners. Specifically, blood pressure was measured using a mercury sphygmomanometer [14]. The arithmetic mean of three systolic and diastolic pressures was used in analysis.

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Supplementary Table 1
Baseline characteristics of selected participants by age group, NHANES III, 1988–1994 [$N = 3,749$ (phase 2:1991–1994); 6,650 (both phases)][a]

Selected participant characteristics	Age group (y)			pe* (Design-based F-test)[b]	
	45–55	55–65	65+		
Unweighted N (both phases)	($N = 1,701$) 25.6%	($N = 1,698$) 25.5%	($N = 3,251$) 48.9%	55–65 versus 45–55	65+ versus 45–55
Socio-economic status	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Poverty income ratio	3.7 ± 0.12	3.3 ± 0.09	2.9 ± 0.09	<0.001	<0.001
Education, y	12.6 ± 0.15	11.7 ± 0.14	11.0 ± 0.16	<0.001	<0.001
Nutritional factors	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
1995-HEI total score	64.1 ± 0.56	67.0 ± 0.61	68.9 ± 0.40	0.001	<0.001
MAR total score	73.9 ± 0.66	73.6 ± 0.53	73.2 ± 0.38	0.65	0.32
Physical activity					
0 = Less, 1 = Same, 2 = more					
Compare activity for past mo to past year	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Less	27.3 ± 2.04	24.8 ± 1.51	23.1 ± 1.13	0.12	0.01
Same	56.3 ± 2.34	62.7 ± 1.62	68.6 ± 1.22	–	–
More	16.3 ± 1.52	12.5 ± 1.07	8.3 ± 0.74	0.007	<0.001
Active compared with men/women your age	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Less	21 ± 1.71	20.5 ± 1.23	14.5 ± 0.98	0.93	0.02
Same	42.8 ± 2.12	41.2 ± 1.81	41.4 ± 1.37	–	–
More	36.3 ± 1.7	38.4 ± 1.96	44.1 ± 1.47	0.38	0.02
Active now compared with self 10 years ago	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Less	55.0 ± 2.2	56.3 ± 1.76	65.6 ± 1.42	0.68 0.05	
Same	30.1 ± 1.6	32.5 ± 1.73	29.0 ± 1.27	–	–
More	14.9 ± 1.38	11.3 ± 1.11	5.4 ± 0.72	0.06	<0.001
Smoking	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
# cigarettes/day	8.5 ± 0.63	7.8 ± 0.48	5.1 ± 0.35	0.35	<0.001
Years smoked cigarettes	6.3 ± 0.45	8.0 ± 0.53	6.4 ± 0.37	0.02	0.89
Alcohol use	8.6 ± 0.81	7.0 ± 0.84	4.8 ± 0.64	0.12	<0.001
Drug use	21.1 ± 0.02	11.1 ± 0.01	6.2 ± 0.01	<0.001	<0.001
Social support	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
(1) In a typical week, how many times do you talk on the telephone with family, friends, or neighbors? 9.6 ± 0.63	10.1 ± 0.38	10.8 ± 0.32	0.52	0.10	
(2) How often do you get together with friends or relatives; I mean things like going out together or visiting in each other's homes? (per year)	93.8 ± 4.17	124.4 ± 7.56	129.3 ± 5.82	<0.001	<0.001
(3) About how often do you visit with any of your other neighbors, either in their homes or in your own? (per year)	51.9 ± 4.97	70.8 ± 9.69	93.2 ± 5.25	0.07	<0.001
(4) How often do you attend church or religious services? (per year)	31.6 ± 2.18	37.2 ± 2.28	43.6 ± 2.33	0.07	<0.001
(5) Altogether, how often do you attend meetings of the clubs or organizations (per year)	11.7 ± 1.04	16.5 ± 1.65	15.2 ± 0.98	0.01	0.01
Health-related factors					
Self-rated health	($N = 1,701$)	($N = 1,698$)	N = 3,251)		
Excellent/Very Good	50.7 ± 2.34	41.7 ± 2.30	37.9 ± 1.46	–	–
Good	33.9 ± 1.55	34.1 ± 1.55	33.3 ± 0.91	0.06	<0.001
Fair/Poor	15.4 ± 1.57	24.2 ± 1.70	28.8 ± 1.22	0.009	<0.001
	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Co-morbidity index	0.7 ± 0.04	1.1 ± 0.05	1.7 ± 0.04	<0.001	<0.001
	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Allostatic load, AL score	2.1 ± 0.07	2.5 ± 0.07	2.8 ± 0.04	<0.001	<0.001
AL components	($N = 1,701$)	($N = 1,698$)	($N = 3,251$)		
Low Albumin	9.6 ± 1.2	13.6 ± 1.4	18.3 ± 1.6	0.01	<0.001

(*Continued*)

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Supplementary Table 1
(*Continued*)

Selected participant characteristics	Age group (y)			pe* (Design-based F-test)[b]	
	45–55	55–65	65+		
Unweighted N (both phases)	(*N* = 1,701) 25.6%	(*N* = 1,698) 25.5%	(*N* = 3,251) 48.9%	55–65 versus 45–55	65+ versus 45–55
High CRP	32.8 ± 1.9	39.8 ± 2.1	40.4 ± 2.0	0.006	<0.001
High waist-hip ratio	73.4 ± 2.1	83.5 ± 1.6	85.4 ± 0.8	0.001	<0.001
High total cholesterol	27.0 ± 1.8	34.6 ± 1.6	32.4 ± 1.4	<0.001	0.03
Low HDL-C	26.3 ± 1.6	26.3 ± 1.7	23.3 ± 1.5	0.98	0.17
High glycated hemoglobin	6.3 ± 0.8	13.0 ± 0.9	14.0 ± 1.0	<0.001	<0.001
High resting heart rate	6.5 ± 0.01	6.9 ± 0.01	4.7 ± 0.00	0.72	0.08
High systolic blood pressure	14.9 ± 1.2	27.9 ± 1.4	51.1 ± 1.3	<0.001	<0.001
High diastolic blood pressure	12.3 ± 1.1	8.5 ± 1.0	5.3 ± 0.5	0.002	<0.001
Weight status	(*N* = 1,701)	(*N* = 1,698)	(*N* = 3,251)		
Underweight	1.2 ± 0.4	1.9 ± 0.4	2.3 ± 0.4	0.18	0.11
Normal	34.8 ± 1.9	29.6 ± 1.6	36.3 ± 1.4	–	–
Overweight	35.1 ± 1.5	37.1 ± 2.1	39.4 ± 1.2	0.05	0.52
Obese	28.9 ± 1.6	31.4 ± 1.8	22.1 ± 0.9	0.04	0.008
Nutritional biomarkers	(*N* = 1,701)	(*N* = 1,698)	(*N* = 3,251)		
25(OH)D	28.7 ± 0.4	27.7 ± 0.4	27.7 ± 0.3	0.013	0.035
Folate	6.8 ± 0.2	8.0 ± 0.3	10.0 ± 0.3	<0.001	<0.001
Vitamin C	151.0 ± 40.1	163.9 ± 46.8	136.3 ± 33.8	0.82	0.77
Vitamin A	60.2 ± 0.6	63.1 ± 0.7	64.2 ± 0.6	0.001	<0.001
Total carotenoids	82.8 ± 1.5	84.3 ± 1.9	87.2 ± 1.6	0.51	0.048
Vitamin E	1273 ± 30	1381 ± 22	1426 ± 13.0	0.007	<0.001
Ferritin	140 ± 6	160 ± 5	158 ± 4	0.006	0.010
Normalized calcium	1.23 ± 0.00	1.24 ± 0.00	1.23 ± 0.00	0.19	0.91
Selenium	126.1 ± 0.84	125.7 ± 0.99	125.4 ± 1.02	0.62	0.39

25(OH)D = 25-hydroxyvitamin D; EU, ELISA units; HEI, Healthy Eating Index; HS, high school; MAR, Mean Adequacy Ratio; NHANES, National Health and Nutrition Examination Surveys.[a]Values are weighted means ± SEM or percent ± SEP, taking into account sampling design complexity (PSU and strata), across 5 imputations with 10 iterations. [b]Design-based F-test took into account design complexity in terms of sampling weights, PSU and stratum. For categorical variables, this was the equivalent of a χ^2 test of independence restricting the sample first to 55–64/45–54, then to 65 + /45–54. For continuous variables, it was the equivalent of a Wald test in a linear regression model with the variable being the outcome predicted by age group and in which 45–54 y was the referent category to which "55–64" and "65+" were compared.

Supplementary Table 2
Periodontal pathogens' serum IgG association with incident all-cause and Alzheimer's Disease dementia in multiple Cox proportional hazards model, overall and restricted by baseline age group and sex: NHANES III, 1988–1994[a,b]

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log^e(HR)	(SE)	p
All-cause dementia															
P. Gingivalis (Pg) mix															
Model 1	–0.01	(0.04)	0.86	–0.03	(0.05)	0.46	–0.02	(0.05)	0.65	–0.04	(0.08)	0.63	+0.01	(0.06)	0.79
Model 2	+0.00	(0.04)	0.99	–0.01	(0.04)	0.83	+0.02	(0.04)	0.61	–0.03	(0.08)	0.70	+0.02	(0.04)	0.63
P. Intermedia (Pi)															
Model 1	–0.06	(0.09)	0.55	–0.05	(0.09)	0.57	–0.06	(0.11)	0.61	+0.17	(0.14)	0.22	*–0.21*	*(0.11)*	*0.070*
Model 2	–0.01	(0.04)	0.71	–0.01	(0.04)	0.85	+0.02	(0.04)	0.60	+0.04	(0.07)	0.54	–0.06	(0.05)	0.20
P. Nigrescens (Pn)															
Model 1	–0.04	(0.07)	0.60	–0.00	(0.07)	1.00	–0.04	(0.09)	0.63	–0.21	(0.16)	0.19	+0.07	(0.09)	0.46
Model 2	–0.04	(0.04)	0.29	–0.02	(0.04)	0.54	–0.02	(0.04)	0.66	–0.01	(0.08)	0.92	–0.07	(0.05)	0.18
T. Forsythia (Tf)															
Model 1	+0.03	(0.08)	0.70	+0.03	(0.08)	0.70	+0.09	(0.09)	0.32	+0.01	(0.13)	0.92	+0.02	(0.08)	0.81
Model 2	+0.03	(0.05)	0.58	+0.03	(0.05)	0.60	+0.07	(0.05)	0.19	+0.03	(0.08)	0.70	+0.03	(0.06)	0.63

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Supplementary Table 2
(*Continued*)

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log^e(HR)	(SE)	p
A. Actinomycetemcomitans (Aa) mix															
Model 1	+0.02	(0.07)	0.75	+0.01	(0.07)	0.84	+0.05	(0.07)	0.53	–0.02	(0.11)	0.88	+0.01	(0.09)	0.88
Model 2	+0.02	(0.05)	0.67	+0.01	(0.04)	0.79	+0.05	(0.04)	0.24	+0.03	(0.08)	0.74	+0.01	(0.05)	0.90
F. Nucleatum (Fn)															
Model 1	–0.00	(0.05)	0.93	–0.01	(0.06)	0.84	+0.09	(0.07)	0.18	–0.01	(0.09)	0.91	+0.01	(0.09)	0.88
Model 2	–0.02	(0.03)	0.49	–0.03	(0.04)	0.47	+0.05	(0.03)	0.14	+0.02	(0.07)	0.79	–0.04	(0.05)	0.29
S. Oralis (So)															
Model 1	+0.07	(0.08)	0.41	+0.06	(0.08)	0.45	+0.12	(0.07)	0.10	**+0.31**	**(0.11)**	**0.007****	–0.04	(0.08)	0.59
Model 2	+0.05	(0.04)	0.22	+0.03	(0.04)	0.39	+0.05	(0.05)	0.24	+0.12	(0.08)	0.15	+0.03	(0.05)	0.56
M. Micros (Mm)															
Model 1	–0.01	(0.07)	0.92	–0.03	(0.06)	0.57	–0.05	(0.06)	0.38	–0.13	(0.08)	0.12	–0.04	(0.11)	0.72
Model 2	–0.01	(0.05)	0.83	–0.03	(0.04)	0.51	–0.00	(0.04)	0.95	–0.06	(0.06)	0.40	+0.04	(0.06)	0.54
C. Rectus (Cr)															
Model 1	**+0.15**	**(0.05)**	**0.003****	**+0.20**	**(0.05)**	**0.001****	**+0.18**	**(0.07)**	**0.017****	+0.09	(0.10)	0.36	+0.11	(0.08)	0.16
Model 2	+0.06	(0.04)	0.20	+0.07	(0.04)	0.13	**+0.10**	**(0.05)**	**0.037**	+0.03	(0.09)	0.74	+0.08	(0.05)	0.14
E. Corrodens (Ec)															
Model 1	+0.05	(0.05)	0.35	+0.02	(0.05)	0.68	+0.09	(0.06)	0.11	–0.04	(0.11)	0.74	**+0.20**	**(0.08)**	**0.011****
Model 2	+0.03	(0.03)	0.37	+0.01	(0.03)	0.79	+0.06	(0.04)	0.11	–0.01	(0.08)	0.93	+0.05	(0.04)	0.21
E. Nodatum (En)															
Model 1	–0.02	(0.08)	0.84	+0.02	(0.08)	0.83	–0.01	(0.09)	0.88	+0.04	(0.11)	0.71	+0.07	(0.06)	0.28
Model 2	–0.02	(0.04)	0.65	–0.01	(0.04)	0.76	–0.01	(0.05)	0.75	+0.00	(0.07)	0.95	–0.02	(0.04)	0.63
S. Intermedius (Si)															
Model 1	–0.08	(0.07)	0.27	–0.08	(0.07)	0.27	–0.07	(0.07)	0.29	+0.08	(0.11)	0.51	*–0.14*	*(0.08)*	*0.079*
Model 2	–0.02	(0.04)	0.65	–0.02	(0.04)	0.54	–0.00	(0.04)	0.98	+0.07	(0.07)	0.37	–0.07	(0.05)	0.21
C. Ochracea (Co)															
Model 1	–0.03	(0.05)	0.47	–0.01	(0.05)	0.84	–0.02	(0.06)	0.70	+0.05	(0.08)	0.52	–0.05	(0.06)	0.47
Model 2	–0.01	(0.03)	0.67	–0.00	(0.03)	0.94	+0.03	(0.04)	0.54	+0.00	(0.06)	0.97	–0.01	(0.04)	0.89
V. Parvula (Vp)															
Model 1	+0.01	(0.07)	0.88	–0.00	(0.07)	0.94	–0.10	(0.07)	0.16	–0.09	(0.09)	0.32	+0.08	(0.09)	0.40
Model 2	+0.00	(0.04)	0.96	–0.01	(0.04)	0.82	–0.00	(0.04)	0.97	–0.01	(0.07)	0.84	+0.02	(0.05)	0.69
A. Naeslundii (An)															
Model 1	–0.04	(0.07)	0.54	–0.07	(0.07)	0.32	–0.06	(0.07)	0.43	–0.05	(0.10)	0.63	–0.04	(0.08)	0.63
Model 2	–0.04	(0.04)	0.25	–0.04	(0.04)	0.24	–0.05	(0.04)	0.19	–0.05	(0.06)	0.42	–0.03	(0.05)	0.51
P. Melaninogenica (Pm)															
Model 1	–0.02	(0.05)	0.70	–0.02	(0.06)	0.76	–0.01	(0.06)	0.61	+0.00	(0.10)	0.97	–0.06	(0.07)	0.44
Model 2	–0.02	(0.03)	0.43	–0.02	(0.03)	0.57	+0.00	(0.03)	0.93	+0.01	(0.07)	0.89	–0.05	(0.04)	0.19
S. Noxia (Sn)															
Model 1	*–0.12*	*(0.07)*	*0.068*	–0.10	(0.07)	0.14	–0.04	(0.08)	0.61	–0.17	(0.12)	0.17	–0.12	(0.09)	0.20
Model 2	–0.04	(0.05)	0.37	–0.03	(0.04)	0.45	+0.01	(0.05)	0.81	–0.07	(0.08)	0.40	–0.02	(0.05)	0.71
T. Denticola (Td)															
Model 1	**–0.10**	**(0.05)**	**0.041**	*–0.10*	*(0.05)*	*0.066*	*–0.13*	*(0.06)*	*0.053*	*–0.14*	*(0.08)*	*0.091*	–0.08	(0.07)	0.32
Model 2	–0.03	(0.04)	0.35	–0.04	(0.04)	0.27	–0.02	(0.04)	0.64	–0.09	(0.07)	0.21	+0.01	(0.05)	0.86
S. Mutans (Sm)															
Model 1	*+0.12*	*(0.07)*	*0.095*	+0.08	(0.07)	0.29	–0.04	(0.07)	0.54	+0.09	(0.13)	0.48	*+0.17*	*(0.09)*	*0.060*
Model 2	+0.05	(0.04)	0.22	+0.02	(0.04)	0.55	+0.02	(0.04)	0.70	+0.07	(0.07)	0.34	+0.05	(0.04)	0.24

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Supplementary Table 2
(*Continued*)

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log^e(HR)	(SE)	p
AD dementia															
P. Gingivalis (Pg) mix															
Model 1	–0.01	(0.06)	0.85	–0.02	(0.06)	0.76	+0.05	(0.07)	0.50	–0.07	(0.12)	0.56	+0.02	(0.08)	0.84
Model 2	+0.02	(0.05)	0.75	+0.00	(0.05)	0.94	+0.07	(0.06)	0.27	–0.06	(0.11)	0.60	+0.04	(0.06)	0.50
P. Intermedia (Pi)															
Model 1	+0.07	(0.11)	0.56	+0.08	(0.11)	0.46	+0.14	(0.13)	0.29	+0.04	(0.19)	0.84	+0.05	(0.08)	0.85
Model 2	+0.05	(0.05)	0.35	+0.04	(0.05)	0.45	+0.08	(0.06)	0.20	+0.10	(0.11)	0.94	+0.03	(0.05)	0.59
P. Nigrescens (Pn)															
Model 1	–0.08	(0.10)	0.45	–0.06	(0.09)	0.49	–0.16	(0.12)	0.17	–0.05	(0.24)	0.83	–0.08	(0.13)	0.74
Model 2	–0.03	(0.05)	0.54	–0.04	(0.04)	0.43	–0.04	(0.05)	0.44	+0.00	(0.14)	0.99	–0.06	(0.06)	0.25
T. Forsythia (Tf)															
Model 1	+0.08	(0.10)	0.45	+0.08	(0.11)	0.45	+0.15	(0.11)	0.18	–0.06	(0.21)	0.77	+0.17	(0.12)	0.17
Model 2	+0.05	(0.06)	0.43	+0.03	(0.06)	0.66	+0.10	(0.07)	0.15	–0.02	(0.14)	0.89	+0.07	(0.07)	0.34
A. Actino-mycetem-comitans (Aa) mix															
Model 1	+0.06	(0.10)	0.86	–0.01	(0.11)	0.94	–0.01	(0.10)	0.95	–0.11	(0.18)	0.56	+0.11	(0.13)	0.38
Model 2	+0.04	(0.06)	0.51	–0.01	(0.06)	0.83	+0.04	(0.07)	0.59	–0.02	(0.15)	0.92	+0.05	(0.06)	0.45
F. Nucleatum (Fn)															
Model 1	–0.02	(0.10)	0.86	+0.01	(0.10)	0.95	+0.13	(0.11)	0.23	–0.08	(0.16)	0.62	–0.01	(0.13)	0.95
Model 2	–0.02	(0.06)	0.71	–0.04	(0.06)	0.53	+0.06	(0.05)	0.28	–0.01	(0.12)	0.96	–0.03	(0.06)	0.60
S. Oralis (So)															
Model 1	–0.05	(0.10)	0.65	–0.03	(0.11)	0.78	–0.02	(0.11)	0.84	*+0.23*	*(0.13)*	*0.069*	–0.17	(0.14)	0.25
Model 2	+0.02	(0.06)	0.67	–0.00	(0.06)	0.96	+0.02	(0.07)	0.78	+0.09	(0.13)	0.51	+0.00	(0.06)	0.97
M. Micros (Mm)															
Model 1	+0.02	(0.08)	0.77	–0.03	(0.08)	0.66	–0.13	(0.10)	0.18	–0.06	(0.12)	0.61	+0.08	(0.11)	0.47
Model 2	+0.01	(0.06)	0.83	–0.03	(0.06)	0.63	–0.02	(0.06)	0.70	–0.02	(0.12)	0.87	+0.03	(0.08)	0.66
C. Rectus (Cr)															
Model 1	*+0.12*	*(0.06)*	*0.063*	**+0.15**	**(0.06)**	**0.024***	+0.05	(0.08)	0.57	+0.15	(0.10)	0.15	+0.09	(0.09)	0.31
Model 2	+0.07	(0.06)	0.23	+0.06	(0.05)	0.29	+0.08	(0.06)	0.20	+0.05	(0.14)	0.75	+0.08	(0.07)	0.28
E. Corrodens (Ec)															
Model 1	–0.01	(0.08)	0.89	–0.02	(0.08)	0.84	+0.07	(0.10)	0.43	–0.03	(0.17)	0.86	–0.03	(0.10)	0.74
Model 2	+0.04	(0.05)	0.45	+0.00	(0.05)	0.93	+0.08	(0.07)	0.28	–0.05	(0.13)	0.72	+0.07	(0.06)	0.25
E. Nodatum (En)															
Model 1	+0.10	(0.09)	0.25	+0.13	(0.09)	0.17	+0.12	(0.10)	0.25	+0.11	(0.13)	0.39	+0.07	(0.11)	0.52
Model 2	–0.00	(0.05)	0.93	–0.02	(0.05)	0.70	–0.00	(0.06)	0.96	–0.02	(0.09)	0.80	–0.00	(0.06)	0.95
S. Intermedius (Si)															
Model 1	–0.14	(0.10)	0.18	*–0.18*	*(0.03)*	*0.077*	–0.11	(0.11)	0.31	+0.14	(0.17)	0.40	**–0.25**	**(0.11)**	**0.030***
Model 2	–0.00	(0.05)	0.93	*–0.08*	*(0.05)*	*0.071*	–0.04	(0.07)	0.51	+0.08	(0.12)	0.51	–0.00	(0.06)	0.95
C. Ochracea (Co)															
Model 1	–0.01	(0.07)	0.87	–0.02	(0.07)	0.82	+0.04	(0.09)	0.64	+0.09	(0.12)	0.45	–0.02	(0.09)	0.84
Model 2	–0.05	(0.05)	0.36	+0.01	(0.05)	0.84	+0.06	(0.06)	0.34	+0.02	(0.11)	0.85	*–0.11*	*(0.06)*	*0.088*
V. Parvula (Vp)															
Model 1	–0.01	(0.11)	0.95	–0.03	(0.11)	0.80	–0.13	(0.13)	0.31	–0.21	(0.17)	0.21	+0.11	(0.13)	0.42
Model 2	+0.01	(0.06)	0.88	–0.02	(0.06)	0.73	+0.01	(0.08)	0.92	–0.07	(0.14)	0.63	+0.02	(0.05)	0.69
A. Naeslundii (An)															
Model 1	*–0.16*	*(0.09)*	*0.074*	*–0.19*	*(0.09)*	*0.051*	*–0.19*	*(0.10)*	*0.07*	–0.15	(0.15)	0.32	*–0.18*	*(0.10)*	*0.074*
Model 2	–0.09	(0.06)	0.11	*–0.11*	*(0.06)*	*0.059*	*–0.12*	*(0.06)*	*0.057*	–0.08	(0.09)	0.37	+0.05	(0.08)	0.52

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Supplementary Table 2
(*Continued*)

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log_e(HR)	(SE)	p	Log^e(HR)	(SE)	p
P. Melaninogenica (Pm)															
Model 1	–0.01	(0.08)	0.88	+0.02	(0.09)	0.82	+0.01	(0.09)	0.90	+0.00	(0.16)	0.99	–0.05	(0.09)	0.54
Model 2	–0.00	(0.05)	1.00	–0.01	(0.05)	0.87	+0.02	(0.06)	0.79	–0.08	(0.09)	0.37	–0.10	(0.07)	0.17
S. Noxia (Sn)															
Model 1	–0.08	(0.10)	0.44	–0.04	(0.10)	0.70	+0.01	(0.10)	0.91	–0.04	(0.14)	0.80	–0.15	(0.11)	0.19
Model 2	–0.02	(0.06)	0.78	–0.03	(0.06)	0.66	+0.02	(0.07)	0.74	–0.01	(0.13)	0.92	–0.03	(0.05)	0.58
T. Denticola (Td)															
Model 1	–0.09	(0.07)	0.18	–0.11	(0.07)	0.15	–0.10	(0.09)	0.28	–0.05	(0.12)	0.69	–0.08	(0.09)	0.38
Model 2	–0.04	(0.05)	0.44	–0.07	(0.05)	0.19	+0.01	(0.06)	0.92	–0.06	(0.12)	0.60	–0.03	(0.06)	0.69
S. Mutans (Sm)															
Model 1	**+0.18**	**(0.08)**	**0.040**	*+0.16*	*(0.08)*	*0.052*	+0.10	(0.09)	0.28	+0.08	(0.13)	0.54	**+0.25**	**(0.12)**	**0.034**
Model 2	+0.05	(0.04)	0.30	+0.02	(0.04)	0.66	+0.03	(0.05)	0.55	+0.06	(0.11)	0.59	+0.04	(0.04)	0.32

BMI, body mass index; HR, hazard ratio; IgG, immunoglobulin G; HS, high school; NHANES, National Health and Nutrition Examination Surveys. [a]Models were adjusted for age, sex, race/ethnicity, poverty income ratio, education (years), urban-rural area of residence, household size, marital status, nutritional factors (HEI, MAR), nutritional biomarkers (25(OH)D, folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium and normalized calcium), lifestyle (smoking, drug use, alcohol, physical activity), health-related factors (self-rated health, co-morbidity index, allostatic load, weight status), dentate status and social support variable, as well as Phase of NHANES III. Covariates (other than exposures) were imputed and analysis is across 5 imputations with 10 iterations. Model 1: adjusted for all other periodontal pathogens; Model 2: one periodontal pathogen at a time. bPeriodontal pathogen exposures were Log_e transformed and then standardized into z-scores. [c]Unweighted $N = 6{,}277$–$6{,}581$, AD incidence weighted mean follow-up time: 197 months, all-cause dementia weighted mean follow-up time: 192 months. [d]Unweighted $N = 4{,}681$–$4{,}912$, AD incidence weighted mean follow-up time: 171 months, All-cause dementia weighted mean follow-up time: 165 months. [e]Unweighted $N = 3{,}077$–$3{,}229$, AD incidence weighted mean follow-up time: 141 months; All-cause dementia weighted mean follow-up time: 133 months; [f]Unweighted $N = 2{,}924$–$3{,}088$, AD incidence weighted mean follow-up time: 193 months, All-cause dementia weighted mean follow-up time: 189 months. [g]Unweighted $N = 3{,}353$–$3{,}517$, AD dementia weighted mean follow-up time: 200 months, All-cause dementia weighted mean follow-up time: 194 months. $*p < 0.033$, marginally significant after correction for multiple testing; $**p < 0.016$, significant after correction for multiple testing.

Supplementary Table 3
Mean attachment loss (AL) and probing pocket depth (PPD) measures and their association with periodontal pathogens: Multiple linear regression models [a,b]

	AL			PPD		
	β	(SE)	p	β	(SE)	p
Both phases periodontal pathogens[c]						
P. Gingivalis (Pg) mix	**+0.20**	**(0.02)**	**<0.001****	**+0.08**	**(0.01)**	**<0.001****
P. Intermedia (Pi)	**+0.09**	**(0.02)**	**<0.001****	**+0.05**	**(0.01)**	**<0.001****
P. Nigrescens (Pn)	+0.03	(0.03)	0.33	+0.02	(0.01)	0.13
T. Forsythia (Tf)	**+0.08**	**(0.02)**	**0.002****	*+0.03*	*(0.01)*	*0.058*
A. Actinomycetemcomitans (Aa) mix	**+0.06**	**(0.02)**	**0.022****	+0.01	(0.01)	0.66
F. Nucleatum (Fn)	+0.04	(0.03)	0.11	+0.02	(0.01)	0.17
S. Oralis (So)	+0.04	(0.02)	0.14	+0.01	(0.02)	0.51
M. Micros (Mm)	**+0.04**	**(0.02)**	**0.042***	+0.02	(0.01)	0.20
C. Rectus (Cr)	**+0.11**	**(0.02)**	**<0.001****	**+0.05**	**(0.02)**	**0.003****
E. Corrodens (Ec)	+0.04	(0.03)	0.15	+0.00	(0.01)	0.73
E. Nodatum (En)	**–0.06**	**(0.02)**	**0.014****	–0.00	(0.01)	1.00
S. Intermedius (Si)	+0.03	(0.02)	0.19	+0.02	(0.01)	0.17
C. Ochracea (Co)	*+0.04*	*(0.02)*	*0.065*	+0.01	(0.01)	0.35
V. Parvula (Vp)	**+0.06**	**(0.02)**	**0.011****	*+0.02*	*(0.01)*	*0.076*
A. Naeslundii (An)	–0.03	(0.02)	0.21	+0.00	(0.01)	0.90
P. Melaninogenica (Pm)	*+0.04*	*(0.02)*	*0.084*	*+0.02*	*(0.01)*	*0.072*
S. Noxia (Sn)	**+0.06**	**(0.02)**	**0.013****	+0.02	(0.01)	0.22
T. Denticola (Td)	**+0.08**	**(0.02)**	**0.001****	**+0.04**	**(0.01)**	**0.001****

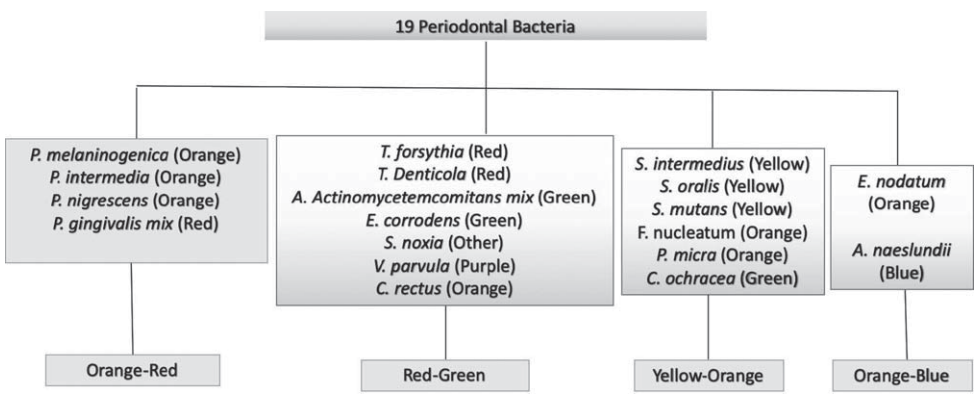
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Supplementary Table 3
(*Continued*)

	AL			PPD		
	β	(SE)	p	β	(SE)	p
S. Mutans (Sm)	+0.02	(0.02)	0.36	+0.01	(0.01)	0.65
Factor scores						
Factor 1: So/Sm/Ec	*+0.05*	*(0.03)*	*0.088*	–0.00	(0.02)	1.00
Factor 2: Pi/Pn/Pm	*+0.06*	*(0.03)*	*0.056*	**+0.04**	**(0.01)**	**0.003****
Factor 3: An/En	**–0.07**	**(0.03)**	**0.014****	–0.00	(0.02)	0.76
Factor 4: Pg/Cr	**+0.22**	**(0.04)**	**<0.001****	**+0.09**	**(0.02)**	**<0.001****
Factor 5: Co/Sn	*0.05*	*(0.03)*	*0.056*	+0.01	(0.01)	0.30
Pre-defined clusters						
Cluster 1: Orange-Red	**+0.12**	**(0.02)**	**<0.001****	**+0.06**	**(0.01)**	**<0.001****
Cluster 2: Red-Green	**+0.08**	**(0.02)**	**<0.001****	*+0.03*	*(0.02)*	*0.078*
Cluster 3: Yellow-Orange	**+0.06**	**(0.02)**	**0.018****	+0.02	(0.01)	0.23
Cluster 4: Orange-Blue	**–0.05**	**(0.02)**	**0.043***	+0.00	(0.01)	0.94
Phase 2 periodontal pathogens[d]						
Pg						
Untransformed	**+0.13**	**(0.06)**	**0.038***	**+0.08**	**(0.03)**	**0.027**
Log$_e$ transformed	**+0.17**	**(0.04)**	**0.001****	**+0.09**	**(0.02)**	**<0.001****
Aa						
Untransformed	+0.04	(0.02)	0.11	+0.01	(0.01)	0.18
Log$_e$ transformed	+0.04	(0.03)	0.14	0.01	(0.01)	0.56

BMI, body mass index; AL, attachment loss; HS, high school; NHANES, National Health and Nutrition Examination Surveys; PPD, Probing Pocket Depth. [a]Models were adjusted for age, sex, race/ethnicity, poverty income ratio, education (years), urban-rural area of residence, household size, marital status, nutritional factors (HEI, MAR), nutritional biomarkers (25(OH)D, folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium and normalized calcium), lifestyle (smoking, drug use, alcohol, physical activity), health-related factors (self-rated health, co-morbidity index, allostatic load, weight status), dentate status and social support variables, as well as Phase of NHANES III. Covariates (other than exposures) were imputed and analysis is across 5 imputations with 10 iterations. Each model included on periodontal pathogen exposure at a time. [b]19 Periodontal pathogen exposures (both phases) were Log$_e$ transformed and then standardized into z-scores. Factor analysis was conducted from which 5 factors were extracted each explaining > 4% of total variance. After varimax rotation, factor 1 loaded highest (λ>0.40) on Tf (0.68), *Aa* (0.65), Fn (0.53), *So* (0.81), *Mm*(0.51), Cr(0.54), *Ec*(0.68), Si(0.63), Vp(0.64), Sn(0.62), *Td*(0.63) and Sm(0.73); factor 2 on Pg (0.42), Pi (0.85), Pn (0.84), Fn (0.44), Cr (0.40), Si(0.42), Co(0.44), *Pm*(0.70); factor 3 on En(0.75), An (0.76); factor 4 on Pg(0.44) and Cr (0.42); and factor 5 on Co (0.45) and Sn (0.41). Factors were labelled based on up to 3 highest loadings. See Supplementary Figure 1 and methods section for definition of each cluster. Two periodontal pathogens (phase 2) were standardized in z-scores. [c]Unweighted $N = 5{,}948–6{,}167$; Weighted $N = 43{,}148{,}724 – 45{,}233{,}568$ [d]Unweighted $N = 3{,}156$; Weighted $N = 48{,}674{,}171$. *$p < 0.05$, marginally significant after correction for multiple testing; **$p < 0.025$, significant after correction for multiple testing.



Supplementary Figure 1. Composition of the four mutually exclusive clusters formed with cluster analysis of z-scores of serum antibody titers against 19 periodontal bacteria. Socransky's color complex is shown in parentheses after each specific periodontal bacterium.

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Supplementary Table 4
Mean Attachment Loss (AL) and Probing Pocket Depth (PD) and their association with AD incidence and all-cause dementia incidence in multiple Cox proportional hazards model, overall and restricted by baseline age group and sex: NHANES III, 1988–1994[a,b]

	≥45 y[c]			≥55 y[d]			≥65 y[e]			Men[f]			Women[g]		
	Log[e](HR)	(SE)	p	Log[e](HR)	(SE)	p	Log[e](HR)	(SE)	p	Log[e](HR)	(SE)	p	Log[e](HR)	(SE)	p
AD incidence															
Model 1: AL	+0.02	(0.06)	0.75	+0.00	(0.05)	0.70	+0.07	(0.07)	0.31	+0.08	(0.08)	0.34	+0.00	(0.07)	0.97
Model 2: PPD	+0.09	(0.10)	0.40	+0.08	(0.09)	0.42	+0.24	(0.11)	0.038*	+0.28	(0.13)	0.045*	+0.01	(0.16)	0.95
All-cause dementia incidence															
Model 1: AL	–0.01	(0.05)	0.82	–0.01	(0.04)	0.83	+0.01	(0.05)	0.83	+0.03	(0.07)	0.65	–0.02	(0.06)	0.70
Model 2: PPD	–0.04	(0.09)	0.64	–0.05	(0.09)	0.58	+0.08	(0.12)	0.49	+0.13	(0.10)	0.22	–0.13	0.13	0.30

25(OH)D = 25-hydroxyvitamin D; AD = Alzheimer's Disease; CAL = Clinical Attachment Loss; HR = Hazard Ratio; NHANES = National Health and Nutrition Examination Surveys. [a]Models were adjusted for age, sex, race/ethnicity, poverty income ratio, education (years), urban-rural area of residence, household size, marital status, nutritional factors (HEI, MAR), nutritional biomarkers (25(OH)D, folate, vitamin C, vitamin A, total carotenoids, vitamin E, ferritin, selenium and normalized calcium), lifestyle (smoking, drug use, alcohol, physical activity), health-related factors (self-rated health, co-morbidity index, allostatic load, weight status), dentate status and social support variables. Covariates (other than exposures) were imputed and analysis is across 5 imputations with 10 iterations. [b]CAL/Probing Depth expressed as is, per 1 mm. [c]Unweighted $N = 5{,}072$; AD weighted mean follow-up time: 197 months; all-cause dementia weighted mean follow-up time: 207 months [d]Unweighted $N = 3{,}468$, AD weighted mean follow-up time: 171 months; all-cause dementia weighted mean follow-up time: 178 months. [e]Unweighted $N = 2{,}080$, AD weighted mean follow-up time: 141 months; all-cause dementia weighted mean follow-up time: 145 months. [f]Unweighted $N = 2{,}511$, AD weighted mean follow-up time: 193 months; all-cause dementia weighted mean follow-up time: 205 months. [g]Unweighted $N = 2{,}576$, AD weighted mean follow-up time: 200 months; all-cause dementia weighted mean follow-up time: 209 months. *$p < 0.05$, marginally significant after correction for multiple testing; **$p < 0.025$, significant after correction for multiple testing.

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Periodontitis Deteriorates Cognitive Function and Impairs Neurons and Glia in a Mouse Model of Alzheimer's Disease

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Abstract.
Background: Although periodontitis is reportedly associated with increased cognitive decline in Alzheimer's disease, the mechanisms underlying this process remain unknown. *Porphyromonas gingivalis* lipopolysaccharide (P.g-LPS) is an endotoxin associated with periodontal disease.
Objective: We investigated the effect of periodontitis on learning capacity and memory of amyloid-β protein precursor (AβPP)/presenilin (PS1) transgenic mice along with the mechanisms underlying these effects.
Methods: Mice were randomly assigned to three groups, namely AβPP/PS1 (control), P.g-LPS Injection, and P.g-LPS Injection + Ligation. Mice from the P.g-LPS Injection group were injected with P.g-LPS in the periodontal tissue three times per week for 8 weeks, while mice from the P.g-LPS Injection + Ligation group were injected with P.g-LPS and subjected to ligation of the gingival sulcus of the maxillary second molar.
Results: Expression of gingival proinflammatory cytokines as well as alveolar bone resorption in P.g-LPS-injected and ligatured mice was increased compared to that in control mice. Mice in the P.g-LPS Injection + Ligation group exhibited cognitive impairment and a significant reduction in the number of neurons. Glial cell activation in the experimental groups with significantly increased amyloid-β (Aβ) levels was more pronounced relative to the control group. Induction of periodontitis was concurrent with an increase in cyclooxygenase-2, inducible nitric oxide synthase, AβPP, and beta-secretase 1 expression and a decrease in A disintegrin and metalloproteinase domain-containing protein 10 expression.
Conclusion: These findings indicated that periodontitis exacerbated learning and memory impairment in AβPP/PS1 mice and augmented Aβ and neuroinflammatory responses. Our study provides a theoretical basis for risk prediction and early intervention of Alzheimer's disease and periodontitis.

Keywords: Alzheimer's disease, cognitive dysfunction, lipopolysaccharide, periodontitis, *Porphyromonas gingivalis*

INTRODUCTION

Periodontitis, an inflammatory disease of the gums and surrounding tissues, is primarily caused by gram-negative anaerobic bacteria [1, 2]. During the development of periodontitis, the effects of bacteria and their toxic products, along with resultant inflammatory responses, are not only localized, but also become systemic throughout the periodontal pocket

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lining, resulting in bacteremia or endotoxemia [3, 4]. Therefore, a localized periodontal infection may spread throughout the body, facilitating the occurrence and development of systemic diseases, such as cardiovascular and cerebrovascular diseases, cancer, and diabetes, in addition to acting as a risk factor for premature birth and low birth weight babies [5]. Furthermore, studies have demonstrated that periodontitis is associated with cognitive decline in Alzheimer's disease (AD) [6, 7].

AD causes progressive mental, behavioral, and cognitive decline, and may lead to reduced learning abilities. It is a progressive degenerative disease of the central nervous system and a major form of dementia, showing characteristic pathological changes including amyloid plaque formation caused by aggregation of extracellular amyloid proteins, formation of neurofibrillary tangles due to abnormal aggregation of intracellular tau proteins, and neuron loss [8, 9]. An imbalance in the production and clearance of amyloid-β (Aβ) is believed to play a key role in AD progression [10]. Evidently overproduction and excessive accumulation of Aβ, caused by cleavage of amyloid-β protein precursor (AβPP) in the brain, are principal contributors to the pathogenesis of AD [11]. Studies have also shown that Aβ is generated during AβPP metabolism, which occurs via two pathways, namely non-amyloid and amyloid pathways. In the non-amyloid pathway, AβPP is catalyzed by α-secretase (a member of a large metallopeptidase family), also known as A disintegrin and metalloprotease (ADAM), and subsequently by γ-secretase under normal physiological conditions, thus preventing Aβ generation. In the amyloid pathway, AβPP is activated by β-site APP cleavage 1 (BACE1), a rate-limiting secretase, leading to the production of toxic Aβ fragments that contribute to AD pathology, following which it is acted upon by γ-secretase, leading to the production of toxic Aβ fragments that contribute to AD pathology [12–14]. Studies have shown that Aβ deposition is associated with overactivation of microglia, which promotes neuroinflammation and neuronal dysfunction [15].

Porphyromonas gingivalis (P.g) is one of the most common pathogens associated with periodontitis, and P.g lipopolysaccharide (P.g-LPS) plays a major pathogenic role in P.g infection [16]. Poole et al. found P.g-LPS in the brains of autopsied AD victims, wherein P.g-LPS levels in brain tissues were significantly higher than those in non-AD subjects [17]. In periodontitis, the balance between bacterial levels and host responses is disrupted, leading to increased inflammatory cell infiltration and production of proinflammatory cytokines, such as interleukin (IL) 1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) [18, 19]. Recent studies have indicated that systemic inflammation plays a central role in AD progression if the acute injury is sufficiently intense (e.g., severe sepsis) or has a long duration (e.g., repeated stimulation by large doses of proinflammatory agents such as LPS) [20]. Sheng et al. observed that intraperitoneal injection of LPS induced neuroinflammation with increasing AβPP expression and Aβ deposition in mice, suggesting that peripheral inflammation might lead to central inflammation and further aggravation of AD [21]. During this process, high concentrations of inflammatory mediators dilate the blood vessels and increase endothelial permeability of the blood-brain barrier, thereby allowing entry of pathogenic factors into the central nervous system, which in turn, activates microglial cells that subsequently release a considerable number of inflammatory factors, thus damaging the functional central nervous system and causing brain inflammation [22, 23]. However, whether periodontitis contributes to cognitive impairment is unclear. In this study, we evaluated the pathogenic manifestations induced by P.g-LPS via the Aβ generation pathway. Notably, we used AβPP/PS1 transgenic mice to examine whether periodontitis induced by P.g-LPS and ligation exacerbated spatial learning and memory impairment. We also examined the biological mechanisms underlying behavioral changes.

MATERIALS AND METHODS

Animals

Thirty 6-month-old AβPP/PS1 double-transgenic male mice were purchased from Nanjing Biomedical Research Institute of Nanjing University-Nanjing Institute of Biomedical Sciences: License Number; SCXK (JiangSun, China) 2015-0001. All mice weighed 35–45 g and were provided *ad libitum* access to water and fed with standard rodent food in a $22 \pm 2^{\circ}$C environment under a 12-h light/12-h dark cycle for 24 h. Next, AβPP/PS1 mice were randomly divided into three groups based on the following treatments: AβPP/PS1 (control group); P.g-LPS (ultrapure, InvivoGen, Inc., San Diego, CA, USA) only; and P.g-LPS combined with ligation. To induce periodontitis, a 4-0 silk ligature was inserted into the gingival sulcus between the second maxillary molar (M2) and first maxillary molar (M1) of the maxilla

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in both sides of each mouse for 8 weeks, followed by interpapillary injection of 2 μL of 1.0 mg/mL P.g-LPS (thrice a week) at the same position in a manner similar to that performed on mice of the P.g-LPS and P.g-LPS + Ligation groups. The injection was administered slowly, and the needle (Gaoge, Shanghai, China) was kept in place for a few seconds to ensure that there was no wastage or loss of P.g-LPS during needle extraction. Age-matched control animals were not injected. Animals were sacrificed 9 weeks after the first injection. Maxillae were dissected and immersed in 10% buffered formalin for 48 h. All efforts were made to minimize animal use as well as animal suffering. All animal experiments were undertaken in accordance with the relevant guidelines and regulations of the State Committee of Science and Technology of the People's Republic of China, Order No. 2 on November 14, 1988 (revised 2011), and approved by the Animal Experimentation Ethics Committee of the Zunyi Medical University, which also meet the NIH guidelines for the care and use of laboratory animals.

Behavioral experiments

Morris water maze test

After 8 weeks of P.g-LPS injection, the Morris water maze test was performed to assess spatial learning and memory. The device consisted of a white plastic round pool (radius = 60 cm, height = 50 cm) filled with water ($23 \pm 2^{\circ}$C) to a depth of 30 cm. The pool consisted of four equal quadrants; a movable platform made of Perspex was positioned under the third quadrant, submerged a centimeter from the water surface. The task consisted of two steps. The first step was direction sailing training. During the first four consecutive days, the mouse was released into the water at one of the four quadrants and allowed 60 s to reach the hidden platform, where it remained for 20 s, and escape latency (i.e., time taken to swim toward the hidden platform, climb, and remain on it for more than 3 s) was recorded. If a mouse failed to reach the platform within 60 s, it was manually guided to the platform and allowed to remain on it for 20 s, and the escape latency was recorded as 60 s [24]. The spatial probe test designed to measure final spatial memory consolidation was scheduled to take place on the 5th day; during this time, the hidden platform was withdrawn. These activities were automatically measured and analyzed using a behavior analyzing system (TopScan Version 3.00, CleverSys, Reston, VA, USA).

Novel object recognition task

The novel object recognition task involved the use of two reaction chambers of the same size: 40 × 40 × 40 cm. Two days of training sessions were conducted to allow the mice to be familiar with to the apparatus. Each session involved placing a mouse in the apparatus containing two similar objects and allowing it to explore for 5 min. The objects were positioned identically in two nearby corners and were similar in texture, color, size, and shape. On the 3rd day (test session), one of the objects was replaced with a new object of the same size but a different shape. The discrimination ratio for each mouse was expressed as $(T_N–T_F)/(T_N + T_F)$ ratio; where T_F = time spent exploring familiar object; T_N = time spent exploring the novel object. The objects were cleaned with 10% ethanol solution between trials [25, 26].

Y-maze test

The Y-maze, which is used to observe the spontaneous alternation rates displayed by mice, allows changes in short-term spatial learning and memory to be determined, according to previously published procedures [27, 28]. The Y-maze consisted of three arms with an angle of 120° between any two arms, which were 40 cm long and 8 cm wide with 15 cm high walls. The setup included a ceiling-mounted infrared camera tracking system attached to a computer with supporting software. The mice were initially placed at the end of one fixed arm and allowed to explore the Y-maze freely for 5 min. Entries into all arms were noted (presence of all four paws inside an arm was considered a valid entry), where consecutive entries into three different arms by an animal was considered as a spontaneous alternation. Next, the mouse was removed from the Y-maze to its house, and the Y-maze was cleaned until dry using 10% ethanol to remove odor [29, 30]. An infrared camera was used to record all trials on a video cassette recorder. The video recordings were later analyzed to determine the number of entries and time spent in each arm. These activities were automatically measured and analyzed using a behavior analysis system (TopScan Version 3.00).

Tissue preparation

Following the behavioral tests, the mice ($n = 4$–5 per group) were anesthetized with sodium pentobarbital and transcardially perfused with chilled 0.1 M phosphate-buffered saline (PBS). The brains were

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divided into left and right hemispheres and placed in pre-cooled 4% buffered paraformaldehyde at 4°C overnight, and subsequently embedded in paraffin. The hippocampi and cerebral cortices of few animals were isolated and immediately stored in Eppendorf micro test tubes at –80°C. Gingival tissues and alveolar bones were stored in 4% buffered paraformaldehyde at –80°C and at 4°C, respectively.

Methylene blue staining

The maxillary molar regions were fixed in 4% neutralized formalin for 24 h. De-fleshed molar samples were stained with 1% aqueous methylene blue (Dalian Meilun Biotechnology, Dalian, China) for 5 min [31]. Digital photographs were obtained using a 500-μm macro lens digital camera (Canon, Tokyo, Japan).

Micro-computed tomography (Micro-CT)

Maxilla specimens were collected and placed in 4% formaldehyde in PBS and subjected to micro-CT analysis using a μ-CT50 scanning system (Scanco Medical vivaCT 40, Brüttisellen, Switzerland) at a resolution of 10.5 μm. The volume of interest was determined using the volume of alveolar bone of root bifurcation, within which trabecular plate thickness, bone volume, and bone volume per total volume were calculated. The volume of interest was drawn with a slice-based method, starting from the bottom of the pulp chamber of the second molar. Images were analyzed using the software μct V6.1 (Scanco Medical vivaCT 40).

Nissl staining

Nissl staining was performed to determine morphological changes occurring in hippocampal neurons as well as the number of surviving neurons, as per previously described methods [32]. Right brain hemispheres ($n = 4$) were serially cut into 35 μm-thick sections in the coronal plane and stained with toluidine blue (Solarbio, Beijing, China). Nissl bodies in the CA1 and DG regions of the hippocampus and cerebral cortex were stained blue-purple. Nissl-positive neurons were counted using a light microscope with 400× magnification (KS300, Zeiss-Kontron, Oberkochen, Germany). Four different visual fields were selected from each section, and the number of surviving neurons in the same area of the hippocampal CA1 and DG regions and cerebral cortex was counted as per previously described protocols [32]. The mean value for each mouse was obtained as the number of neurons in the mouse.

Western blotting

The hippocampal and cerebral cortical tissues ($n = 4$ per group) were homogenized using a protein extraction solution containing a complete protease inhibitor mixture. The homogenized samples were centrifuged at 12,000 rpm for 15 min, and the supernatant was extracted, aliquoted, and finally stored at –80°C. Protein concentrations of each sample were determined using the BCA protein assay kit (Biocolor Biotechnology, Antrim, UK). Equal amounts of proteins (25–30 μg) were heated at 100°C for 5 min, separated by electrophoresis using a 10% SDS or 15% polyacrylamide gel, and transferred onto polyvinylidene difluoride membranes (0.45 μm in thickness). Membranes were blocked for 2–3 h with 5% defatted milk in 1× Tris-buffered saline Tween-20 and probed with the corresponding primary antibodies against AβPP (1:5000, ab32136, Abcam, Cambridge, UK), BACE1 (1:2000, D220305, BBI Life Sciences, Shanghai, China), ADAM10 (1:2000, 25900-1-AP, Proteintech, Rosemont, IL, USA), inducible nitric oxide synthase (iNOS, 1:1000, bs-0162R, Bioss, Woburn, MA, USA), cyclooxygenase-2 (COX-2, 1:1000, 12375-1-AP, Proteintech), IL-6 (1:500, 21865-1-AP, Proteintech), TNF-α (1:2000, 60291-1-lg, Proteintech), glial fibrillary acidic protein (GFAP; 1:1000, #3670, Cell Signaling Technology, Danvers, MA, USA), ionized calcium-binding adaptor molecule 1 (Iba1; 1:1000, ab178847, Abcam), $A\beta_{40}$ (1:2000, MAB2675, Abnova, Taipei, Taiwan), and $A\beta_{42}$ (1:2000, #14974, Cell Signaling Technology) at 4°C overnight. The blot was then incubated with the corresponding anti-mouse or rabbit immunoglobulin horseradish peroxidase-conjugated antibodies for 1–2 h at 37°C. Next, ECL Reagent (Millipore Corporation, Billerica, MA, USA) and Image Lab (Bio-Rad, Hercules, CA, USA) were used to analyze the protein bands [25].

Immunohistochemistry and immunofluorescence

Brain samples were processed for immunohistochemistry and immunofluorescence according to previously reported procedures [33]. Briefly, brain samples were immersed in xylene for 10 min twice and rehydrated by serially diluting in a descending ethanol gradient (absolute alcohol to 95%, to 80%, to

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70%) for 5 min and washed thrice in distilled water for 5 min. Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) in a microwave for 6 min at a high temperature [34]. To quench endogenous peroxidase activity, sections were incubated with 3% H_2O_2 in PBS for 30 min. After rinsing thrice with PBS, the sections were blocked with 10% normal goat serum in PBS for 30 min and incubated with primary antibodies against GFAP and Iba1 overnight at 4°C. After washing with PBS thrice, the sections were probed with goat anti-rabbit lgG/horseradish peroxidase polymer or goat anti-mouse lgG/horseradish peroxidase polymer (1:500, Beyotime, Shanghai, China) at 37°C for 1 h. Sections were washed with PBS and visualized using the DAB chromogen solution (Dako, Glostrup, Denmark) for a few seconds. After counterstaining with hematoxylin, the sections were dehydrated with alcohol and cleared in xylene before being mounted under a glass coverslip using neutral balsam mounting medium [34]. The immunostained sections were then imaged using a microscope (Olympus, Tokyo, Japan). Images of the hippocampal and cortical regions were recorded, and the results are expressed as the average intensity of positive immunoreactive cells of GFAP and Iba1 immunohistochemistry. Immunofluorescence assays were performed to detect Aβ location [25]. Briefly, the sections were washed thrice in PBS and incubated with 10% normal goat serum in PBS for 1 h at 37°C. The sections were then incubated with the primary rabbit Polyclonal Aβ antibody (Cell Signaling Technology, 15126S) overnight at 4°C. After washing in PBS, the sections were incubated with the corresponding secondary antibodies for 2 h at 37°C and then incubated with DAPI for 5 min at 37°C.

Quantitative real-time PCR (qRT-PCR)

The gene expression of IL-6 and TNF-α genes was detected via qRT-PCR. Total mRNA from the gingiva was extracted using RNAiso Plus (Takara, Shiga, Japan) according to the manufacturer's instructions. The quality and quantity of RNA were determined using Nano Drop (ND-2000, Thermo Fisher Scientific, Waltham, MA, USA), with a 260/280 ratio of 1.8/2.1. A total of 50 ng of extracted mRNA was reverse-transcribed into cDNA using the High Capacity Reverse Transcriptase Kit (Takara). Thereafter, a PCR sample of 15 μL was prepared with 3 μL cDNA (10 ng/ μL), 7.5 μL TB Green Premix EX Taq II (Takara), 0.5 μL primer mix (10 μM each), and 4 μL ddH_2O. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 50 cycles of denaturation at 95°C for 5 min; annealing at 60°C for 10 s; elongation at 60°C for 30 s [35]. Primer sequences (Table 1) of the selected genes were designed using the Primer 3 software. For data normalization purposes, mRNA expression of actin was determined, and the relative units were calculated using the comparative $2^{-\Delta\Delta CT}$ method [25].

Statistical analysis

All statistical analyses were conducted out using the SPSS software, version 19.0 (SPSS, Inc., Chicago, IL, USA). Normality of the data was checked using the Kolmogorov-Smirnov test. Normally distributed continuous variables are expressed as mean ± SEM. One-way analysis of variance (ANOVA), followed by Dunnett's test, was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of periodontitis on alveolar bone loss and expression of proinflammatory cytokines in the gingiva

Methylene blue staining results showed that alveolar bone resorption in the P.g-LPS and P.g-LPS + Ligation groups was enhanced compared with that in the AβPP/PS1 mice (Fig. 1a). Micro-CT images showed severe alveolar bone loss had occurred in the P.g-LPS and P.g-LPS + Ligation groups compared to that in the control group (Fig. 1b). Furthermore, the trabecular plate thickness, bone volume, and bone volume per total volume values in the ligation group were significantly different compared to those in the control and P.g-LPS groups ($p < 0.01$; Fig. 1c–e). We also assessed levels of proinflammatory cytokines by evaluating mRNA expression of IL-6 and TNF-α in the gingiva tissues via qRT-PCR. The levels of both

Table 1
Primer sequences used for qRT-PCR analysis

Gene	Forward (5′–3′)	Reverse (5′–3′)
TNF-α	GCGACGTGGAACTGGCAGAAG	GCCACAAGCAGGAATGAGAAGAGG
IL-6	AGGAGTGGCTAAGGACCAAGACC	CTGACCACAGTGAGGAATGTCCAC
β-actin	GGCCAACCGTGAAAAGATGA	CAGCCTGGATGGCTACGTACA

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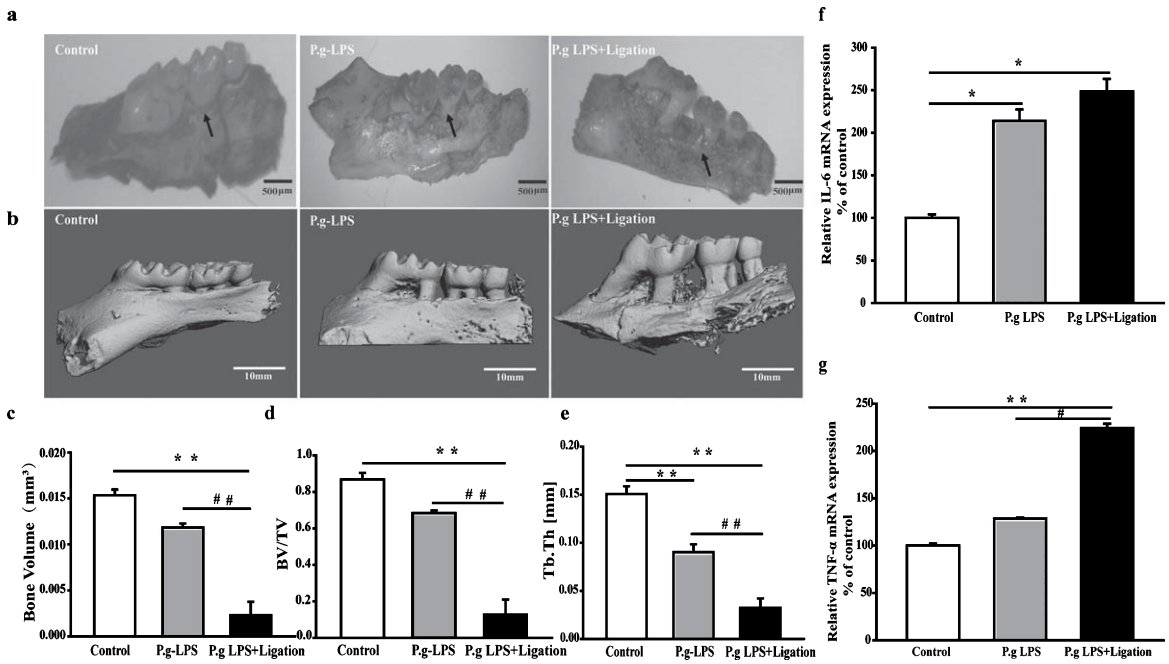


Fig. 1. Effects of periodontitis on alveolar bone loss and proinflammatory cytokines in the gingiva. (a) Methylene blue staining, (b) Micro-CT, (c) bone volume, (d) bone volume per total volume, and (e) trabecular thickness in the region of interest in mouse alveolar bone. The mRNA levels of (f) *IL-6* and (g) *TNF*-α in the gingiva of AβPP/PS1 mice. Values are expressed as mean ± SEM ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ P.g-LPS versus P.g-LPS + Ligation.

proinflammatory cytokines were notably enhanced in the P.g-LPS + Ligation group compared to those in the AβPP/PS1 mice group ($p < 0.05$ or $p < 0.01$; Figs. 1f, g).

Effects of periodontitis on spatial learning and memory impairment in AβPP/PS1 transgenic mice

To study the effects of periodontitis on learning and memory, as well as spatial learning and memory abilities of mice, the animals were subjected to behavioral tests 8 weeks after surgery. In the navigation test, escape latency of all mice was gradually shortened, while their capability to locate the platform was enhanced after training (Fig. 2a). A decrease in the mean escape latencies of AβPP/PS1 transgenic mice was observed during days 2–4. No distinct differences were observed between the mean escape latencies of P.g-LPS + Ligation and AβPP/PS1 groups on days 1 and 2 ($p =$ 0.76 and 0.113). However, marked differences were observed on days 3 and 4 ($p = 0.001$ and 0.002). The percentage of time spent in the target quadrant by animals of the P.g-LPS + Ligation group while searching for the platform was lower than that spent by animals of the AβPP/PS1 control group, suggesting cognitive impairment in the former (Fig. 2b).

Spontaneous alternation shown by the P.g-LPS + Ligation group during the Y-maze test was notably decreased compared to that shown by the AβPP/PS1 group ($p < 0.01$, Fig. 2c). A similar result was obtained for the novel object recognition assay. Generally, the mice spent more time with the novel object compared to the time spent with the familiar object. Relative to behavior at consecutive training sessions, a notable decrease was observed in the new object discrimination index during the experimental session for the P.g-LPS + Ligation group compared to that observed for the control group ($p < 0.05$; Fig. 2d).

Effects of P.g-LPS and ligation-induced periodontitis on neuronal loss

The number of neuronal cells in the CA1 and DG regions of the hippocampus and cerebral cortex was determined to study the effects of P.g-LPS- and ligation-induced periodontitis on neuronal morphology and boundary. Structurally intact and highly dense pyramidal layer neurons were observed in the control group (Fig. 3). By contrast, the number of

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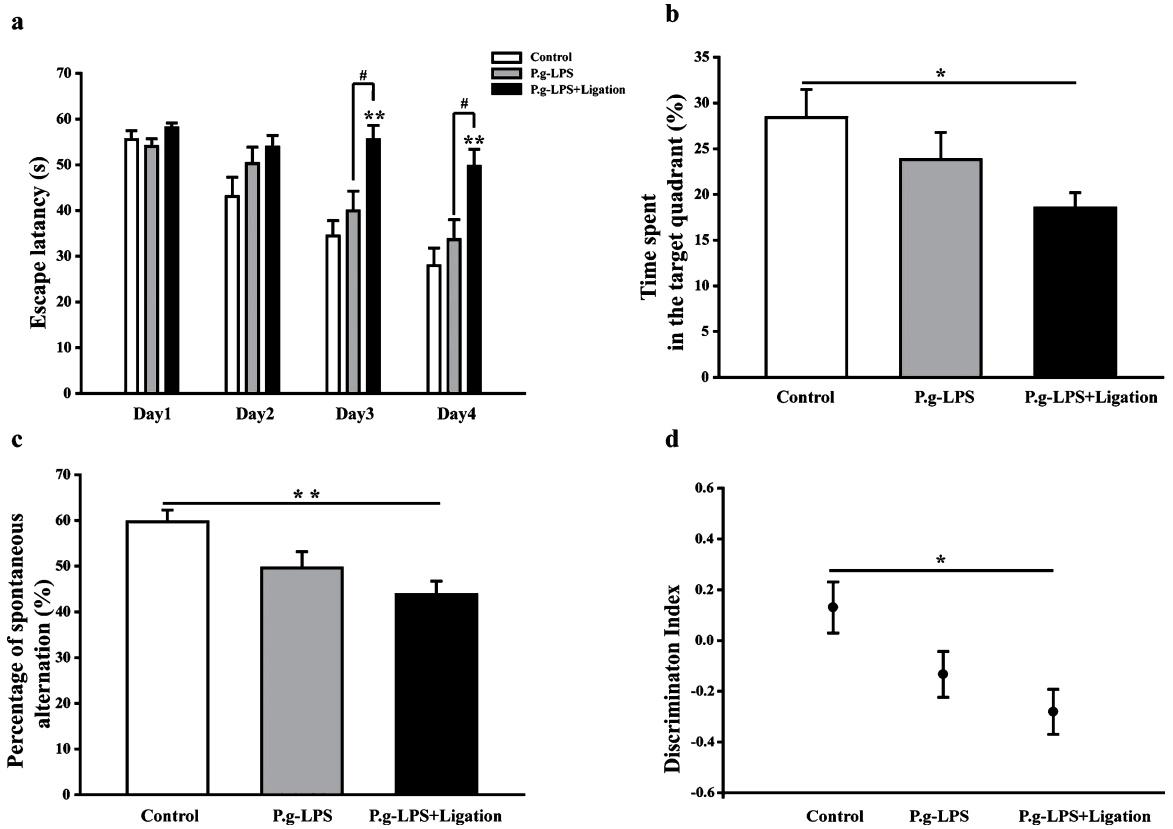


Fig. 2. Effects of periodontitis on learning and memory functions of AβPP/PS1 mice. (a) Escape latency of mice to reach the hidden platform from day 1 to 4. (b) Percentage of time utilized by the mice for swimming in the target quadrant. (c) Spontaneous alternation rate of mice in the Y-maze assay. (d) New object discrimination index. Values are expressed as the mean ± SEM ($n = 10$), $^{*}p < 0.05$, $^{**}p < 0.01$ versus control. $^{\#}p < 0.05$ P.g-LPS versus P.g-LPS + Ligation.

atrophied and pyknotic neurons in the P.g-LPS and P.g-LPS + Ligation groups was reduced.

Effects of periodontitis on the levels of $A\beta_{42}$ and $A\beta_{40}$ in the hippocampus and cerebral cortex

Immunofluorescence staining with anti-Aβ antibody was used to assess general Aβ levels in the hippocampi and cerebral cortices of mouse brains (Fig. 4a–c). Aβ deposition in the hippocampal regions and cerebral cortices of mice from the P.g-LPS and P.g-LPS + Ligation groups was significantly increased relative to that in mice form the AβPP/PS1 control group.

Western blotting yielded similar results (Fig. 4d–i). Compared with the control group, a dramatic increase in the Aβ level was observed in the P.g-LPS and P.g-LPS + Ligation groups, including the level of $A\beta_{40/42}$ ($p < 0.05$ or $p < 0.01$). Interestingly, significant differences were also observed between the P.g-LPS and P.g-LPS + Ligation groups ($p < 0.05$ or $p < 0.01$).

Effects of periodontitis on Aβ production pathway in the hippocampus and cerebral cortex

Abnormally increased Aβ production correlates closely with the activity of secretases of the AβPP cleavage process. Therefore, we evaluated the changes in AβPP that might contribute to increased Aβ production (Fig. 5). AβPP protein levels were significantly elevated in the brains of mice of the P.g-LPS + Ligation group compared to those in mice of the control group ($p < 0.01$). Notably, there was a significant difference between P.g-LPS and P.g-LPS + Ligation groups ($p < 0.05$), indicating that ligation increased inflammation, leading to increased expression of AβPP. We further analyzed the roles of two crucial enzymes, ADAM10 and BACE1, in the cleavage of AβPP [28]. BACE1 and ADAM10 expression increased and decreased significantly, respectively, in the P.g-LPS + Ligation group compared with that in the AβPP/PS1 group ($p < 0.05$ or $p < 0.01$) in both cerebral cortices and hippocampi.

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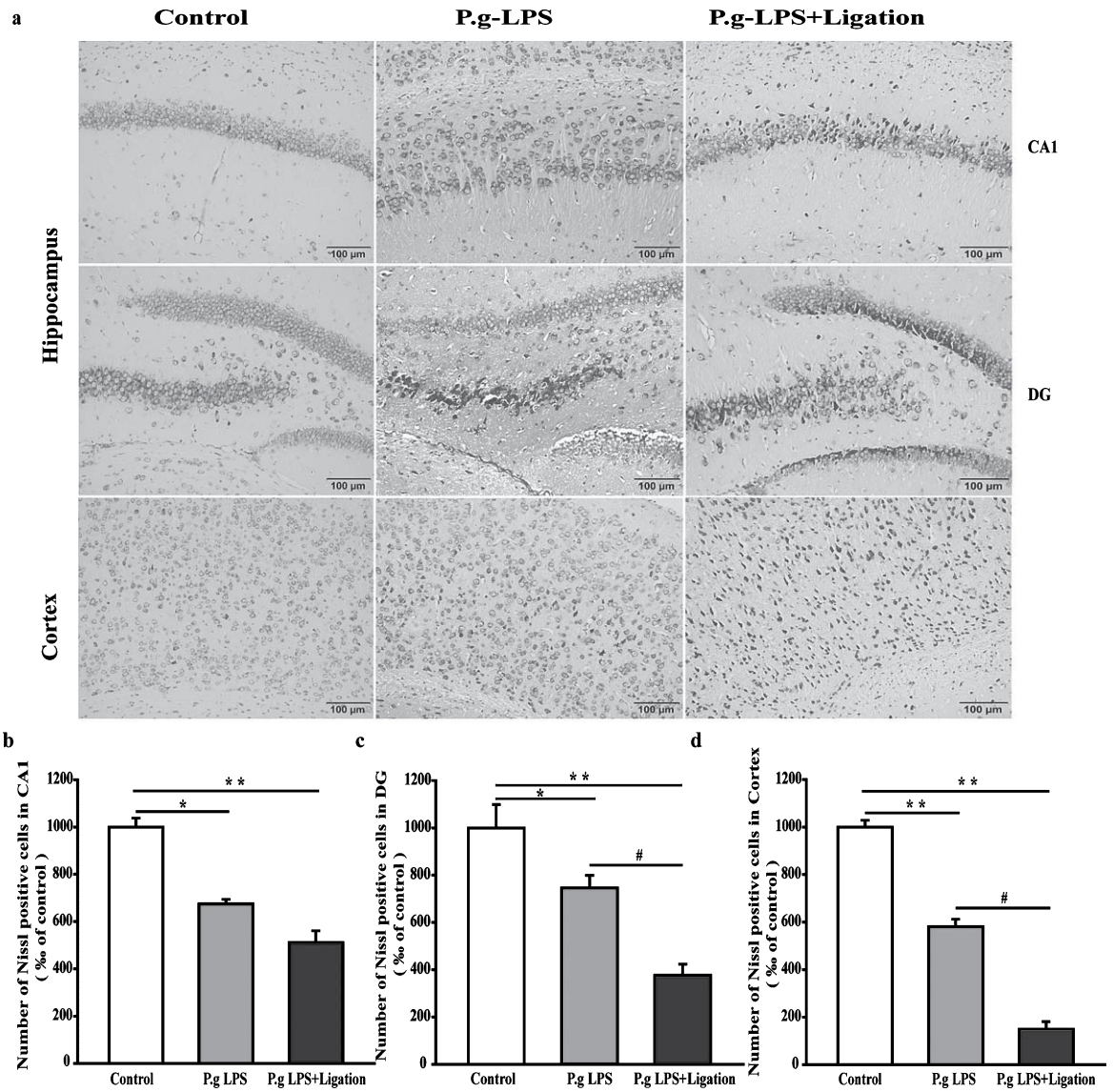


Fig. 3. Effects of periodontitis on neurons in the hippocampus and cerebral cortex of mice. (a) Representative photomicrographs of Nissl staining. (b-d) Quantitative analysis of the number of neurons. Values are expressed as mean ± SEM ($n = 4$), $^{*}p < 0.05$ versus control, $^{**}p < 0.01$ versus control, $^{\#}p < 0.01$ P.g-LPS versus P.g-LPS + Ligation.

Periodontitis promotes astrocyte and microglial activation

Intraperitoneal injection of LPS may induce neuroinflammation [21, 36]. Therefore, we determined whether P.g-LPS also caused neuroinflammation, by evaluating microglial and astrocytic responses via western blotting and immunohistochemistry staining of Iba1 and GFAP, respectively (Fig. 6). Iba1 immunostaining intensity in the P.g-LPS and P.g-LPS + Ligation groups increased compared to that in the AβPP/PS1 control group. Furthermore, the number of Iba1-immunoreactive microglia in these groups was increased compared with that in the control group. Additionally, GFAP-positive staining intensity in the hippocampal regions and the cerebral cortices increased significantly in the P.g-LPS and P.g-LPS + Ligation groups compared with that in the control group. Notably, the mean intensity of positive immunoreactive cells as well as the number of astrocytes in the hippocampal and cortical regions of mice with periodontitis increased. Similar results were observed via western blotting (Fig. 6g–l). Iba1 and GFAP expression in the hippocampi of the

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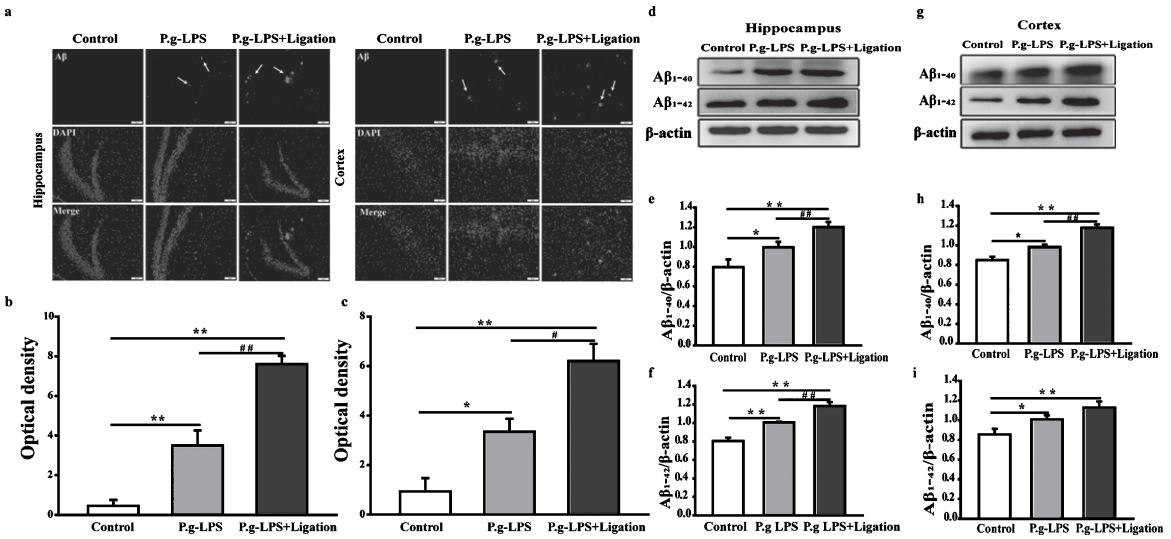


Fig. 4. Effects of periodontitis on the levels of $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus and cerebral cortex of mice. (a) Images of immunofluorescence in the hippocampus and cerebral cortex of AβPP/PS1 mice (magnification 200 ×). (b, c) Optical density statistics for Aβ deposition in the hippocampus and cerebral cortex. (d, g) Representative bands of $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus and cerebral cortex of mice. (e, i) Quantitative analysis based on normalized with β-actin expression levels. Values are expressed as mean ± SEM ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ P.g-LPS versus P.g-LPS + Ligation.

P.g-LPS + Ligation group was significantly increased compared to that in the control ($p < 0.01$ and $p < 0.05$, respectively) and cortex ($p < 0.01$) groups. Interestingly, Iba1 expression in the cerebral cortex of mice from the P.g-LPS group differed significantly from that of mice from the P.g-LPS + Ligation group ($p < 0.05$).

Periodontitis increased the expression of proinflammatory cytokines in the hippocampus and cerebral cortex

Next, we explored whether P.g-LPS and ligation-induced periodontitis affected the proinflammatory response of glia by assessing the protein expression of iNOS, COX-2, interleukin 1 beta and TNF-α via western blotting. As shown in Fig. 7, the expression of inflammatory proteins in the hippocampal and cortical tissues of both P.g-LPS and P.g-LPS + Ligation groups was increased compared to that in the control group. Interestingly, a notable difference was observed between the cerebral cortices of mice from the P.g-LPS and P.g-LPS + Ligation groups.

DISCUSSION

Periodontitis is a chronic infectious disease caused by the formation of a plaque biofilm. Notably, periodontal pathogens and associated virulence factors, such as LPS, induce the secretion of proinflammatory cytokines, thereby triggering inflammatory responses that result in alveolar bone loss [37]. In the current study, periodontitis was induced by applying a silk ligature around the second maxillary molars, together with interpapillary injections of P.g-LPS, wherein insertion of the silk ligature into the gingival sulcus initiated plaque-induced marginal periodontitis, while the injections maintained an effective LPS concentration [38]. Therefore, ligation combined with P.g-LPS injection may better mimic the pathology and symptoms of periodontitis in the human mouth. In this study, compared to P.g-LPS injection alone, ligation combined with P.g-LPS injection exerted increased toxic effects, suggesting that continuous LPS stimulation exerted greater toxicity under plaque colonization. Additionally, examination of the relevant proinflammatory factors in the gingival tissue, along with the micro-CT results, indicated that the periodontitis model was successfully established.

In recent years, increasing evidence has confirmed that periodontitis is remarkably associated with cardiovascular and cerebrovascular diseases, cancer, diabetes, premature birth and low birth weight, and respiratory tract infections [5, 39]. Moreover, a 2017 meta-analysis study showed that patients with periodontitis were nearly three times more likely to develop AD than those without periodontitis. Moreover,

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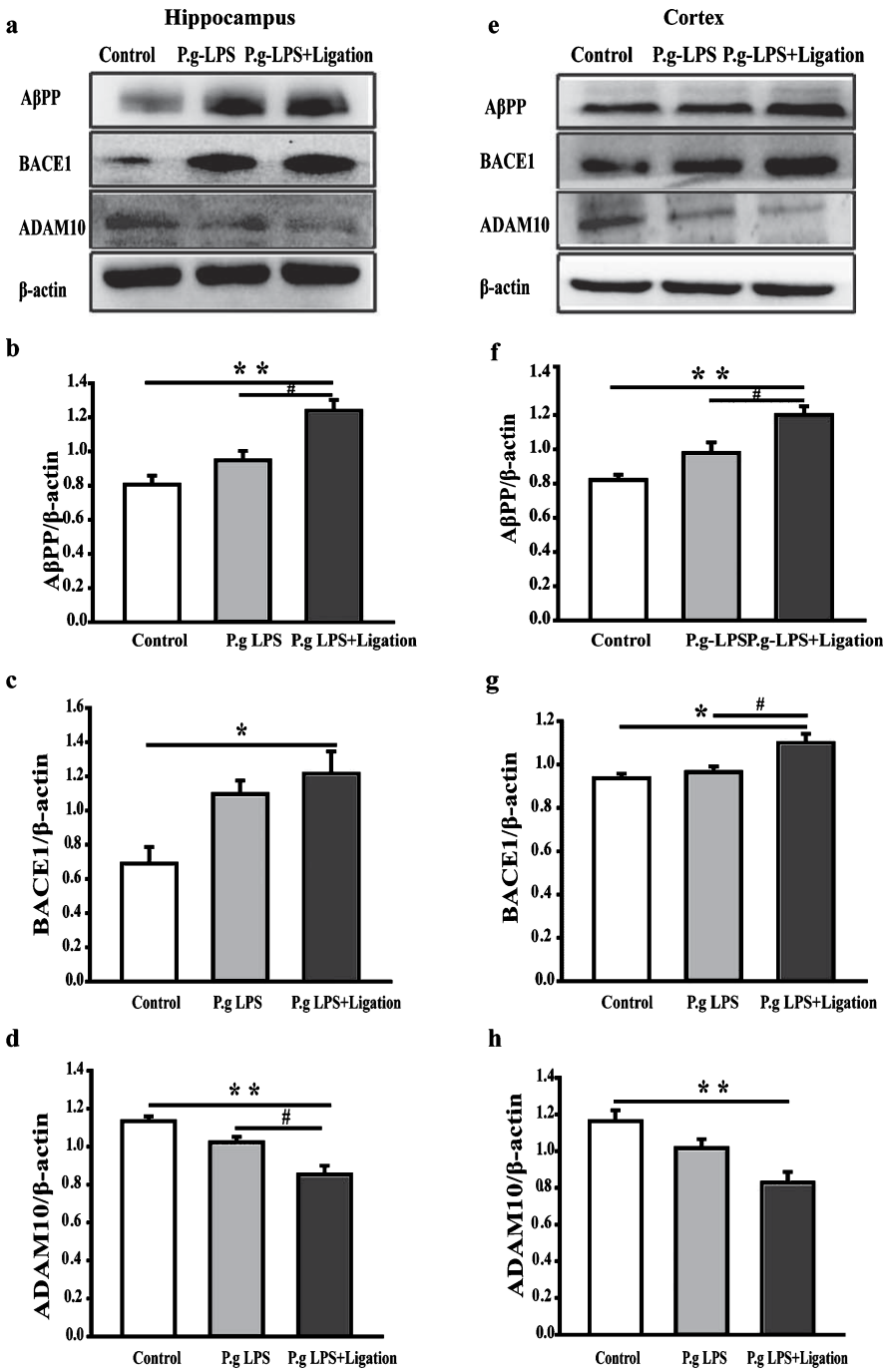


Fig. 5. Effect of periodontitis on the process of amyloid-β production in the hippocampus and cerebral cortex of mice. (a, e) Antibody-reactive bands of AβPP, BACE1, and ADAM10 in the hippocampus and cerebral cortex of mice. (b–h) Quantitative analysis based on normalized with β-actin expression levels. Values are expressed as mean ± SEM ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$ versus control. $^{\#}p < 0.05$ P.g-LPS versus P.g-LPS + Ligation.

the oral health of patients with AD was worse than that of patients without AD [40]. Previous studies have demonstrated that the direct inflammatory effect exerted by P.g-LPS aggravates neuroinflammation in the mouse brain [41]. Experimental studies have also shown that bacteria-induced periodontitis may

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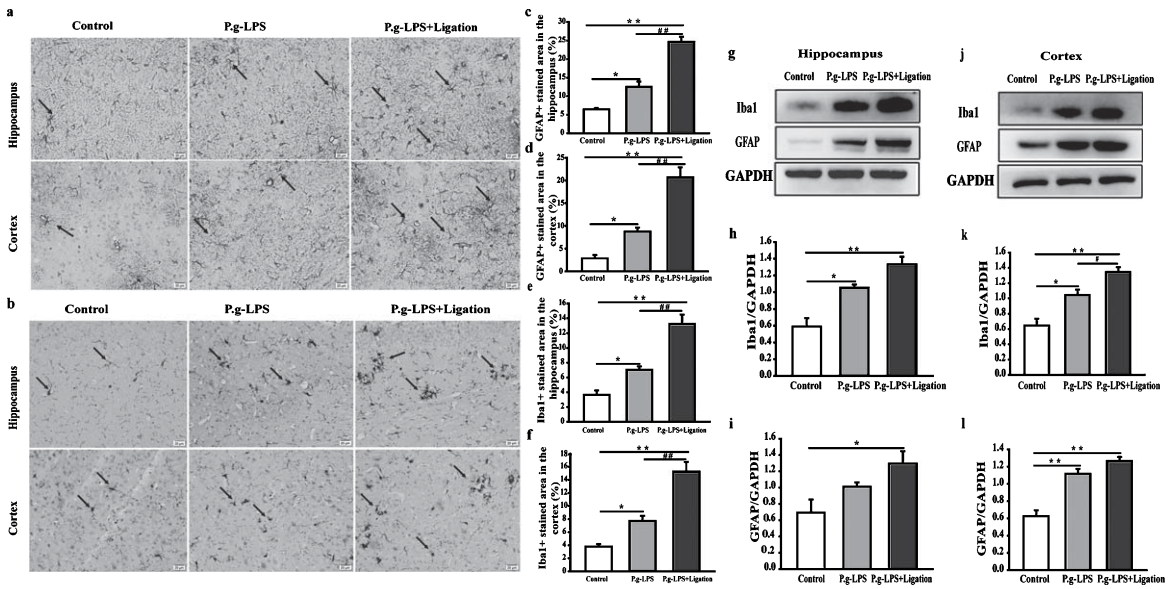


Fig. 6. Periodontitis promotes astrocytes and microglial activation. (a, b) Micrographs of GFAP and Iba1 immunoreactivity in the hippocampus and cerebral cortex (magnification 200 ×, scale bar 200 μm). (c, e) Quantitative analysis. (g, j) Antibody-reactive bands of Iba1 and GFAP in the hippocampus and cerebral cortex of mice, respectively. Quantitative analysis of (h, k) Iba1 and (i, l) GFAP expression in the hippocampus and cerebral cortex. The relative optical density was normalized to GAPDH values. Values are expressed as the mean ± SEM ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ P.g-LPS versus P.g-LPS + Ligation.

be related to the exacerbation of AD pathology [42, 43]. There have also been reports that these changes may be related to age [44]. Dominy et al. identified P.g in the brain of AD patients and confirmed through mouse experiments that the entry of P.g into the brain facilitated the release of gingipain, which promoted AD. Notably, inhibition of this protein alleviated AD symptoms, thereby revealing a possible mechanism underlying the effect of P.g in AD [45]. Since it has been confirmed that P.g may be an effector of gingipain, we investigated whether P.g was also an important effector of LPS.

AβPP/PS1 mice, a widely recognized AD model, were used to verify whether periodontitis aggravated the progression of dementia in AD mice. From approximately 9 months of age, AβPP/PS1 mice exhibited the formation of senile plaques that increased with age, in a manner similar to human Aβ stain material [46, 47]. Therefore, we used 6-month-old male AβPP/PS1 mice to detect whether intervention via periodontitis induced early AD-like pathology in AD mice. The results indicated that following 8 weeks of P.g-LPS injection combined with ligation, spatial and non-spatial learning as well as memory of mice decreased, indicating that the degree of dementia was more severe in mice with periodontitis. Although a previous study [35] showed that intraperitoneal injection of P.g-LPS might aggravate cognitive dysfunction in mice, the results of our study indicated that periodontal intervention via P.g-LPS injection alone did not cause any apparent cognitive impairment. Other studies have reported that insufficient or incomplete absorption of P.g-LPS, local anatomical structure and physiology of gingiva, or the biological effects of repeated injections of low-dose P.g-LPS over a long period leading to tolerance, may prevent significant impairment [48, 49].

Neuron loss, a pathological feature of AD, is markedly associated with declining learning capacity and reduced memory. Owing to neuronal loss compounded by other factors, the brain undergoes typical changes, such as thinning of the cortex and widening of gyri, during the late stages of AD. The primary function of Nissl bodies, which are characteristic functional units in neuronal cell bodies and dendrites, is protein synthesis [50]. Upon toluidine blue staining, a Nissl body is observed as a blue granular mass. Upon neuronal damage Nissl bodies undergo morphological changes and may dissolve leading to a decrease in their number [51]. Therefore, changes in Nissl body morphology and quantity may indirectly reflect nerve cell damage. Thus, we studied the effect of periodontitis on the number of neurons by staining Nissl bodies. In animals, neurons in the cerebral cortex and the CA1 and DG areas of the hippocampus are remarkably associated with learning and mem-

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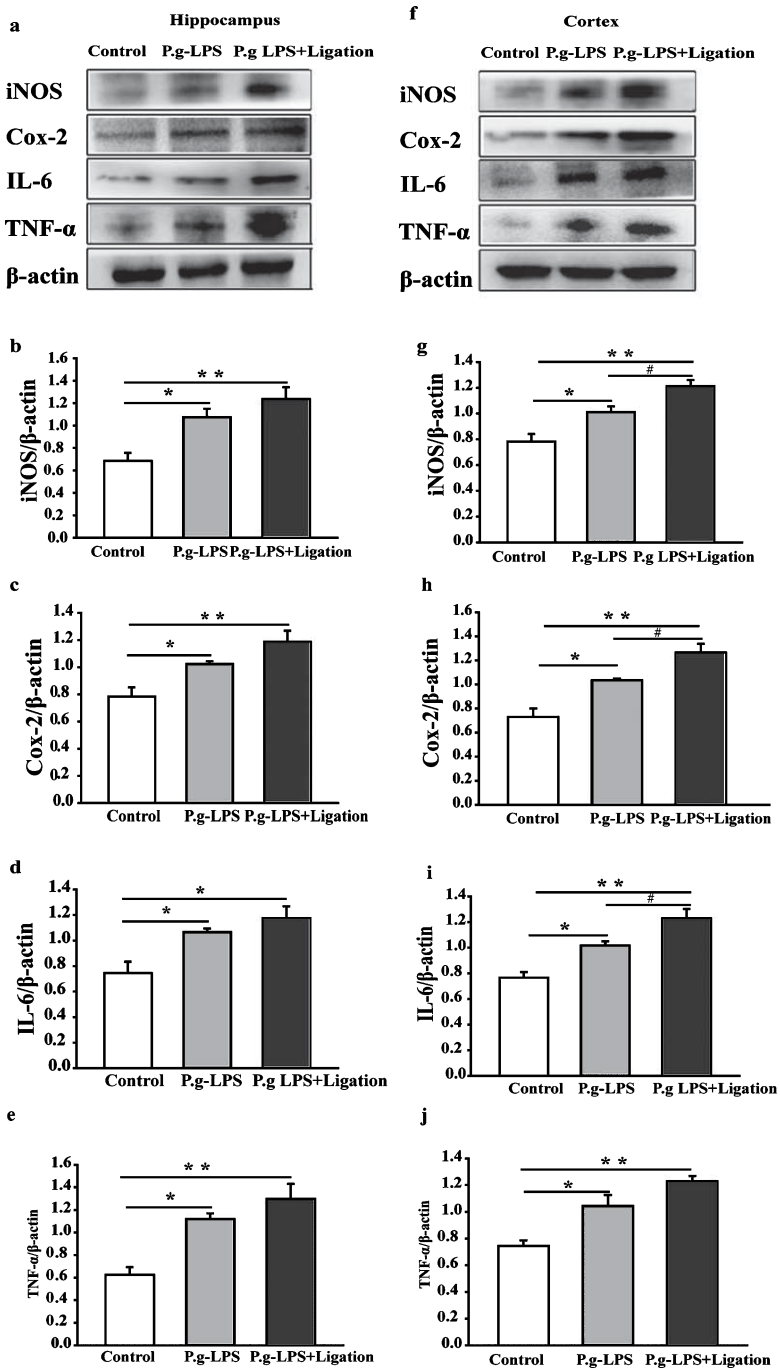


Fig. 7. Effects of periodontitis on iNOS, COX-2, IL-6, and TNF-α expression in the hippocampus and cerebral cortex of mice. (a, f) Antibody-reactive bands of iNOS, COX-2, IL-6, and TNF-α in the hippocampus and cortex of mice, respectively. Quantitative analysis of (b, g) iNOS, (c, h) COX-2, (d, i) IL-6, and (e, j) TNF-α expression in the hippocampus and cerebral cortex. Relative optical density was normalized to β-actin. The values are expressed as mean ± SEM ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{\#}p < 0.05$ P.g-LPS versus P.g-LPS + Ligation.

ory [52]. Therefore, in this study, changes in the specific brain areas of AD mice were evaluated to assess injuries caused by periodontitis. The results showed that the number of viable neurons in the cerebral cortex as well as in the CA1 and DG regions in the hippocampi of mice in P.g-LPS and

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P.g-LPS + Ligation groups were significantly reduced due to atrophy or dissolution of Nissl bodies, indicating that periodontitis-induced damage and loss of neurons had occurred in the brains of AD mice.

Aβ oligomers, including $A\beta_{40}$ and $A\beta_{42}$, have been shown to exert neurotoxic effects [53, 54]. Aβ, which is generated during AβPP metabolism, is cleared via enzymatic degradation pathways [55, 56]. Aβ deposition caused by the processing of AβPP by β- and γ-secretases, results in the accumulation of $A\beta_{42}$ and $A\beta_{40}$ in the hippocampus and cortical areas, thereby leading to neurotoxic effects [57–59]. Our results showed that the Aβ content in the hippocampi and cerebral cortices of the periodontitis model group was significantly increased compared with that in the control, suggesting that induction of periodontitis had effectively upregulated Aβ expression levels, via activation of the Aβ generation pathway.

AβPP is metabolized via non-amyloid and amyloid pathways under the regulation of α-, β-, and γ-secretase, wherein the amyloid pathway (comprising β- and γ-secretase) produces full-length Aβ [60]. Of note, α-secretases are members of the ADAM family of proteins, among which ADAM9, ADAM10, and ADAM17 demonstrate α-secretase activity [61]. Of these, reduced protein expression of ADAM10 is markedly associated with AD, thereby indicating that it plays an important role in the pathological process of AD [62]. β-secretase is the rate-limiting enzyme that initiates Aβ production. Of the β-secretase enzymes, BACE1, which is the key rate-limiting factor in Aβ production, plays a vital role in AD pathogenesis, according to the Aβ theory [63]. Thus, selective activation of α-secretase or inhibition of β- and γ-secretase reduces Aβ production. In our study, ligation-induced periodontitis increased the expression of AβPP and BACE1. Furthermore, it reduced the expression of ADAM10 in the hippocampi and cerebral cortices of AβPP/PS1 mice, suggesting that deposition of Aβ induced by periodontitis occurred via promotion of the AβPP amyloid pathway and inhibition of the non-amyloid pathway. However, the results of P.g-LPS injection alone showed no significant difference, compared to the control. Therefore, Aβ deposition caused by P.g-LPS may not be attributed to enhancement of the amyloid pathway but may be attributed to an insufficient clearance of Aβ [64].

Studies have shown that Aβ deposition is related to excessive activation of microglia and astrocytes [65, 66]. Neuropathological studies of transgenic models and subjects with AD have indicated that amyloid plaques are surrounded by multiple inflammatory proteins, such as complement factors, acute-phase proteins, and proinflammatory cytokines, along with clusters of activated microglia [67]. Similar to microglia, reactive astrocytes also tend to aggregate around fibroid amyloid plaques. When exposed to Aβ, astrocytes release cytokines and other potentially cytotoxic molecules that induce neuroinflammatory responses [68]. We examined the activation of microglia and astrocytes. A significant increase in the expression levels of Iba1 and GFAP suggested that periodontal inflammation activated immune cells in the brain, which exerted multiple toxic effects that aggravated AD. Several studies have shown that LPS induces neuroinflammation, as indicated by significant increases levels of proinflammatory mediators, such as COX-2, iNOS, IL-6, and TNF-α, *in vitro* in neuroglial cells *in vivo* in animal models [69]. In the current study, the co-expression of inflammatory proteins was higher in LPS-treated (P.g-LPS and P.g-LPS + Ligation groups) mouse brains, suggesting that microglia and astrocytes had been activated with a consequent increase in iNOS, COX-2, TNF-α, and IL-6 expression. These findings were consistent with increased amyloidogenesis.

The current study revealed that in AD mice, activated glial cells and upregulated expression of inflammatory mediators in the brain, as well as activated neuroinflammation, were positively correlated with $A\beta_{40}$ and $A\beta_{42}$ deposition. As glial cells serve as the brain's resident immune cells, the vicious circle between dysfunctional glial cells and Aβ is a crucial pathological event that accelerates the progression of AD. Excessive accumulation and aggregation of Aβ result in neurotoxicity, which eventually leads to neuronal degeneration and neuronal death [14]. Our research demonstrated that induction of Aβ deposition and neuroinflammation by P.g-LPS played an important role in the virulent effect exerted by periodontitis on the development of AD. Furthermore, combining ligation with P.g-LPS not only stimulated the clinical pathogenic effect of periodontitis more effectively but also demonstrated that the inflammatory lesions caused by synergistic colonization of periodontal pathogens were worse than those caused by LPS injection alone. This suggested that, in addition to LPS, P.g and other periodontal pathogens may function as virulence factors or resort to more complex mechanisms that promote AD. This is further substantiated by the results of a recent study

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conducted by Dominy et al. in which the effect of P.g gingiva on AD was investigated [45].

The current study was beset with certain limitations. Direct evidence indicating that periodontitis aggravates Aβ by affecting AβPP, as well as α-, β-, and γ-secretase may be necessary. Additional analyses of the effects of periodontitis on the amyloid and non-amyloid pathways should be conducted. Moreover, the activation of glial cells and the release of inflammatory cytokines in periodontitis, as observed in this study, may have been induced via various mechanisms. Therefore, the precise mechanism(s) underlying glial cell activation and inflammatory cytokine enhancement warrants further elucidation. Many *in vitro* and *in vivo* studies have indicated that LPS-induced inflammation is mediated by Toll-like receptor-4, which activates signaling pathways that upregulate expression of various proinflammatory cytokines and chemokines [70, 71]. Therefore, it may be useful to investigate the role played by the Toll-like receptor-4 pathway in the association between periodontitis and cognitive dysfunction in mice. Furthermore, mounting evidence indicates that gastrointestinal microorganisms may be related to AD [72, 73]. Dysbiosis of biofilm flora in the oral cavity, which serves as the portal to the digestive tract, exerts adverse effects on intestinal flora. Hence, the composition of intestinal flora as well as the intestinal environment (intestinal barrier function) and intestinal immunity are regulated by oral bacteria, either directly or indirectly [74]. Finally, all animal models are limited by the fact that no single model can represent all aspects of human periodontal disease or AD. Therefore, clarifying whether a selected model is suitable for the study of a specific hypothesis is vital [75]. Although the current study did not prove that periodontitis was a risk factor for AD, our experimental results suggested that periodontitis might exert an effect on the development of AD.

In conclusion, our experiment revealed that periodontitis exacerbated learning and memory impairment of AβPP/PS1 transgenic mice, and induced Aβ generation, activated glial cells, and promoted proinflammatory cytokine release.

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Do Periodontal Pathogens or Associated Virulence Factors Have a Deleterious Effect on the Blood-Brain Barrier, Contributing to Alzheimer's Disease?

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Abstract. The central nervous system (CNS) is protected by a highly selective barrier, the blood-brain barrier (BBB), that regulates the exchange and homeostasis of bloodborne molecules, excluding xenobiotics. This barrier forms the first line of defense by prohibiting pathogens from crossing to the CNS. Aging and chronic exposure of the BBB to pathogens renders it permeable, and this may give rise to pathology in the CNS such as Alzheimer's disease (AD). Researchers have linked pathogens associated with periodontitis to neuroinflammation and AD-like pathology *in vivo* and *in vitro*. Although the presence of periodontitis-associated bacteria has been linked to AD in several clinical studies as DNA and virulence factors were confirmed in brain samples of human AD subjects, the mechanism by which the bacteria traverse to the brain and potentially influences neuropathology is unknown. In this review, we present current knowledge about the association between periodontitis and AD, the mechanism whereby periodontal pathogens might provoke neuroinflammation and how periodontal pathogens could affect the BBB. We suggest future studies, with emphasis on the use of human *in vitro* models of cells associated with the BBB to unravel the pathway of entry for these bacteria to the CNS and to reveal the molecular and cellular pathways involved in initiating the AD-like pathology. In conclusion, evidence demonstrates that bacteria associated with periodontitis and their virulence factors are capable of inflecting damage to the BBB and have a role in giving rise to pathology similar to that found in AD.

Keywords: Alzheimer's disease, bacteria, blood-brain barrier, periodontitis, virulence factors

INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder that affects the elderly population resulting in reduced function of the brain and cognitive impairment. Histopathologically, AD is characterized by the presence of insoluble extracellular amyloid-β

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(Aβ) plaques and intracellular aggregates of hyperphosphorylated tau proteins (neurofibrillary tangles) [1]. For a long time, researchers have dwelled on the amyloid cascade hypothesis, which claimed that the accumulation of $A\beta_{42}$, a toxic polypeptide that forms aggregated amyloid plaques, was the cause of synaptic death, neuronal loss, and cognitive impairment observed in AD [2]. Aβ plaques, however, were also found in brain tissue of healthy subjects with no dementia; additionally there was no correlation observed between the load of Aβ and the severity of AD [3]. Subsequently, alternative hypotheses have arisen, including the inflammatory and the pathogen hypothesis.

The inflammatory AD hypothesis suggested that an inflammatory response in the brain parenchyma could perpetuate and provoke synaptic dysfunction and neurodegeneration [4, 5]. Inflammation has also been proposed to arise as a result of Aβ plaques, neurofibrillary tangles, and a number of other related constituents [6]. Conversely, Aβ plaques and neurofibrillary tangles have also been considered as byproducts of inflammation. It has been shown that an inflammatory response induces both astrocytes and microglial to produce pro-inflammatory cytokines; tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), as well as C-reactive protein (CRP) [7], both *in vitro* and in AD tissues [8]. Additionally, *in vitro* studies reported that pro-inflammatory cytokines, TNF- α, IL-1β, and IL-6, can induce the production of $A\beta_{42}$ and tau protein phosphorylation [9, 10], which in turn stimulate microglial cells to produce a range of pro-inflammatory mediators [10]. Of relevance to the theme of this review, it has also been reported that systemic events created by injecting mice with lipopolysaccharide (LPS), results in an indirect increase in brain influx of Aβ and decrease in brain efflux of Aβ concomitant in the presence of elevated serum levels of IL-6, IL-10, and IL-13 [11].

The central tenet of the pathogen AD hypothesis is that viral, bacterial, or fungal pathogens could play a role in triggering AD pathology [12]. This hypothesis focuses on the fact that pathogens such as herpes simplex viruses (HSV) have been found in the brains of AD patients [13], and that Aβ accumulation may represent an anti-viral response, given some support by a recent study which reported that $A\beta_{42}$ shares a similar structure to that of antimicrobial peptides [14–16]. While Aβ could be a defense mechanism that counters infectious agents, its long-term impact on brain tissue integrity could be harmful [17].

AD, the main cause of dementia, is projected to influence 115 million individuals all over the globe [1]. The estimated number of individuals with dementia in the UK in 2019 was 885,000 [18], and this figure is projected to rise to roughly 1.14 million in 2025 and about 2.1 million in 2051 [19]. During the next few decades, low- and middle-income countries are expected to witness the greatest increase of dementia cases compared to high-income countries due to the increasing number of older people [20, 21]. After taking into consideration the increasing number of older people in low- to middle-income countries driving the increased prevalence of worldwide dementia cases, the WHO found that there is an increased prevalence of dementia in these countries compared to age-standardized high income countries [22].

Periodontal disease

One of the most common chronic infectious states in the human body is periodontal disease (POD), prevalent in 20–50 % of the adult population [23]. Individuals from low-income countries face 1.8-fold increased risk of developing severe periodontitis compared to that of high-income individuals [24]. It is an inflammatory condition that affects the supporting tissues of teeth, namely the periodontium including the gingiva, surrounding bone and the collagen fibers of the suspensory periodontal ligament (PDL), that penetrate the cementum to anchor the tooth to the bone [25] (Fig. 1). The hallmark of POD is microbial dysbiosis leading to tissue loss that encompasses both soft and hard tissues of the periodontium [26], resulting in the formation of periodontal pockets and loss of attachment of the junctional epithelium [27], and constitutes one of the main culprits of tooth loss in adults [28]. In the periodontal health, anaerobic gram-negative bacteria associated with periodontitis, make up a small proportion of the subgingival commensal bacteria. However, with the progression of the disease and the initial formation of pockets due to swelling of the gingiva, an increased number of anaerobic gram-negative bacteria starts to establish colonies in the pockets [27]. The headline microorganisms associated with POD are the anaerobic Gram-negative bacteria: *Porphyromonas Gingivalis (Pg), Tannerella Forsythia (Tf), Treponema denticola (Td)*, and *Aggregatibacter actinomycetemcomitans (Aa)*, and *Pg* is considered pivotal for disease development [29, 30]. *Pg* possesses numerous virulence factors capable of inducing damage to host cells and averting and

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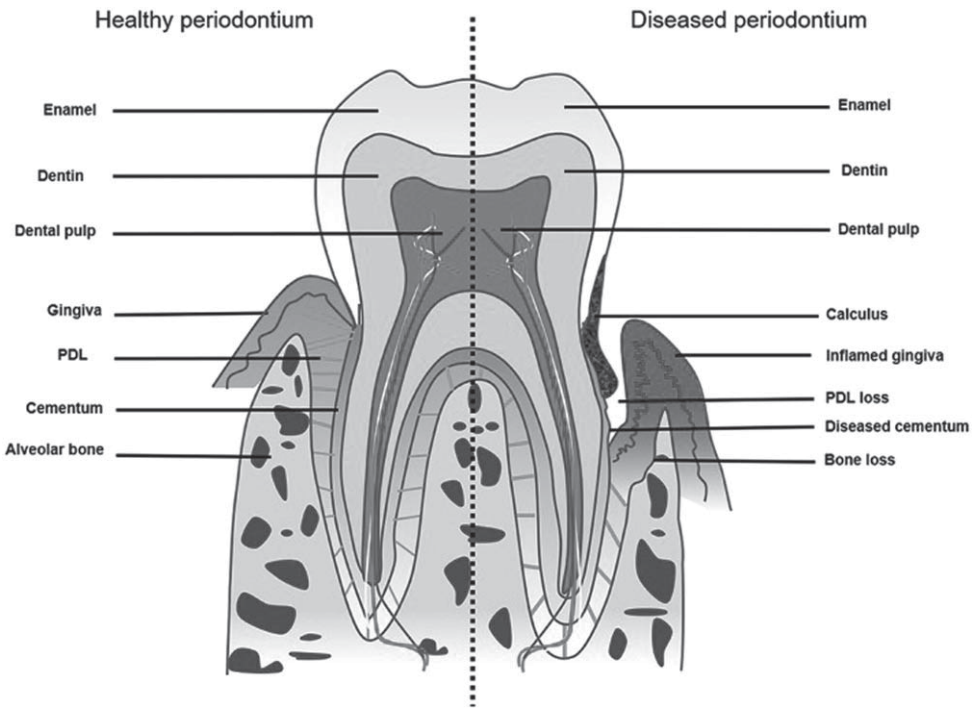


Fig. 1. A diagram that compares the healthy periodontium with the diseased one. The accumulation of calculus, which is mineralized plaque renders the gingival tissue inflamed and could lead to loss of PDL and bone [40].

subverting the host immune response. These include LPS, gingipains, fimbriae, lectins, proteases and capsule, the first three being the most virulent [31]. *Pg* releases LPS either after the bacterium is lysed or in the form of microvesicles produced from the membrane of the vital bacterium [32]. Gingipains are proteases secreted by *Pg* found in outer membrane vesicles, and while they play a role in the interactivity between the host and *Pg* [33], their ability to subvert the host's immune system stems from their capability of degrading proteins of the complement system, such as C5 [34]. Fimbriae are prominent filamentous organelles that exist on the surface of a bacterium [35], that mediate the attachment of *Pg* to the host cells [36], and provoke the production of pro-inflammatory cytokines, IL-1β and IL-6, from macrophages [37]. The key issue in respect of the pathogen or inflammatory AD hypotheses is that pro-inflammatory cytokines produced in the periodontal tissues, IL-1β, IL-6, TNF-α, chemokines IL-8, and the bacteria initiating and maintaining the inflammatory state, can access the systemic circulation through the ulcerated periodontal tissues and eventually interact with or even colonize distant organs [38]. This systemic invasion of bacteria and pro-inflammatory mediators constitute risk factors for various diseases such as cardiovascular disease, diabetes, atherosclerosis but of interest for this review on AD [7, 38, 39].

Alzheimer's disease and periodontal disease

When exploring the link between AD and POD, a number of human clinical studies have suggested that patients with periodontitis were more prone to developing dementia [41–47]. Poor cognitive performance, impaired memory, and development of AD has been associated with serum antibodies (IgG) to *Pg* [43, 48]. Stein et al. (2012) found that antibodies to *Pg* and *Td*, were elevated at baseline prior to the development of AD compared to participants who remained cognitively healthy [49]. Another study found patients receiving no periodontal treatment had a higher tendency to develop dementia compared to those who received treatment when comparing patients matched for gender, age, income, educational level, and smoking [44].

The data from such studies should be interpreted with caution as the association between the two diseases, could be bi-directional [50]. AD might predispose to POD, as patients who suffer from cognitive decline could lack the facility to practice oral hygiene instructions, which may result in

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development of POD [49]. But the author's group support the concept that since periodontitis is a low-grade chronic source of systemic inflammation [51], it could promote neurodegenerative diseases like AD, by a persistent release of systemic circulatory pro-inflammatory molecules or associated bacteria, with potential access to brain tissues.

A third factor to consider that provides possible links between AD and POD is genetic susceptibility. A comparison of genome-wide association study datasets in human populations following pathogen exposure in early-life and from AD patients found that upregulated genes in the AD hippocampal transcriptome were related to immune and inflammatory processes upregulated during infection. Resistance to previous infections in early-life led to an immune mediated gain of function in APOE4, CR1, and TREM2, which then links to deposition of Aβ and AD in later life [52], indicating chronic low grade infection and an antimicrobial immune response in the brain may result in neurodegeneration.

Another mechanism by which POD could be linked to AD is by inducing dysbiosis in the gut microbiota. The alteration of gut microbiota has been found to be related to elevated Aβ in the CNS, impaired memory, and spatial learning in APP/PS1 mouse model [53]. Similarly, the prolonged use of wide-spectrum antibiotics in an AD mouse model to alter the composition and diversity of the gut microbiota has led to an increase of the levels of peripheral cytokines, CCL11 and CXCL16, and of the levels of soluble Aβ, while Aβ plaques have decreased [54]. In addition, the use of clarithromycin and amoxicillin or metronidazole to treat *Heliobacter pylori* infection in humans could lead to neuropsychiatric disorders such as anxiety and delirium [55]. As for the role of POD in this regard, oral administration of *Pg* to C57BL/6 mice has led to a change in the composition of the gut microbiota and upregulation of IL-6 in the small intestine as well as compromising the gut barrier function [56]. In another study employing C57BL/6N mouse model, it has been reported that administrating *Pg* orally resulted in a significant increase of bacteroidales [57]. Furthermore, ligature-induced periodontitis in C57BL/6 J mice has led to developing periodontitis, glial activation, and cognitive dysfunction as well as oral and gut microbiota dysbiosis. The gut microbiota dysbiosis that arose following POD was thought to lead to reduced expression of tight junction proteins, claudin-1, occludin, and ZO-1, in the intestinal barrier in addition to a decrease in claudin-5, occludin, and ZO-1 in the BBB [58].

Numerous postmortem studies have also explored the relationship between AD and POD. *Pg* LPS was detected in the brain tissues of AD patients while they were absent in postmortem samples from aged-matched non-AD subjects [59]. Dominy et al. (2019) reported the existence of gingipains antigens, proteases secreted by *Pg*, in higher loads in the brains of patients with AD cases, in comparison to brain tissues from non-AD patients. They also reported the presence of *Pg* DNA in the brain and CSF of AD patients [60]. Another study investigating whether *Treponema* invades the brain has shown that the majority of specimens (14 out of 16) from subjects with AD had *Treponema*, compared to a minority in controls (4 out of 18) [61].

On a similar note, the DNA of *Td* and *Pg* was detected in the hippocampus of C57BL/6 mice 24 weeks post oral injection of either of these pathogens, and this provoked the production of $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus with no difference between the pathogens [62]. Since *Td* belongs to the same genre taxonomy as *Treponema pallidum*, which is the pathogen involved in syphilis, it is no wonder that it is capable of translocating to the brain [63].

Before accessing brain parenchyma, pro-inflammatory mediators, virulence factors, or microorganisms would have to cross or interact with the BBB, as a prelude to provoking inflammation-based changes.

ANATOMICAL COMPONENTS OF THE NEUROVASCULAR UNIT AND BBB FUNCTION

The BBB serves as a protector for the brain parenchyma, by being a highly selective interface that facilitates transport of nutrients into the brain whilst preventing access to pathogenic blood-borne agents [64]. The structural and functional relationship between the brain and blood vessels, known as the BBB does not include just the brain endothelium barrier, it also incorporates components such as the neurons, perivascular astrocytes, pericytes, microglia, and the extracellular matrix, collectively known as the neurovascular unit (NVU) [65] (Fig. 2A). Pericytes are embedded in the basement membrane of the microvessels, where they envelop the outer surface of the vessels by establishing gap junctions with endothelial cells [66]. Pericytes play a key role by contributing to the stability of microvascular permeability and angiogenesis by producing growth factors and extracellular matrix [67].

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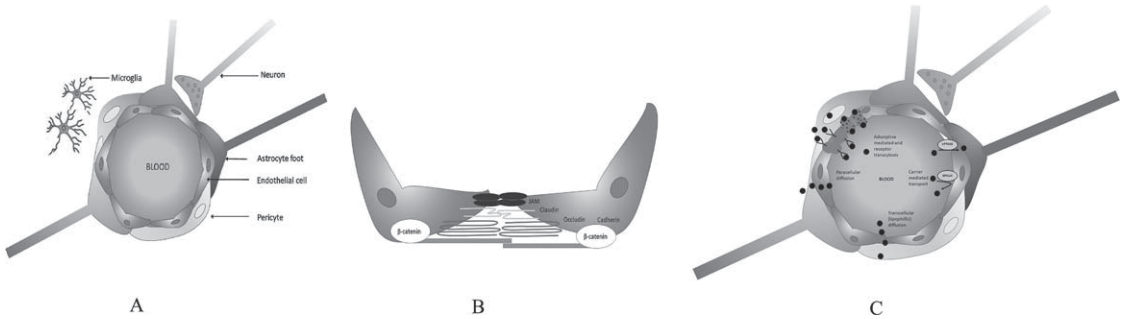


Fig. 2. The blood-brain barrier is formed by (A) a network of cells (B) tight junction and adherens junction proteins form a physical barrier and (C) major transport pathways constitute the metabolic barrier.

In addition, they also possess phagocytotic features that help in the clearance of soluble small molecules using pinocytosis, such as dextran [68]. The end-feet of astrocytes, which enclose the capillary basement membrane, facilitate an intimate connection between CNS neurons and the blood vessels of the NVU, in which astrocytes control blood flow after receiving signals from adjacent neurons [69]. The signaling mechanism involves neurons producing and releasing glutamate, inducing astrocytes to the release of either potassium that serves as a vasodilator agent or ATP that results in vasoconstriction [70]. In addition, astrocytic end-feet establish an additional barrier known as the glia limitans by sheathing the blood vessels and the CNS surface [71]. Alongside the barrier function, astrocytes play a vital role in maintaining the integrity of the BBB in addition to regulating water homeostasis to and from the brain tissues [72, 73]. Microglia have also been demonstrated to play an important role in maintaining vascular integrity and modulating tight junction protein expression during inflammation [74]. Interestingly when in close proximity to the microvasculature, microglia were found to interact interdependently with NVU endothelial cells [75], such that activated endothelial cells have been shown to play a role in the activation of microglia [76], via release of chemokines [77]. Further, pro-inflammatory cytokines, IL-1β and TNF-α, produced by microglia were found to downregulate tight junction protein expression (ZO-1 and occludin), resulting in an increase of BBB permeability to sodium fluorescein [78, 79]. The interactions between the NVU and surrounding cells is further exemplified by work which demonstrates that microglia activated by LPS to the M1 phenotype, produce cytokines capable of inducing reactive astrocytosis to the A1 phenotype [80]. Microglia have also been shown to be highly responsive to pathogens [81], in contrast to astrocytes, which were not found to be activated following LPS treatment [82]. Similarly, microglia activated to the M2 phenotype, promoted A2 astrocytes reactivation through the production of anti-inflammatory cytokine IL-10 [83].

Further barrier properties are provided by the basement membrane, which consists of extracellular matrix proteins, laminin, nidogen, type IV collagen, and heparan sulphate proteoglycans, produced by the endothelial cells, pericytes and astrocytes [84–86].

A key element in creating the barrier function is to maintain tight endothelial cell adherence. This is achieved by 1) Tight junctions, formed by primary proteins (claudin, occludin, junctional adhesion molecules (JAMs) and accessory proteins of the zona occludens (ZO-1, ZO-2, ZO-3). They form intramembranous strands which compose a net-like meshwork surrounding the cells [87]. They adhere the endothelial cells tightly together thus modulating the paracellular permeability, maintaining the integrity of the BBB and conferring high electrical resistance [88]; 2) Adherens junctions (AJs), connect adjacent endothelial cells via proteins of the cadherin superfamily, which are linked to the endothelial cytoskeleton by proteins of the cytoplasmic plaque, catenins [89] (Fig. 2B).

The BBB is not an impassable barrier though. Small lipid soluble molecules travel across by passive diffusion, while other substances, such as IGF, glucose and vitamins, have to traverse the BBB via specific systems namely receptor-mediated transport, carrier mediated transport, efflux transport system and adsorptive endocytosis [90]. The carrier-mediated transporters could be active or passive [91] and can be located on the luminal and / or abluminal surfaces [92], designed to carry molecules from the circulation to the brain (uptake transporters) or in the opposite direction (efflux transporters) [93] (Fig. 2C).

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Table 1
Changes at the blood-brain barrier in AD

Author (year)	Effect on BBB tissues	Model	Measure	AD stage
Yamada (2002) [105], Zlokovic (2005) [106], and Thomsen et al. (2017) [107]	BBB Basement membrane and could be toxic to BBB Endothelial cells	Human	Aβ accumulation- cerebral amyloid angiopathy developed in more than 80% AD patients	Postmortem biopsies
Lepelletier et al. (2017) [108]	BBB Basement membrane	Human	Increased thickness and altered composition	Subclinical AD and AD patients
Yates et al. (2014) [109]	BBB Endothelium/ brain parenchyma	Human	Cerebral microbleeds and cortical siderosis	AD patients
Vogelsang et al. (2018) [110]	BBB Transporters	Human brain derived circulating endothelial cells	Downregulation of GLUT-1	Blood collected from mild AD patients

WHAT HAPPENS TO THE BLOOD-BRAIN BARRIER FUNCTION IN ALZHEIMER'S DISEASE?

There is evidence that changes in the BBB integrity occur in AD [94, 95]. From a therapeutic perspective, it would be helpful to fully understand the mechanism by which the disruption arises, how BBB integrity is repaired and how this integrity can be enhanced to prevent or reverse resultant neuropathology at the early stages of disease. The question whether the dysfunction of the barrier happens because of the neuropathological changes or the latter emerges because of the former remains an important but difficult question to answer definitively [96]. BBB disruption has been observed in early-stage AD patients compared to age-matched controls, where dynamic contrast enhanced MRI has shown leakage of contrast agents such as gadobutrol, that has been injected into the circulation, and clearly identified BBB located cerebral microbleeds [97]. In the healthy brain parenchyma there is an almost complete absence of neutrophils and lymphocytes as the endothelial tight junctions prohibit these cells from accessing the brain tissue [84]. The breakdown of the BBB has been reported using advanced dynamic contrast-enhanced MRI to commence in the hippocampus, which has a key role in learning and memory, and it becomes atrophic in AD despite possessing the highest barrier properties, during normal aging. This dysfunction of the BBB was greater in subjects with mild cognitive impairment in comparison with age-matched controls, and this breakdown of the BBB could contribute to cognitive impairment as the authors suggested [98]. Activated microglia and astrocytes have been shown to release pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and elevated levels of these molecules have been found to accompany AD lesions in brain tissues of AD [8], and these inflammatory molecules have the potential to increase the BBB permeability [64, 99–101]. Neuroinflammation has also been shown to upregulate endothelial leukocyte adhesion molecule expression, facilitating leukocyte transmigration into the CNS [102, 103]. The passage of such immune cells into the brain can result in the intracerebral production of pro-inflammatory cytokines, IL-17, IL-22, TNF-α, and IFN-γ, which further compromises the integrity of the BBB by altering the expression of junctional proteins [104]. This can result in oedema, immune infiltration, ionic homeostasis disturbance, and modified signaling and ultimately degradation of neurons [84]. A summary of other changes observed at the BBB in AD patients in shown in Table 1.

PROPOSED MECHANISM LINKING PERIODONTAL DISEASE, PERIPHERAL INFLAMMATION, AND BBB DISRUPTION

The entry of pathogens and/ or associated virulence factors into brain parenchyma may be facilitated by disruption of the BBB in AD, possibly an early feature of the disease. Exploration of the possible mechanisms by which POD may induce changes at the BBB have been conducted by both *in vivo* and *in vitro* studies (Table 2). This evidence is supported by the knowledge that POD-associated bacteria, such as *Pg*, contribute to significant bacteraemia following dental procedures, such as root surface debridement [111], tooth brushing [112], and even chewing [111]. Could POD provide a chronic source of pathogens and thus a chronic assault on the integrity of the BBB?

Systemic cytokines, initiated by the chronic inflammatory changes associated with *Pg* infected periodontal pockets, could translocate to the brain

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Table 2
In vitro and *in vivo* models investigating the effects of peripheral inflammation and/ or periodontal pathogen exposure on molecular and cellular components of the BBB

Author (year)	Model	Species	Mechanism	Findings
Basuroy et al. (2006) [113]	*In vitro* cerebral vascular endothelial cells	Porcine Murine	TNFα induced peripheral Inflammation	Decrease in occludin, increase BBB permeability
McCollet al. (2008) [115]	*In vivo* ischemic brain injury	Murine	IL-1β induced peripheral inflammation	Disruption to claudin-5 and collagen IV by MMP9
Furutama et al. (2020) [117]	*In vivo* ligature around molars to induce POD	Murine	POD induced peripheral and CNS inflammation IL-1β	Decrease in claudin-5, increase in endothelial permeability with POD
Rokad et al. (2017) [121]	*In vivo:* 1) TNF-α transgenic mice; 2) wild-type mice; 3) *ApoE* knockout sham infected mice and 4) *ApoE* knockout *Pg* infected	Murine	POD induced cerebral oxidative stress in hippocampal microvasculature	Tight junction proteins degradation, leading to a loss of functional BBB integrity with POD

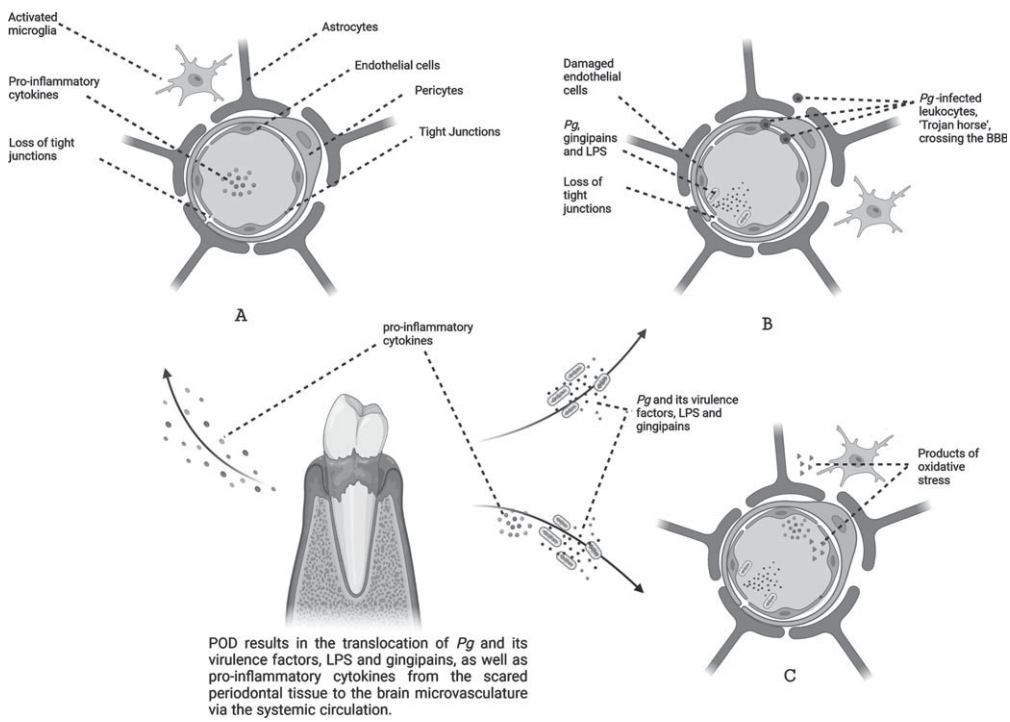


Fig. 3. The translocation of (A) pro-inflammatory cytokines from the periodontal tissue to the brain microvasculature might lead to a loss of tight junctions and impair the BBB, which could activate microglia and astrocytes to produce inflammatory molecules. B) Pg or its virulence factors, gingipains and LPS, can induce direct damage to endothelial cells and could lead to loss of tight junctions as well. Pg might also be able to infect leukocytes and use them as 'Trojan horse' to cross the BBB. C) Pg, its virulence factors and pro-inflammatory cytokines produced because of POD can provoke the release of oxidative stress products from endothelial cells and microglia, which could compromise the function of the BBB. Created with BioRender.com.

either as a result of increased permeability of the BBB [113], or by using existing saturable transporters [114]. Peripheral TNF-α and IL-β have been shown to increase the permeability of the BBB by decreasing expression of occludin thus disrupting the integrity of tight junctions [113, 115] (Fig. 3A). After crossing the BBB, cytokines then have the potential to activate both endothelial and glial cells to produce other pro-inflammatory cytokines [116].

Support for this proposal can be found in studies such as Furutama et al. (2020) whereby POD was induced by placing ligatures on the second upper molars bilaterally of C57BL/6J WT mice. This led to an increase in serum IL-6, which in turn resulted in an

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increase of IL-1β in the hippocampus. This study also demonstrated that the mRNA expression and protein levels of claudin 5 were decreased. Moreover, the gap area between endothelial cells in the hippocampus was also increased, enhancing the permeability of the BBB but only for small molecules such as 3-KDa dextran [117]. A source of weakness in this study was that the authors did not confirm the development of periodontitis after placing the ligature.

Oxidative stress was reported to be the earliest event during AD as shown in a study that examined postmortem AD brain tissues [118], and it has been shown to even occur before and lead to AD lesions [119]. In addition, throughout the inflammatory process, microglia and astrocytes not only produce cytokines, but also they produce NO^{-}, $O2^{-}$, and H_2O_2 [120]. In a study looking at the oxidative stress arising following a *Pg* infection, four groups of mice were studied: 1) transgenic mice that overexpress *TNF*-α, 2) wild-type mice, 3) *ApoE* knockout sham infected mice, and 4) *ApoE* knockout *Pg* infected mice [121]. The *Pg* infected model showed the highest protein carbonyl content, a biomarker for oxidative stress, in the microvasculature of the hippocampus compared to the other groups, including the transgenic mice group. These findings suggested that oxidative protein damage caused by infection with *Pg* led to a compromise in BBB integrity in this model [121]. Moreover, *Pg* or endothelial microvesicles EMVs shed from human umbilical vein endothelial cells (HUVECs) following Pg infection were found to induce endothelial dysfunction, and this dysfunction was accompanied by oxidative stress [122]. Activating microglia by *Pg*LPS led to an increase in the production of reactive oxygen species, $O2^{-}$, and $O2^{-}$ was reported to compromise the BBB and increase its permeability [123] (Fig. 3C).

Reviewing the effect of *Pg* reveals that it has also been shown to affect endothelial cells of the BBB directly, through the release of the proteolytic enzymes gingipains, which reduces the adhesion of human microvasculature endothelial cells (HMVEC), as well as inducing apoptosis [124] (Fig. 3). Furthermore, gingipains have been shown capable of activating the blood clotting system producing thrombin, which has been shown to increase the permeability of the BBB *in vitro* [125, 126]. Thrombin has also been demonstrated to decrease the expression of ZO-1 and occludin in cultures of rat brain microvascular endothelial cells [127]. Additionally, *Pg* was found to adhere to HUVECs using E-selectin [128], a membrane protein used in the initial capture and recruitment of leukocytes in inflammatory responses [60, 129]. Khlgatian et al. demonstrated that *Pg* invaded HUVECs using fimbriae and provoked the expression of cell adhesion molecules, capable of facilitating leukocyte transendothelial migration [130]. In human coronary artery endothelial cell cultures, it was observed that *Pg* invades the cells to inhibit phagosome formation and function and thus evades the attention of lysosomes [131]. Furthermore, gingipains may play a role in evading the immune response by breaking down receptors responsible for the detection of LPS by host cells [132], which could increase the level of *Pg* circulating the blood. *Pg* was also found to move from one vascular cell to another thus maintaining the inflammatory state [133]. *Pg* LPS was also shown to stimulate the expression of vascular cell adhesion molecule-1 (VCAM-1), in human aortic endothelial cells [134], giving further support to the possibility of leucocyte capture prior to transmigration. One interesting mechanism proposed is the "Trojan horse" (Fig. 3B). This is described as the paracellular pathway, whereby pathogens infect leukocytes and transmigrate the BBB by using infected leukocytes, e.g., macrophages as a "Trojan horse" [135]. Here, bacteria either induce mutations in leukocytes so they possess the ability of translocating through the BBB endothelium, or they infect leukocytes that already possess this characteristic [136]. This might also be utilized by *Pg* to exploit macrophages to get further access to different systems [137]. The potential for *Pg* to target endothelial cells of the NVU via tight junctions and leukocyte adhesion molecules raises the possibility of increasing the inflammatory traffic across the BBB.

Another mechanism by which POD could affect the BBB is by increasing the expression of receptors on endothelial cells, which in turn boost the influx of Aβ from the periphery into the CNS. Zeng et al. (2020) treated cultures of human temporal lobe microvessels (hCMEC/D3) with *Pg* and reported a Cat B dependent increase of the expression of the receptor for advanced glycation end products (RAGE). RAGE was demonstrated to mediate the transportation of $A\beta_{42}$ by seeding hCMEC/D3 on transwell inserts in 24-well plate and measuring the fluorescent signal of $A\beta_{42}$ appearance in the basolateral side of the well [138]. The peripheral Aβ induced by intraperitoneal injections of *Pg* in mice was the central focus of a study done by Nie et al. (2019) whereby the expression of IL-1β, Cat B, $A\beta_{1\text{-}42}$, $A\beta_{3\text{-}42}$, and $A\beta PP_{770}$, the precursor of Aβ

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in the periphery, were found to be increased in the liver-resident macrophages following *Pg* infection [139]. $A\beta_{3\text{-}42}$ has been found to accumulate in greater quantities in the brain of AD patients [140], while remaining absent in healthy specimens [141]. An increase in the expression of Cat B, $A\beta_{1\text{-}42}$, $A\beta_{3\text{-}42}$, and $A\beta PP_{770}$ in the gingiva-resident macrophages was detected in samples of patients with POD [141]. The Zeng group also investigated the role of *Pg* in increasing the influx of $A\beta_{42}$ into the brain following *Pg* infection. They reported an increase of RAGE expression in endothelial cells, immunofluorescent staining, of C57BL/6J WT mice after intraperitoneal injections with Pg, as well as increased signal of $A\beta_{42}$ around the stained endothelial cells [138].

DISCUSSION

The BBB is maintained by the NVU with its cellular components, endothelial cells that adhere to each other by tight junction proteins, pericytes, neurons microglia and astrocytes, and extracellular components, basement membrane, to selectively filter the blood-borne substances and prevent deleterious agents from entering the brain and inflecting any damage. Disruption of any component of the NVU can lead to an impairment of the barrier function and, in turn, harmful agents might cross to the brain. The BBB is disrupted following ischemic stroke, and this impairment takes place immediately after stroke and could last for several weeks [142]. About a third of patients develop dementia three years after the incidence of a stroke [143]. It is worth mentioning that TNF and IL-1β as well as IL-6 are increased in the blood and CSF of human subjects following the onset of a stroke [144]. IL-1β mRNA was found to be increased 42 times in the brain of mouse models 24 hours post stroke, and that of TNF reached a peak of 57.7 folds 12 hours following stroke [145]. On the basis that the overexpression of certain protein, heat shock protein 27, in endothelial cells of the BBB results in the protection of the barrier following stroke, which in turn protects the brain parenchyma [146], it could be concluded that the impairment of the BBB might lead to damaging the brain parenchyma not the other way around.

Researchers have been trying to elucidate the adverse effect of POD on components of the NVU that renders the BBB permeable, which can give rise to AD pathology or contribute to an existent one in the brain.

Clinical studies over the last two decades have provided a growing body of evidence associating POD with cognitive impairment and AD, but no causal link has been established yet. Supporting evidence comes from the detection of periodontal pathogen DNA and virulence factors in the post-mortem brain tissue of patients with AD [59–61]. In addition, correlations have emerged with upregulated genes in the AD hippocampal transcriptome overlapping with genes upregulated during infection, related to immune and inflammatory processes [52]. There are several mechanisms proposed for this association between AD and POD that to date have only been studied in *in vitro* or *in vivo* models: 1) the entry of periodontal pathogens across the BBB may be facilitated by disruption of barriers which occurs during aging and early AD; 2) periodontal pathogens such as *Pg* inflict damage to the CNS indirectly via peripheral inflammation, provoking systemic release of pro-inflammatory molecules which may compromise the integrity of the BBB and contribute to neuroinflammation, and 3) periodontal pathogens cause direct damage to endothelial cells of the BBB through the release of virulence factors such as gingipains secreted by *Pg*; 4) POD and the pathogens associated with it lead to a decrease of tight junction proteins expression in the intestinal barrier and the BBB; 5) periodontal pathogens yield an imbalance of the gut microbiota, which in turn results in increased levels of Aβ in the CNS; 6) periodontal pathogens give rise to oxidative stress, the earliest event in AD, which compromises the BBB; 7) Pg could infect and use leukocytes as 'Trojan horse' to cross the BBB and infiltrate the CNS.

Once *Pg* invades the endothelial cell, it inhibits phagosomes, and evades lysosomes, *Pg* can hijack the endosomal recycling pathway, spreading from one cell to another to propagate inflammation and potentially traverse to the CNS. Proinflammatory cytokines produced contribute to the loss of CNS barrier integrity and disruption of tight junctions which results in barriers becoming more permeable. Neuroinflammation causes the upregulation of leukocytes adhesion molecules expression, which allows leukocytes to transmigrate to the CNS, via the endothelial cells to assist the microglia [102]. The activated microglia and astrocytes then contribute to the developing neuropathology or aggravating an existent one.

However, caution in interpreting the evidence is required. All the mechanistic studies investigating the link between AD and POD have been conducted in *in vitro* non-human or *in vivo* models. Species

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differences exist between immune and inflammatory response to pathogens and neurotoxic Aβ plaques, and two-dimensional mono cell cultures do not reflect the complex signaling that exists between cells of the CNS barriers and basement membrane. For instance, Toll-like receptor 4 (TLR 4), which is one of the receptors expressed by microglia and other cells in the body that allows the binding of LPS to the cells, is not expressed as highly in human microglia compared to rodents [147]. The findings of a recent study by Hayashi et al. (2019) did not support the previous research, as no effect on cognitive skills was observed following continuous intracerebroventricular injection of *Pg*LPS 2 μg/day in 5xFAD mice [148]. They attributed this discrepancy to the low dose of *Pg*LPS compared to other studies that used 1 mg/kg [149]. Results obtained from animal models could not be fully extrapolated to humans because of the various resilience levels exhibited by animals and humans to LPS. For example, the dosage of *Ec*LPS required to promote cytokine synthesis in mice was found to be 1000–10,000 fold of that which led to septic shock in human subjects, and this was attributed to cellular variations between human and mice [150].

Another limitation of current studies is the focus on one pathogen or virulence factor, rather than considering the dynamic and polymicrobial oral microbiome and how it reacts with the host microenvironment [151]. Using *Pg* LPS only and disregarding other virulence factors such as gingipains except for the study by Nonaka and Nakanishi (2020) [152] might have yielded inaccurate results. To elaborate, as shown above, *Pg* LPS resulted in microglia secreting different cytokines such as TNF-α [153], but gingipains were found to cleave the soluble and membrane forms of TNF-α [154, 155]. IL-6 was shown to be produced by microglia after treating with PgLPS [153], but gingipains were reported to inactivate IL-6 as well [156]. Evidently, it is vital to test the effects of all the virulence factors produced by *Pg*.

It has been reported that immunostaining of cyclic adenoses monophosphate (cAMP), the second messenger involved in signal transduction pathways, was increased in the cortical microvasculature, especially the hippocampus, of tissues from AD patients compared to controls, and this upregulation was concomitant with increased staining of Aβ [157]. These findings indicate that cAMP might play a role in the accumulation of amyloid-β protein precursor intracellularly [157]. Moreover, higher concentrations of cAMP were found in the CSF of AD patients compared to controls [158].

However, it has been established that cAMP has numerous effects on the BBB based on its production. cAMP synthesis is mediated by the adenylyl cyclases (AC), which is a family of enzymes that have 10 isoforms, one of which is located in the cytoplasm and the rest are located in the membrane [159]. If the production of cAMP is mediated by transmembrane AC, then the resulted cAMP enhances the endothelial barrier functions. Conversely, the production of cAMP mediated by the soluble AC located in the cytoplasm leads to disruption of the barrier [160]. cAMP was found to increase the expression of tight junction protein claudin-5 in porcine endothelial cells [161]. Additionally, the fall of cAMP production in neurons due to chronic stress leads to compromised function of the BBB [162]. On the other hand, some bacteria can produce cytoplasmic AC, which in turn mediates the generation of cAMP that renders the endothelial cells permeable [163].

With regards to the mechanism of this messenger, high intracellular levels of cAMP result in the activation of protein kinase A (PKA) which in turn leads to the phosphorylation of cAMP-responsive element binding protein (CREB), and the latter provokes the synthesis anti-inflammatory cytokines that promote resolution of inflammation, and activation of macrophages into the M2 phenotype. Additionally, the activity of nuclear factor κB (NF-κB) is decreased, which decreases the production of pro-inflammatory cytokines. Activation of exchange protein directly activated (Epac) by cAMP also inhibits the release of pro-inflammatory cytokines [164]. Also, cAMP contributes to regulating the pro- and anti-inflammatory responses in immune cells [165]. The intracellular increase of cAMP in immune cells plays a role in dampening the anti-inflammatory response by inhibition the production of several pro-inflammatory cytokines such as TNF-α and IL-12 [166, 167]. In addition, the elevation of intracellular cAMP in vascular endothelial cells curbs IL-6 receptor signaling [168]. Roflumilast, a cAMP elevator used for the treatment of peripheral inflammatory diseases, has led to a shift in the activation state of microglia towards the neuroprotective phenotype, M2 [169], and cAMP has inhibited the production of TNF-α from LPS-treated microglia [170].

Recognizing that no *in vitro* model would ever be able to replicate the complexities of the *in vivo* brain microenvironment in humans; cell culture systems have advanced considerably and now some limitations can be addressed and *in vivo*

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extrapolations improved. For example, the use of primary derived human cells is often superior to immortalized cell-lines where phenotypic expression of key proteins is often diminished when compared to primary derived cell cultures. Also, three-dimensional, perfused multi-cellular cell cultures should be considered for future research when cell-cell signaling is such a pivotal part of the propagation of neuropathology. The presence of three-dimensional architecture, sheer stress and co-culture with multiple cell types improves the phenotypic expression of tight-junction proteins and transporters at the BBB. Three-dimensional cultures systems have been shown to have the potential to reproduce different characteristics of the brain physiology in AD disease [171].

It should be noted that there is evidence suggesting a link between POD and other neurodegenerative diseases such as Parkinson's disease (PD), and this evidence stems from several population-based studies. In a retrospective cohort study in Taiwan, POD was found to be accompanied with higher propensity to developing PD when compared with matched controls [172]. Similarly, Jeong et al. reported a weak correlation between POD and PD in a cohort study after adjusting for age, sex, smoking, drinking, exercising, stroke, and depression [173]. This association could be attributed to neuroinflammation, which is a prominent feature of PD [174], and as described in this review, POD has the potential to give rise to it. Moreover, peripheral inflammation is a contributor to PD pathology since the levels of IL-1β, IL-6, and TNF-α are elevated in the periphery during the disease progression [175]. Furthermore, it has been suggested that dysbiosis of the gut microbiota plays a role in PD [176], and as aforementioned here, *Pg* can induce gut microbiota dysbiosis.

CONCLUSION

In summary, the pathogens and virulence factors associated with POD and/or the pro-inflammatory cytokines produced following POD have been shown to potentially affect the BBB and contribute to loss of BBB integrity and disruption of tight junctions, which could be the key to pathogens gaining entry to the CNS. The pathogens possibly target the endothelial cells to propagate inflammation and recruit immune cells, developing neuropathology or aggravating an existent pathology. Research should ideally be conducted in a clinically relevant model, representative of the disease state to assess potential therapeutics for antimicrobial treatment of chronic periodontal disease, dampening of the chronic peripheral and neuroinflammatory state, promotion of clearance of neurotoxins. Ideally this would be conducted in a fully characterized human, multicellular model, representative of the CNS barriers and the associated macrophages.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/21-5103r1).

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An Investigation into the Effects of Outer Membrane Vesicles and Lipopolysaccharide of *Porphyromonas gingivalis* on Blood-Brain Barrier Integrity, Permeability, and Disruption of Scaffolding Proteins in a Human *in vitro* Model

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Abstract.
Background: The effects of the key pathogens and virulence factors associated with gum disease such as *Porphyromonas gingivalis* (*P. gingivalis*) on the central nervous system is of great interest with respect to development of neuropathologies and hence therapeutics and preventative strategies. Chronic infections and associated inflammation are known to weaken the first line of defense for the brain, the blood-brain barrier (BBB).
Objective: The focus of this study is to utilize an established human *in vitro* BBB model to evaluate the effects of *P. gingivalis* virulence factors lipopolysaccharide (LPS) and outer membrane vesicles (OMVs) on a primary-derived human model representing the neurovascular unit of the BBB.
Methods: Changes to the integrity of the BBB after application of *P. gingivalis* LPS and OMVs were investigated and correlated with transport of LPS. Additionally, the effect of *P. gingivalis* LPS and OMVs on human brain microvascular endothelial cells in monolayer was evaluated using immunofluorescence microscopy.
Results: The integrity of the BBB model was weakened by application of *P. gingivalis* LPS and OMVs, as measured by a decrease in electrical resistance and a recovery deficit was seen in comparison to the controls. Application of *P. gingivalis* OMVs to a monoculture of human brain microvascular endothelial cells showed disruption of the tight junction zona occludens protein (ZO-1) compared to controls.

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Conclusion: These findings show that the integrity of tight junctions of the human BBB could be weakened by association with *P. gingivalis* virulence factors LPS and OMVs containing proteolytic enzymes (gingipains).

Keywords: Alzheimer's disease, blood-brain barrier, human brain microvascular endothelial cells, *in vitro* BBB model, microbiome, periodontal disease

INTRODUCTION

The concept that microbial infections may be a risk factor for neurodegenerating disease such as sporadic Alzheimer's disease (AD) has gathered momentum in the past decade. Evidence has emerged from epidemiological, postmortem, and experimental studies suggesting that microbials potentially linked to AD may be viral, bacterial, or fungal in origin [1–4]. There are multiple risk factors for developing sporadic AD, the most significant being advanced age [4]; however, the possibility of a microbial role as a causative factor for AD opens new possibilities for the discovery of novel preventative measures and therapeutic targets. Pivotal to the discovery of such novel drug targets for AD is the need to create experimental models that are representative of human physiology and disease state.

AD is a chronic neurodegenerative condition which develops over decades, displaying histological hallmarks of extracellular amyloid-β (Aβ) plaques and hyperphosphorylated intracellular tau tangles within the brain parenchyma [5]. AD can also be considered as a chronic inflammatory disease and has been linked to inflammatory events such as traumatic brain injury or vascular disease, with subsequent activation of the immune system and release of inflammatory mediators to protect the brain [6]. Individuals may be predisposed either through age, genetics, illness, or lifestyle habits (smoking, diet, exercise) [7] and then experience a persistent inflammatory trigger, which can establish a chronic inflammatory reaction with devastating toxic effects at a cellular level [8] and even initiating AD pathology [6].

The notion that Aβ can behave as an antimicrobial peptide [9, 10] raises the possibility that an external, chronic assault on brain tissues, for example infection by the oral periodontal disease bacteria *Porphyromonas gingivalis* (*P. gingivalis*), could cumulatively damage brain tissue and lead to low level neurodegenerative changes decades before a clinical diagnosis of AD presents [8]. Researchers have suggested that antibodies against external pathogens cross react with Aβ binding to the pathogens, inducing autoimmune complexes and reactivity via a molecular mimicry mechanism [11]. This could potentially weaken the anti-microbial effect of Aβ, cause Aβ plaques, and allow further pathogenic influx. Voidani et al. (2018) found that monoclonal antibodies to $A\beta_{42}$, tau protein, and α-synuclein crossreacted to 25 different pathogens associated with AD, but no reaction was seen to *P. gingivalis* ATCC 33277. This highlights the importance of investigating *P. gingivalis* virulence factors, such as lipopolysaccharide (LPS) and outer membrane vesicles (OMVs), as these molecules could be more significant than the bacterium [12].

Periodontal disease (PD) is a chronic infection caused by bacteria in the gums around teeth represented by microbial dysbiosis and tissue destruction [13, 14]. PD has been linked to other organ specific disease such as AD, atherosclerosis, and diabetes mellitus [15, 16]. The progression of PD in humans is determined by microbiological, environmental, and genetic factors (multiple polymorphisms) [17]. PD can go undiagnosed for years and even if oral hygiene measures are improved, there is a potential of daily bacteremia of periodontal pathogens when chewing foods or cleaning teeth [18]. It is this chronic bacterial load in the circulation which is proposed to contribute to a chronic systemic inflammatory state.

P. gingivalis, an anaerobic Gram-negative coccobacillus, is a key pathogen of PD and has numerous mechanisms which can affect the surroundings tissues including 1) endotoxin or LPS mainly located on the outer membrane and 2) via release of OMVs by the Type IX secretion system. It is hypothesized that circulating periodontal pathogens and/or associated virulence factors could lead to disruption of the integrity of the blood-brain barrier (BBB). While there is some evidence to support this concept, *P. gingivalis* cells have not yet been found in the brain of AD patients or clinical models [15]; however, postmortem studies have found *P. gingivalis* DNA, virulence factors, LPS, and proteases secreted by *P. gingivalis* (gingipains), in the human brains of individuals with AD [19, 20]. These findings were supported by an investigation of patients

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with Crohn's disease and ulcerative colitis showing that significantly elevated LPS antibodies correlated with elevated gut barrier and BBB protein antibodies, indicating the systemic disruption of LPS on the BBB and GI tract integrity compared to healthy controls [21]. *In vivo* animal studies, investigating the administration of *P. gingivalis* associated virulence factors have shown that these substances settle in the animal's brain [22, 23]. Illievski et al. (2018) [23] showed that infection of mice with *P. gingivalis* induced neuroinflammation and appeared to induce the deposition of intracerebral Aβ protein, similar to the human AD pathology. It remains unclear, however, whether Aβ in the brain was due to a direct cerebral invasion of intact *P. gingivalis*, the virulence factors such as gingipains or an indirect effect from the inflammatory mediators of systemic infection [23]. It is proposed that two pathways for *P. gingivalis* induce neurodegenerative changes, either 1) *P. gingivalis* can access the brain directly or 2) the bacteria can orchestrate the neurological changes from a distant site of infection, such as the periodontal pockets in the oral cavity. The key question remains if or how *P. gingivalis* and its virulence factors cross the BBB and access brain tissue?

P. gingivalis and associated virulence factors

The virulence or invasive ability of a *P. gingivalis* strain can be classified according to the expression of fimbriae, capsule, LPS, and gingipains release [24]. These virulence factors originate from the mother cell but are disseminated wider by release of OMVs. For example, the non-capsulated laboratory strain FDC 381 has been shown to invade carcinoma cells 10^3 times more than other *P. gingivalis* strains [24], though FDC 381 is classed as a less virulent type causing only mild localized abscesses [25]. These findings highlight two key aspects of *P. gingivalis* pathogenic behavior, the ability of the bacterium to invade tissues and modulate the subsequent immune response of the host. Furthermore, a variance between different invasive abilities of OMVs from *P. gingivalis* strains has been attributed to the expression of long fimbriae (not FimA) and the gingipain adhesive domains in the outer membrane [26]. OMVs of *P. gingivalis* are highly enriched in the proteolytic gingipains (RgpA/B and Kgp) and the nano sized spheres also incorporate high concentrations of LPS and fimbriae [27], which are sustainable in human tissues such as the brain [28]. *P. gingivalis* OMVs, containing a high concentration of enzymes, are considered to have both harmful and beneficial roles, enabling *P. gingivalis* to regulate its microenvironment [29]. Internalized OMVs are associated with cell degradation [30] and induction of an innate immune response with a greater intensity than that initiated by the bacteria itself. Gingipains are also believed to help *P. gingivalis* evade the reach of the immune system, making *P. gingivalis* so successful in establishing a chronic diseased state [31].

P. gingivalis has also been shown to dysregulate dendritic cells by disturbing autophagy and apoptosis [32] endowing this pathogen with an exceptional ability for self-preservation. Labelled *P. gingivalis* OMVs have also been shown to be taken up by cortical microglial cells in mice, 24–48 h after peripheral injection highlighting the potential reach of this virulence factor. It has even been suggested that OMVs may act as a decoy to the host immune system, diverting attention and thus protecting the mother cell from elimination [33], forming another element of immune evasive strategy of *P. gingivalis*. The size and proteolytic capacity of the OMVs makes the dispersal into tissues easier than for the intact bacteria and OMVs are more likely to survive transport to remote organs.

LPS from the outer membrane of Gram-negative bacteria is a powerful pro-inflammatory pathogen associated molecular pattern and previous studies support the capabilities of oral bacterial LPS as an inducer of peripheral inflammatory responses and as an initiating factor in intracerebral inflammatory activity [19, 23]. The LPS of *P. gingivalis* has been extensively studied in relation to its pathogenicity and is found in both a soluble and membrane bound form and bound to TLR4 enhanced by sCD14 [34] activating pro-inflammatory pathways. *P. gingivalis* LPS is differently recognized by mouse and human TLR4 receptors both *in vitro* and *in vivo*, only weakly inducing proinflammatory cytokine secretion in mouse models and therefore caution should be taken when extrapolating data from mouse systems exposed to *P.gingivalis* or its LPS to humans [34].

Human blood-brain barrier

The cells of the BBB, also described as the neurovascular unit (NVU), comprise of endothelial cells, pericytes, astrocytes, and neurons [35]. The total length of capillaries in the human brain is 400 miles [36], which makes this the largest potential entry point for pathogens to the central nervous system (CNS), but its intimate integrity affords a significant barrier. This integrity arises from endothelial cell 1)

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intercellular tight junctions displaying high electrical resistance, limiting any transcytosis compared to peripheral endothelial cells [37], 2) lack of fenestrae (transcellular pores), and 3) shared basement membrane with pericytes with reduced pinocytic activity. Human *in vitro* BBB models are used to investigate both disease and drug interactions of this interface providing a very valuable tool to assess permeability, transport and integrity via transendothelial electrical resistance (TEER) measurements, as well as expression of proteins [38]. The benefits of using a human primary-derived cell-based model with combinations of cells of the NVU are numerous (for a comprehensive review, see [38]).

The integrity of the BBB is reduced with age [39], and multiple neurological conditions, including AD and Parkinson's disease, have been associated with a "leaky" BBB [37] and raised level of systemic pro-inflammatory cytokines thought to weaken the barrier integument [40, 41]. This implies the importance of the role of the BBB in brain homeostasis and risk of age-related neurodegeneration [15].

Studies in mouse apolipoprotein E (ApoE) knock-out models [42] and humans who express the E4 isoform of *APOE4*, the most prevalent predisposed genetic risk factor for AD, also show accelerated breakdown of the BBB structure and degeneration of brain capillary pericytes required for barrier integrity [43].

CNS permeation by P. gingivalis and associated virulence factors

The journey for *P. gingivalis* and associated virulence factors, from the periodontal pocket to the CNS could follow a number of routes, such as tracking along the trigeminal or olfactory nerves [44], internalization to the CNS by peripheral immune cells, or arriving in the systemic circulation at the BBB or the blood-cerebrospinal fluid barrier [45].

Both Gram negative and positive bacteria can cross at the BBB and blood-cerebrospinal fluid barrier interfaces to the CNS by transcytosis. Bacteria such as *Neisseria meningitidis* are able to open endothelial intercellular junctions to cross the CNS barriers in acute infection [45], and *P. gingivalis* gingipains have been shown to degrade the epithelial JAM-1 protein [46] and induce cell adhesion molecule cleavage and apoptosis in human microvascular endothelial cells [47]. *P. gingivalis* has also been demonstrated to induce apoptosis and tight junction disruption in cultured human lung epithelial cells [48]. It is not clear however, whether these findings were caused by the bacteria or virulence factors.

Neuroinflammation

If *P. gingivalis* was shown to induce damage to the BBB of an individual, some time before any neuroinflammation becomes clinically detectable, then the authors propose that bacteria or associated virulence factors must be capable of damaging the BBB enough to trigger a change either to its integrity allowing an influx of inflammagens and/or weakening the clearance strategies of the barrier. If the initial causal factor for the pro-inflammatory state is not resolved, then any subsequent effects of chronic oxidative stress on the NVU cells could lead to loss of redox balance, alterations in numbers and differentiation of T-cells subpopulations and subsequent loss of regulation of the neuroinflammatory response [49]. Animal studies for example have demonstrated that LPS can incite oxidative stress, activate glial cells and degrade tight junctions in the NVU and surrounding cells [50, 51].

Though much research has been undertaken to understand the events at the BBB in individuals with neuropathological diseases [50–52] and the effects of *P. gingivalis* and its virulence factors on tissues, very little is known about what effect this bacterium and its virulence factors exert on the cells of the blood-brain interface especially in the pre-clinical stages.

The authors thus pose the question, could a persistent level of *P. gingivalis* virulence factors in the circulation, renewed daily by chewing or tooth brushing [18] be sufficient to cause damage to the BBB in an otherwise healthy state?

The aim of this study was to investigate how the *P. gingivalis* virulence factors LPS and OMVs affected primary human cells in an *in vitro* BBB in the absence of the bacterial mother cell. This was assessed by investigating 1) BBB integrity by TEER across the barrier, 2) how barrier permeability to fluorescent labelled LPS and dextrans was altered by the presence of *P. gingivalis* LPS and OMVs, and 3) how LPS and OMVs interacted with the human brain microvascular endothelial cells.

METHODS

BBB model

Primary-derived cell lines of human brain microvascular endothelial cells (HBMECs) (Neuromics

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USA) at passage 3, human brain vascular pericytes (HBVP) (ScienCell, USA) at passage 3 and human astrocytes (HA) cells (ScienCell, USA) at passage 3 were grown in flasks pre-coated with either AlphaBioCoat solution (Neuromics, USA) or Poly-L-Lysine 10 mg/ml (Sciencell, USA). Cells were cultured in complete endothelial cell growth basal medium (EBM) (Lonza, Switzerland), complete pericyte medium (PM) (Sciencell, USA) and astrocyte basal medium (ABM) (Lonza, Switzerland) with addition of human serum (Life Science Group, UK). The cells were grown to a confluency of 85–90% in humidified incubator at 37°C, 5% CO_2. The media was changed in all culture vessels every 48 h until 50% confluent after this point every 24 h. Trypsin (TrypLeexpress, Gibco, Thermofisher, USA) and Hank's Balanced Salt Solution (Gibco, Thermosfisher, USA) were used for passaging cells. All observations of the cells were carried out under an inverted light microscope (Leica DMIL light microscope from Leica Microsystems GmbH, Germany). The viability and counting of the cells were assessed by using 1:1 Trypan Blue 0.4% (Sigma-Aldrich) and a haemocytometer [53, 54]. Transwell™ multiple well plate with permeable polycarbonate membrane inserts (6.5 mm, 8.0 μm pore) (Corning, Fisher, UK) were coated with fibronectin (Sigma, UK) The HA and HBVP were seeded on the basolateral side of the insert and the HBMEC on the apical side [53]. The cells were maintained with medium of equal volume of PM, ABM and EBM (Lonza, Switzerland and Sciencell, USA) in a 37°C humidified incubator under 5% CO_2.

BBB integrity

After 4 days, the integrity of the barrier model was tested by measuring the TEER with an EVOM-2 instrument (WPI, UK). As the barrier becomes established, the TEER value rise expressed in Ohm/cm^2 [55]. The triculture *in vitro* BBB model was considered ready for testing when the TEER values reached an average of 260 Ohm/cm^2 [53, 56]. The triculture barrier integrity were also assessed by application of FITC dextran 3–5 kD, which was added to the apical compartment of the inserts and incubated for specific time periods according to the test protocols in the 37°C humidified incubator under 5% CO_2. At the specified time points in the test protocol samples were removed from the basolateral compartment and measured in a GENios Pro plate reader (Tecan, Austria) at 490 nm excitation and 520 nm emission (gain 40, 22°C). Standard curves were produced from standard solutions of FITC 3–5 kDa and FITC-conjugated LPS in the range 0.04 to 100 μg/ml.

Culture of bacteria

P. gingivalis ATCC-BAA-1703 (strain FDC 381) was purchased from LGC limited (UK) in freeze dried vials. The bacteria were cultured according to the supplier's instructions. Briefly the *P. gingivalis* FDC 381 were cultured in ATCC medium 2722, supplemented tryptic soy broth (TSB) (TSB 3%, Yeast extract 0.5%, L-cystein hydrochloride 0.05%, Hemin (5 mcg/ml) with K_2HPO_4, Vitamin K1 (1 mcg/ml) (Sigma Aldrich, UK)) on TSA with 5% sheep blood (Thermo Scientific, UK) and FAA agar with 7% horse blood Neomycin (75 mg/l) (E and O, UK). All cultures were incubated at 37°C in an anaerobic chamber (Bactron, USA) using an anaerobic gas mixture of 5% H_2, 5% CO_2, and 90% N_2. See Supplementary Figure 1 for an example. The cultures were grown for 3 days. The optical density (OD) of the broth cultures were measured daily and selected for use when between OD_{655} 0.1 and 1 [57]. The cultures were Gram stained and imaged for quality control daily to ensure monoculture samples utilizing a Gram staining kit (Merck, UK) and inverted light microscope using a X40 and X100 objective (Leica DMIL, Germany) and a Nicon DS-L4 camera and software.

Isolation of outer membrane vesicles of P. gingivalis

The outer membrane vesicles of *P. gingivalis* FDC 381 were isolated following the protocol used by Seyama et al. (2020) [57]. The bacterial culture in TSB was centrifuged at 2800×g for 15 min at 4°C to separate the vesicles from the bacterial cells. The supernatant was passed through a 0.2 μm syringe filter (Millipore, UK) and then concentrated to under 1 mL by using an Ultra-15 Centrifugal Filter for the nominal molecular weight limit (NMWL) 100K (Sigma-Aldrich, UK). The concentrate was mixed with total exosome isolation reagent for culture (Life technologies, UK) and this was incubated at 4°C overnight. The samples were centrifuged at 10,000×g for 60 min at 4°C. The vesicles were eluted in 100 μL X1PBS. The TSB without bacteria was treated by the same method as a negative control. The diameter of the outer membrane vesicles was measured and mono-dispersity ensured using a Zetasizer (Malvern Zetasizer Nano, Panalytical

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Instalment Ltd., UK) [58] and the concentrations of the samples was measured on the NanoDrop Spectrophotometer (280 nm) (Nanodrop 2000, Thermo Scientific, UK).

BBB response to P. gingivalis virulence factors

The *in vitro* BBB model response to virulence factors of *P. gingivalis* was tested by incubations with various concentrations (0.1 μg/ml, 0.3 μg/ml, 1 μg/ml, 10 μg/ml, 50 μg/ml, and 100 μg/ml) of *P. gingivalis* LPS (Invivogen, France) and *P. gingivalis* outer membrane vesicles (OMV) and 1 μg/ml, 10 μg/ml, 50 μg/ml, and 100 μg/ml of *P. gingivalis* LPS-FITC conjugate (Nanocs, USA). An experiment was also conducted with application of *P. gingivalis* LPS-FITC conjugate (Nanocs, USA) in combination with OMVs at 10 μg/ml. Control wells included no treatment (cell and media alone) and a cell blank (fibronectin insert, no cells). All measurements were made from each well five times (TEER) or three times (Permeability, Papp), each plate had three wells (intraassay variability check). The test samples were diluted in complete medium of equal measures of EBM, PM, and ABM. The test samples were placed in the apical compartment of the transwell and the TEER was measured at set time points (0.5, 1, 2, 4, 24, 48, and 72 h). At each time point the TEER values of the wells were measured 5 times and triplicate samples were collected from the basolateral compartment.

Apparent permeability of the BBB to P. gingivalis virulence factors

Samples from the basolateral side of the BBB model were measured in the GENios Pro plate reader (Tecan, Austria) at 490 nm excitation and 520 nm emission (gain 40, 22°C) [55], to quantify the appearance of fluorescent labelled LPS or FITC-dextran. The appearance from the apical to the basolateral compartment was calculated from standard curves of known concentrations of both FITC- dextran (3–5 kDa) (Sigma-Aldrich, UK) and the *P. gingivalis* LPS-FITC conjugate (Nanocs, USA) and the data from the standard curves were used to calculate the permeability (Papp) values in each experiment as shown in Equation 1.

$$P_{app} = \left(\frac{V}{A \times C_0}\right) \times \left(\frac{dQ}{dt}\right) \qquad (1)$$

where:

V = Volume of basolateral compartment ($V = 0.6\,cm^3$)
A = surface area of the polycarbonate membrane ($0.3\,cm^2$)
C_0 = Initial concentration of the *P. gingivalis* LPS-FITC conjugate or FITC-Dextran in the apical well
dQ = concentration of *P. gingivalis* LPS-FITC conjugate or FITC-Dextran collected from the basolateral part (μg/ml) (passing across the cell layer to basolateral side).
dt = Change in time (s)

Human IL6 ELISA

As a quality assurance measure to check all virulence factors (LPS, LPS-FITC conjugate, and OMVs) at 100 or 200 μg/ml were capable of producing an inflammatory response, all test reagents were evaluated for inflammasome activity by incubation with HBVP for 4 h in triplicate and the spent cell culture media was assayed for human IL-6 release using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, USA). Test samples (spent media), controls (sample diluent buffer), and human IL-6 standards (100 μL) were added to the precoated 96 well ELISA plate. This was incubated at 4°C for 24 h with gentle shaking. After removal of the samples the wells washed with X1 wash buffer four times. Biotinylated detection antibody was added and incubated for 1 h at room temperature with gentle shaking. The wells were washed four times with X1 wash buffer and HRP-streptavidin solution was incubated for 45 min at room temperature with gentle shaking. After washing four times with X1 wash buffer, ELISA colorimetric TMB reagent was added. This was incubated for 30 min at room temperature covered to exclude light while gently shaking. Then stop solution was added and the absorbance was measured in the 96-well plate immediately at 450 nm (Tecan, Austria). A standard curve was produced, the concentration of IL-6 in the samples was calculated.

Immunofluorescent imaging of P. gingivalis virulence factors interactions with tight junction proteins of HBMEC cells

HBMECs (Neuromics, USA) were seeded at a density of 250000 cells/ml in black, tissue culture

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treated 24-well μ-plates (IBIDI at Thistle scientific, UK) and grown in EBM (Lonza, Switzerland) in a 37°C humidified incubator under 5% CO_2 for 8 days. The cells were tested for viability with Trypan Blue (Sigma-Aldrich, UK) and by visual daily inspection. On day 7 the cells were treated with 0.1 μg/ml and 0.3 μg/ml of unconjugated *P. gingivalis* LPS (Invivogen, France) or *P. gingivalis* outer membrane vesicles (OMV) diluted in EBM (Lonza, Switzerland) and incubated for 24 h. After this incubation period the cells were fixed for IF protocol described below.

After incubation with the test samples the cells were washed in x1 PBS and fixed with 4% formaldehyde, washed and permeabilized with x1 PBS and 0.1% Triton-X (Sigma- Aldrich, UK) and blocked with 20% normal goat serum (Stratech, UK) in 1x PBS with 0.1% Triton-X for 60 min. The cells were incubated with the primary antibody ZO-1 (D6L1E) Rabbit mAb (1:400) (Cell signaling, NL) at 4°C for 12 h and shaking and the secondary antibody Cy™5 AffiniPure Goat Anti-Rabbit IgG (1:800) (Jacsonimmuno, USA) for 1 h at 4°C. The cells were counter stained with DAPI (1:3500) (Stratech, UK) and imaged in a Zeiss Cell Observer system featuring the Zeiss definite focus, Colibri LED illumination and AxioVision 4 digital image processing software (Carl Zeiss Microscopy, Germany) detecting the signal for DAPI at ex:358 nm em:463 nm and Cy5 ex:646 nm em:664 nm. The images were viewed and processed using Zen 2.3 Lite software.

Statistical analysis

The TEER and the permeability (Papp) data obtained from the *in vitro* BBB model was tested for homogeneity of variances and normality using the Shapiro-Wilk test. Difference between treatment groups was analyzed using an ANOVA with Dunnett's *post-hoc* analysis or an independent *t*-test for comparison between independent experiments. The analysis was performed using the Statistical Package SPSS Version 26 and 27 (IBM, USA). Statistical significance was defined when $^{*}p < 0.05$, and highly significant when $^{**}p < 0.01$ and $^{***}p < 0.001$.

RESULTS

Baseline variability and modelling TEER changes in the BBB model

The baseline of the experimental model and optimization was carried out to establish whether the primary cells and the 3-layer model were suitable for the planned experiments and not affected by the presence of the tracer compound. Prior to the start of the experiment, TEER values were consistent from day 7, with a typical variation of $\pm 10\ \Omega/cm^2$ over a 4-h time period. Upon addition of either unconjugated *P. gingivalis* LPS (test wells), media (blank wells), or FITC (control wells) to the apical side of the BBB, there was a small initial dip in TEER at the start of the experiment that was attributable to movement artefact and slight disturbance of the BBB in all wells, typically this was $\pm 25\ \Omega/cm^2$ and recovery to baseline was observed in the control wells within 2 h. The response of TEER in test wells upon addition of unconjugated LPS often showed a lower drop in TEER and that did not always recover to pre-baseline TEER. To determine whether the change in TEER was significantly different from baseline variation or movement artefacts, the pattern of response was modelled and the magnitude (ΔTEER), recovery time and rate of change were defined as shown in Fig. 1 and were then used for statistical comparison between the control and test wells at different LPS concentrations.

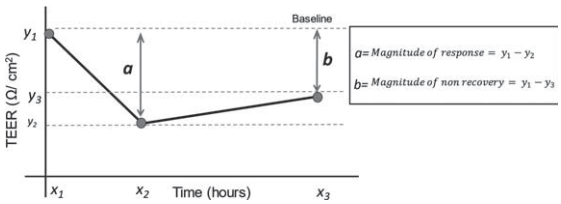


Fig. 1. Modelling the magnitude and rate of change in TEER after application of test sample to the *in vitro* BBB model.

Confirmation of inflammatory response of virulence factors tested, measured by human IL6 ELISA

In order to show that the virulence factors applied to the BBB model (unconjugated *P. gingivalis* LPS, FITC *P. gingivalis* LPS, and *P. gingivalis* OMVs) had a biological response all samples were tested for induction of IL6 in HBPC prior to use. The OMVs isolated from *P. gingivalis* grown in TSB were suspended in PBS and the concentration was measured on the nanodrop 2000 (Protein A280) (Thermo Scientific, UK). The samples ranged from 0.4–1.8 mg/ml. The OMV samples particle size was assessed by dynamic light scattering analysis (DLS) in a Zetasizer (Malvern Panalytical, UK). The OMV samples showed variations in particle sizes (sample heterogeneity) within the expected range (20–500 nm) for

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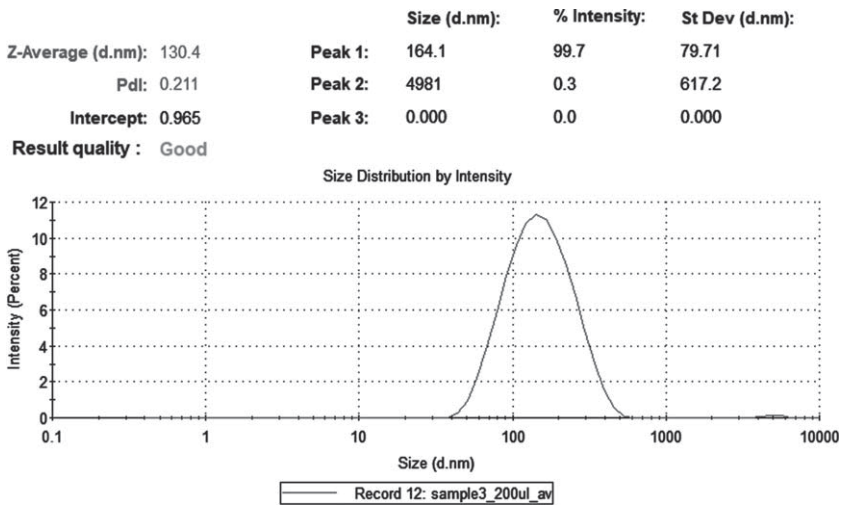


Fig. 2. Zetasizer reading of particle size in *P. gingivalis* OMV isolated from broth culture. The data shown is the average of 3 readings ($n = 3$).

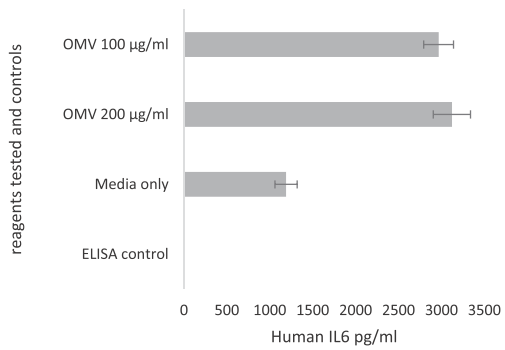


Fig. 3. Levels of IL6 secreted by HBPC after incubation with *P. gingivalis* OMV and PM only. The data shown is mean ± SD. ($n = 9$).

FDC 381 [12, 59] (Fig. 2) and test samples for cell application were selected because of monodispersity with a polydispersity index (PdI) ≤ 0.323 [58].

The inflammatory response of HBPC cells was measured following exposure to unconjugated *P. gingivalis* LPS, FITC *P. gingivalis* LPS conjugated, and *P. gingivalis* OMVs (100 or 200 µg/ml) after 4 h of co-incubation, negative controls were media only. A standard curve was prepared and used to determine the concentrations of IL6 secreted by the cells in the test wells. This test was repeated every time a new conjugate reagent was used for the first time in triplicates. The results showed an elevated level of IL6 in the test samples compared to controls (Fig. 3). These results were seen as a positive control of the virulence activity in the samples tested.

Effect of unconjugated P. gingivalis LPS on BBB integrity

The *in vitro* model was tested with unconjugated *P. gingivalis* LPS to assess the barrier response to this virulence factor. Application of unconjugated *P. gingivalis* LPS to the BBB caused a significant decrease in TEER for 0.3 µg/ml ($p \leq 0.05$), 10 µg/ml ($p \leq 0.05$), and 100 µg/ml ($p \leq 0.05$) when compared the magnitude of change to the control well, where FITC alone or media alone were administered (Fig. 4A). The magnitude of recovery of TEER values determined as the maximum TEER measured during the recovery phase. For all wells treated with unconjugated *P. gingivalis* LPS, there was still a deficit in recovery of the BBB integrity compared to the pre-incubation phase, as indicated by the deficit in TEER 72-h post incubation relative to the baseline at time zero. The magnitude of deficit in TEER was significantly greater in the test wells where unconjugated *P. gingivalis* LPS was applied at 0.3 µg/ml ($p \leq 0.05$), 10 µg/ml ($p \leq 0.05$), and highly significant with 100 µg/ml ($p \leq 0.01$) compared to the control wells where FITC-alone was applied to the BBB (Fig. 4B).

The integrity of the *in vitro* BBB model was also assessed by testing the wells with FITC-Dextran 3–5 kD as a marker of tight junction permeability. After incubation with unconjugated *P. gingivalis.* LPS or media (blank) for set time points. FITC-Dextran 3–5 kD was added to the wells and the fluorescent appearance of FITC-dextran on the

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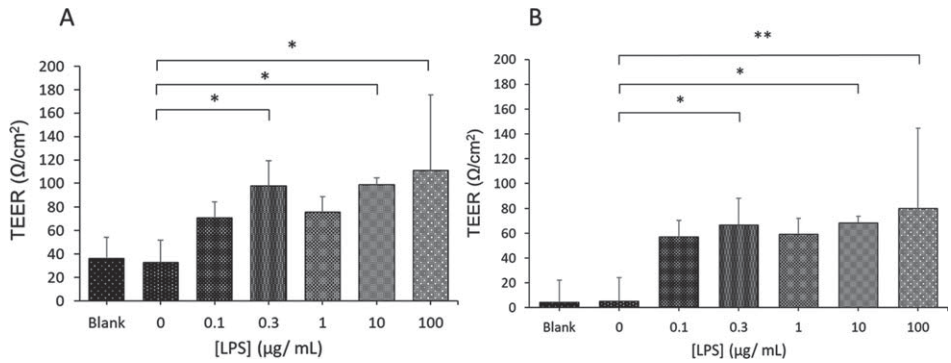


Fig. 4. Changes in BBB integrity measured by calculating the magnitude of decrease in TEER in response to application of unconjugated *P. gingivalis* LPS (A) and the magnitude of deficit in recovery of TEER 72-h post application of unconjugated *P. gingivalis* LPS relative to initial baseline TEER (B). Statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone) where $^{*}p < 0.05$ and $^{**}p < 0.01$. Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

basolateral side of the BBB was measured. It was observed that the FITC-Dextran appeared earlier in the wells with 10 and 100 μg/ml of unconjugated *P. gingivalis* LPS following pre-incubation; however, this was not statistically significant relative to the blank wells and no significant concentration dependent effect in *P. gingivalis* LPS treatment related to FITC-dextran appearance was observed for the complete test period. The percentage appearance of FITC-dextran appeared to increase following longer exposure (24–72 h) to *P. gingivalis LPS*, as shown in Fig. 5C–E.

The apparent permeability (Papp) of FITC-dextran (100 μg/mL) after incubation for 30 min was calculated for three time points (60, 120, and 240 min) as described previously. Final Papp values at 60 min, 120 min, and 240 min were found to be $1.04 \times 10^{-8} \pm 2.3 \times 10^{-8}$ cm/s, $8.7 \times 10^{-8} \pm 1.7 \times 10^{-7}$ cm/s, and $4.8 \times 10^{-8} \pm 4.7 \times 10^{-8}$ cm/s.

Effect of FITC-conjugated P. gingivalis LPS on BBB integrity

To investigate potential transport across the *in vitro* BBB model, a FITC labelled *P. gingivalis* LPS was applied to the established model and appearance of the conjugate was measured with the model's integrity. It was shown that there were no significant differences in the magnitude of TEER response between the wells with application of all concentrations of FITC-*P. gingivalis* LPS conjugate and the control (FITC alone); however, a decrease in TEER was observed in all wells after application of 1,10,50 and 100 μg/ml (Fig. 6A). These wells did not appear to recover as well compared to controls (Fig. 6B).

The appearance of the FITC *P. gingivalis* LPS was greatest in the highest concentration (100 μg/ml) at 1 and 4 h (Fig. 7A, B), and an increase of percentage appearance was observed with the concentrations 10 and 50 μg/ml as the experiment progressed at 24 and 48 h (Fig. 7C, D). The percentage appearance of the FITC *P. gingivalis* LPS in the basolateral compartment of the model did not exceed 5% during the duration of the experiments. A drop in the TEER values were seen to correlate with the appearance of the conjugate in all the wells of the higher concentrations (50 μg/ml and 100 μg/ml LPS) (data not shown). All the wells were tested with FITC-Dextran at the end of each experiment to assess the final integrity of the barrier (data not shown).

Effect of P. gingivalis OMVS on BBB integrity

The application of *P. gingivalis* OMVs showed similar patterns to the LPS study. The magnitude of decrease in TEER observed in response to treatment with OMVs was significantly different from the control group for the 0.1 μL/mL ($p < 0.01$); 0.3 μL/mL ($p < 0.05$); 50 μL/mL ($p < 0.05$), and 100 μL/mL ($p < 0.01$) (Fig. 8A). This decrease in TEER did not recover to pre-treatment baseline for the wells treated with 50 and 100 μL/mL *P. gingivalis* OMVs as the magnitude of deficit was highly significantly different to the control group ($p < 0.001$), as shown in Fig. 8B.

Figure 9 shows the appearance of FITC-Dextran permeation following incubation of the *in vitro* BBB with increasing concentrations of *P. gingivalis*. OMV

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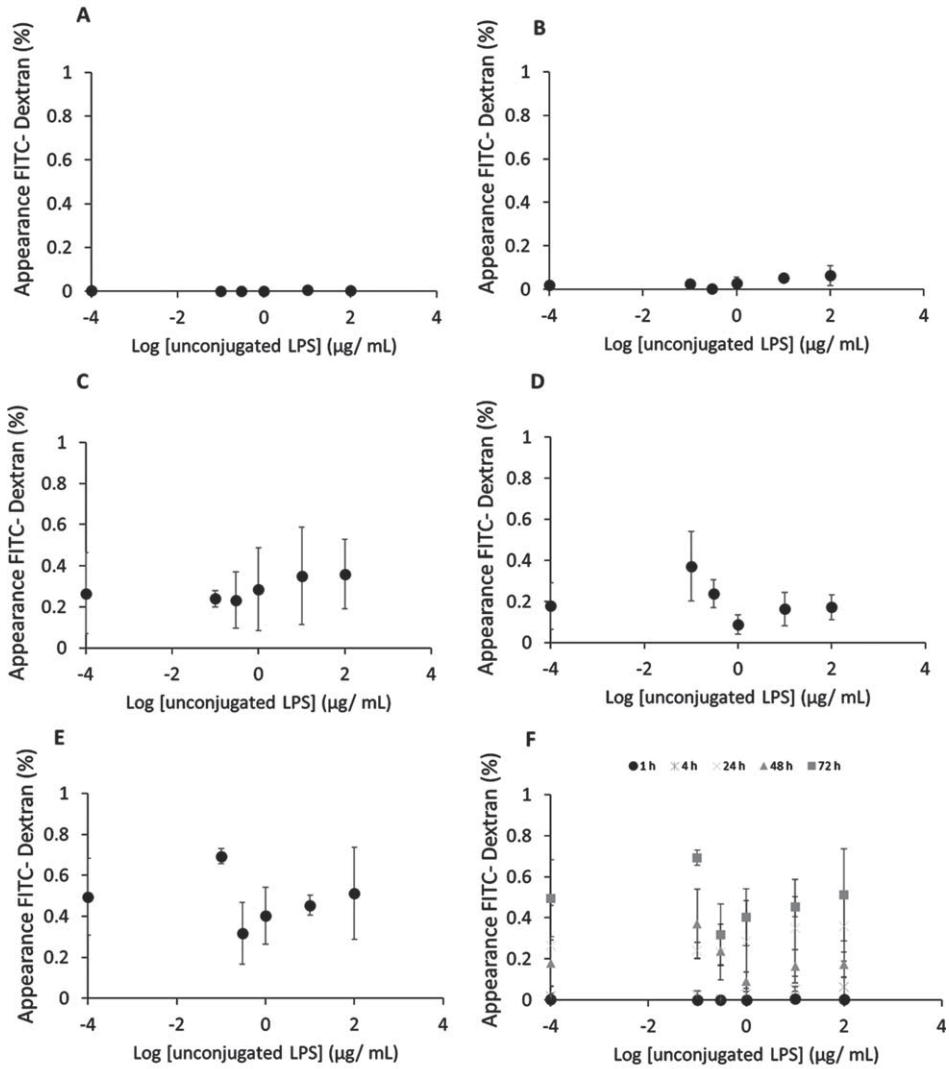


Fig. 5. Shows the percentage of FITC-dextran (3–5 kDa) permeating through the *in vitro* BBB after incubation with increasing concentrations of unconjugated *P. gingivalis* LPS (0–100 µg/mL) for 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 h (E) and all exposure times compared together (F). Each data point represents mean ± SD from three wells and two experimental repeats ($n = 6$).

exposed for varying durations. Similar to the unconjugated LPS, the effect of OMV treatment on the extent of FITC-dextran permeation was fairly constant after the 24-h exposure, but this time the permeation did appear to increase as the concentration of OMV increased (Fig. 9C-E).

Effect of P. gingivalis OMV and LPS on BBB integrity

The *in vitro* BBB model was tested with FITC conjugated *P. gingivalis* LPS in the presence of a constant concentration of OMV to assess the effect the OMVs could potentially have on the appearance of the conjugated LPS in the basolateral compartment. The concentration of OMV was chosen (10 µg/ml) as this was the lowest which showed an effect in the BBB models integrity in the OMV only experiment previously (Fig. 9D). The controls for the combined experiment were media only and OMV (10 µg/ml) only. The data was compared to the previous experiments with FITC *P. gingivalis* LPS only (Fig. 7). The application of *P. gingivalis* LPS-FITC conjugate in conjunction with 10 µg/ml *P. gingivalis* OMVs showed a similar pattern in terms of response in the BBB model as seen in the previous experiments.

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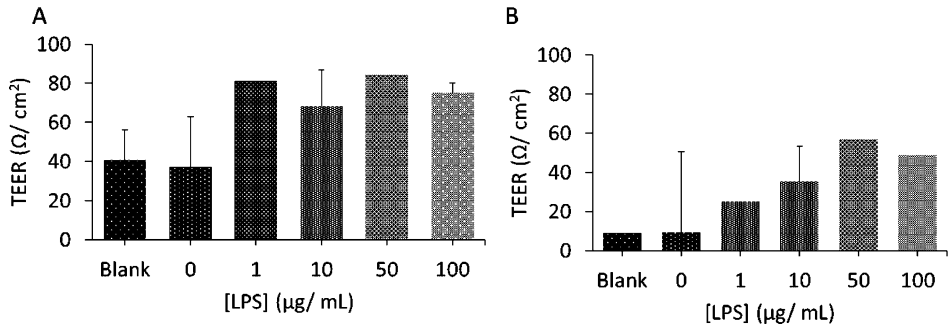


Fig. 6. Changes in BBB integrity measured by calculating the magnitude of decrease in TEER in response to application of conjugated FITC-*P. gingivalis* LPS (A) and the magnitude of deficit in recovery of TEER 72-h post application of conjugated FITC-*P. gingivalis* LPS relative to initial baseline TEER (B). No statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone). Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

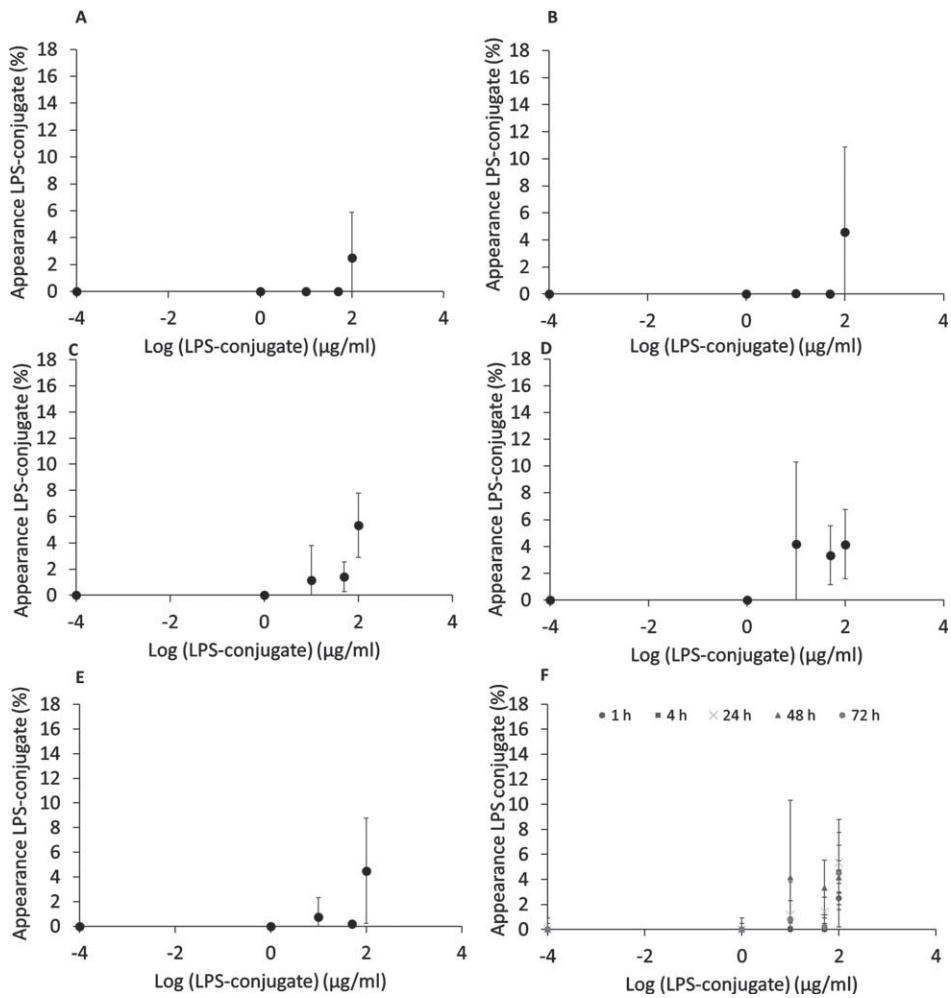


Fig. 7. Percentage appearance of FITC *P. gingivalis* LPS conjugate on the apical side of the *in vitro* BBB model relative to the stock FITC *P. gingivalis* LPS administered to the basolateral side after 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 (E) and a comparison of all time points (F). Statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone) where $^{*}p < 0.05$ and $^{**}p < 0.01$. Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

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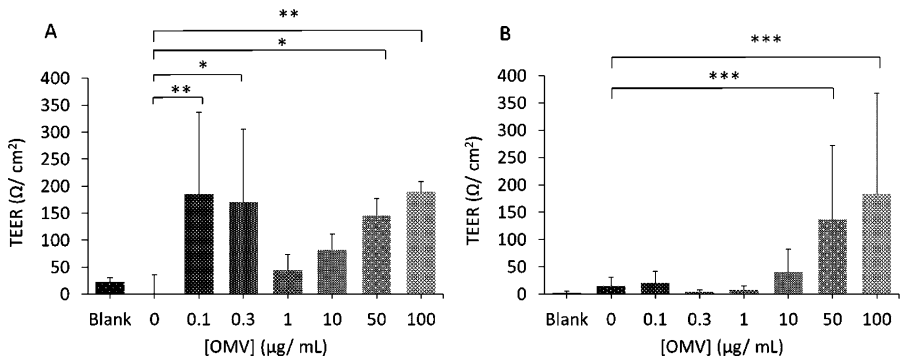


Fig. 8. Changes in BBB integrity measured by calculating the magnitude of decrease in TEER in response to application of *P. gingivalis* OMVs (A) and the magnitude of deficit in recovery of TEER 72-h post application of conjugated *P. gingivalis* OMVs relative to initial baseline TEER (B). Statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone) where $^{*}p < 0.05$, $^{**}p < 0.01$ and, and $^{***}p < 0.001$. Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

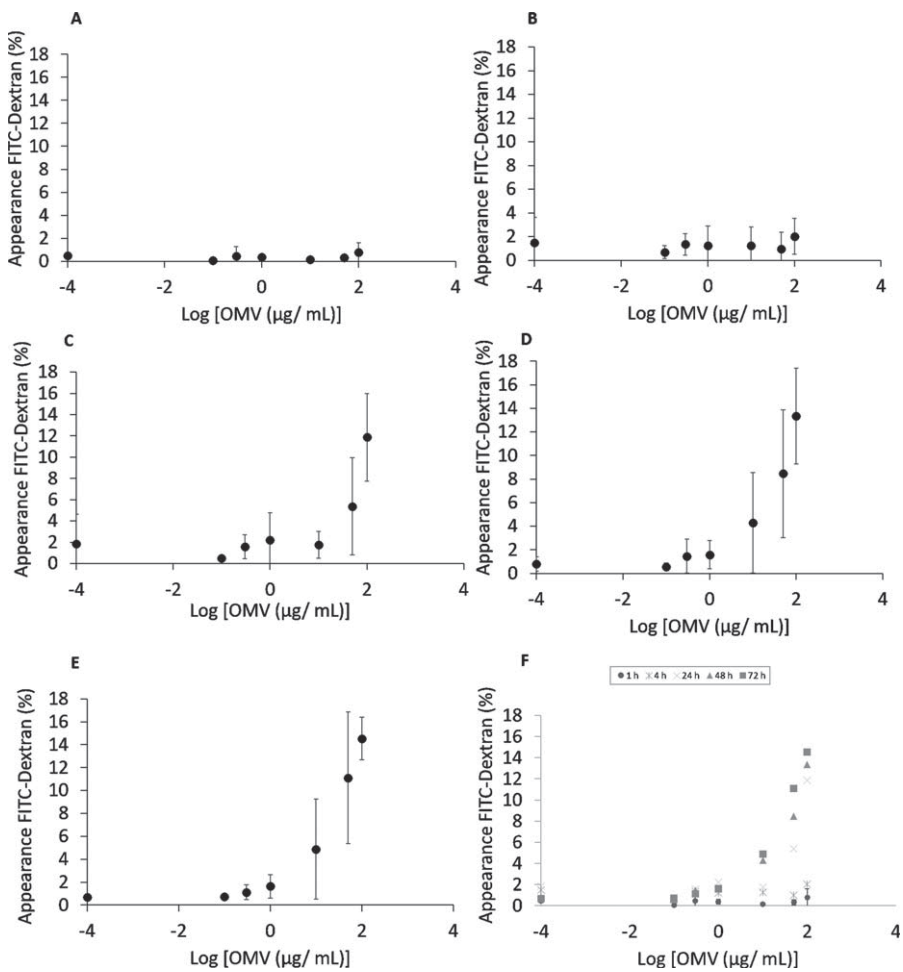


Fig. 9. Percentage appearance of FITC-dextran (3–5 kDa) on the apical side of the *in vitro* BBB model after application of *P. gingivalis* OMVs, percentage appearance relative to the stock FITC-dextran administered to the basolateral side after 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 (E) and a comparison of all time points (F). Statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone) where $^{*}p < 0.05$ and $^{**}p < 0.01$. Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

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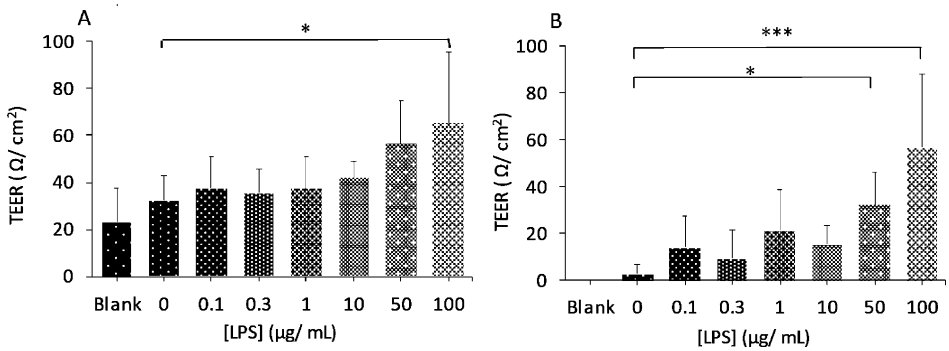


Fig. 10. Changes in BBB integrity measured by calculating the magnitude of decrease in TEER in response to application of FITC *P. gingivalis* LPS conjugate and 10 μg/ml *P. gingivalis* OMVs (A) and the magnitude of deficit in recovery of TEER 72-h post application of FITC *P. gingivalis* LPS conjugate and 10 μg/ml *P. gingivalis* OMVs relative to initial baseline TEER (B). Statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone) where $^{*}p < 0.05$ and $^{***}p < 0.001$. Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

The TEER responses in these experiments showed a significant difference in the 100 μg/mL FITC *P. gingivalis* LPS conjugate with larger magnitudes of change in TEER compared to the controls ($p < 0.05$) (Fig. 10A). The higher the concentration of FITC *P. gingivalis* LPS with OMV, a reduced recovery was observed, although this was only significant in the highest concentration of 50 μg/mL and highly significant in the 100 μg/mL FITC *P. gingivalis* LPS with 10 μg/ml OMVs ($p < 0.05$ and $p < 0.001$) (Fig. 10B). An increase in the permeability was seen especially after 24 h where some of the increases were 5-fold compared to the experiment with *P. gingivalis* LPS-FITC conjugate application only, though this increase was not significant (Figs. 7 and 11C).

Assessment of virulence factors interactions with HBMEC monolayers measured by immunofluorescence microscopy

The experiments of application of virulence factors to the BBB model indicated a potential disruption of the barrier. These findings lead to the experiment with application of 1) unconjugated *P. gingivalis* LPS (0.1 and 0.3 μg/ml) and 2) OMVs (0.1 and 0.3 μg/ml) to HBMEC cells in monolayer with an aim to determine how the observed BBB model disruption happened, all the experiments were repeated 3 times independently. The HBMEC cells were chosen for this experiment as these are the first cells coming into contact with the virulence factors in the *in vitro* model. The negative control cells (no virulence factors applied) showed the expected position of the ZO-1 protein at the cell-cell junctions and the signal of the ZO-1 protein appeared clear and well organized in these controls (Fig. 12B, C). The concentrations for these experiments were based on observations after application of virulence factors of higher concentrations in the optimization stages showed the HBMEC cells viability were consistently acceptable at these concentrations. All the test wells and controls were imaged using the same exposure times and all post-exposure modifications were carried out to the same level with the Zen software. The HBMEC monolayer with application of *P. gingivalis* LPS showed no noticeable effect on the ZO-1 signal (Fig. 12D-F) (only 0.3 μg/ml shown) and similar observations were made in the wells with 0.1 μg/ml *P. gingivalis* OMV application (Fig. 12G-I) compared to the untreated controls (media only). The wells with application of 0.3 μg/ml *P. gingivalis* OMVs showed a more diffused signal from the ZO-1 proteins compared to untreated controls, which could appear as a reduction in the signal (Fig. 12J-L) though a change in the signal from the application of 0.3 μg/ml *P. gingivalis* OMVs were seen in all 3 repeat experiments it was not clear if the change was a displacement of the ZO-1 protein or reduced numbers as the experiment here did not quantify the protein. All experiments were conducted with controls both for treatments, antibodies and counterstains and all showed the correct results.

DISCUSSION

In this study, we investigated the effect of *P. gingivalis* virulence factors on the cells in a human *in vitro* BBB model. The movement of *P. gingivalis* LPS

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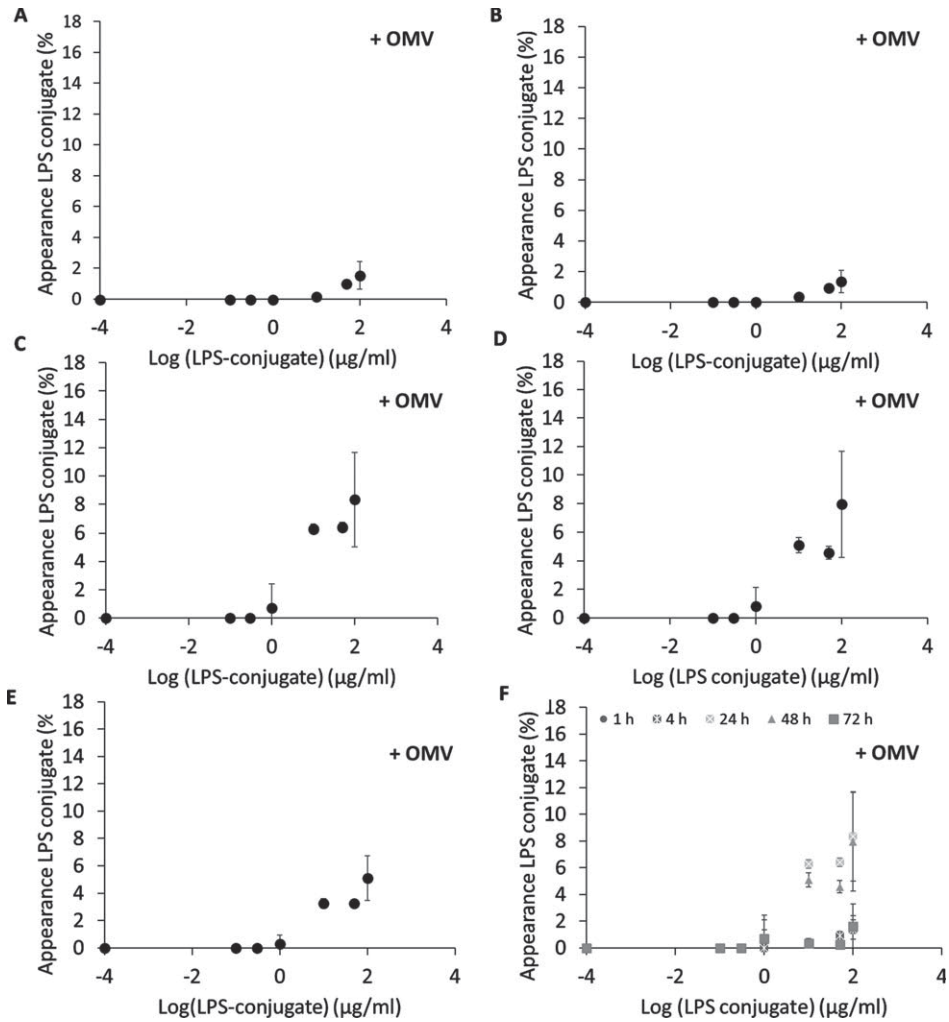


Fig. 11. Percentage appearance of FITC *P. gingivalis* LPS conjugate on the basolateral side of the *in vitro* BBB model relative to the stock administered to the apical side after 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 (E) and a comparison of all time points (F). Statistical significance of response was measured using an ANOVA with Dunnett's *post-hoc* relative to the control (administration of FITC alone) where $^{*}p < 0.05$ and $^{**}p < 0.01$. Data represents mean ± SD from three wells and two experimental repeats ($n = 6$).

across to the CNS side of the model (Fig. 7) and the enhancement of LPS appearance in the basolateral compartment in the presence of OMVs (Fig. 11), supports findings from animal and other *in vitro* studies that virulence factors are capable of traversing the BBB [19, 21, 23, 30]. Designing human studies of chronic bacterial infection at the BBB is challenging and making a link to sporadic AD must be carried out cautiously. This is due to the latent period before pathological changes of AD are clinically symptomatic and measurable in brain tissue of patients and the difficulty of attributing changes to microbial infection rather than the interaction of multiple other risk factors associated with AD development. *In vitro* BBB models have been widely used for decades to investigate drug transport and individual disease processes, including Aβ clearance mechanisms in relation to AD [52, 60]. Most BBB model studies investigating bacterial related interactions have focused on acute events [61, 62].

For the experiments reported here, it was vital to ensure that barrier integrity was stable for the duration of testing and not affected by the application of FITC tracer compounds, as magnitudes of change in TEER was a key marker of changes in BBB integrity. A number of controls were included and TEER measurements were made for 24 h prior to any application of virulence factors. An initial small drop in TEER values was observed for every experiment in both test and control wells (average of 25 ohms/cm^2) and this

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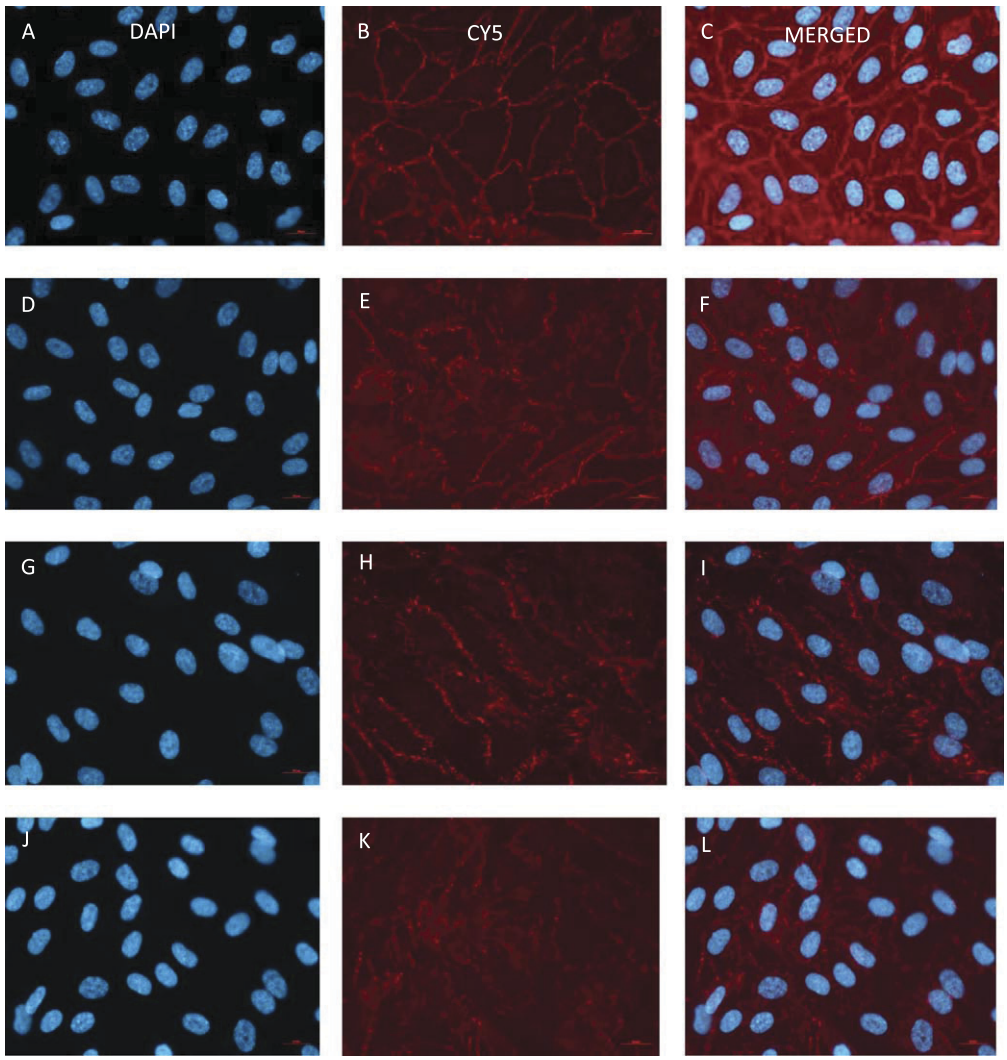


Fig. 12. Immunofluorescent study of application of *P. gingivalis* virulence factors to HBMEC cells. Virulence factors of *P. gingivalis* disturb ZO-1 localization in HBMEC cells. HBMEC cells in monolayer were treated with EBM (A-C), 0.3 μg/ml *P. gingivalis* LPS (D-F), 0.1 μg/ml *P. gingivalis* OMVs (G-I) or 0.3 μg/ml *P. gingivalis* OMVs (J-L) for 24 h. Panels A, D, G, and J show the nuclei stained with DAPI and detected at 358 nm (blue). Panels B, E, H, and K show Cy5 signal detected at 646 nm (red) detecting the primary ZO-1 (D6L1E) Rabbit mAb. Panels C, F, I, and L represent the composite pictures. Images were taken with 60x objective oil lens with the same exposure times. Scale bar 20 μm.

was regarded as an artefact explained as a short-lived disturbance in the conductance across the BBB model following the application of the Evom electrodes or fresh media. The first experiment assessed whether there was a dose-response relationship between the concentration of endotoxin and OMVs and BBB response, which would indicate the resilience of the BBB model to these virulence factors. The concentrations of virulence factors applied were deduced from previous studies [7, 63]. After application of unconjugated *P. gingivalis* LPS to the BBB, a clear pattern emerged (Fig. 1), of an initial drop in TEER values including the controls; however, the decrease in TEER in the BBB which had endotoxin applied were both significantly greater in magnitude and had failed to recover when compared to the control BBB, indicating a lasting measurable effect in the BBB model barrier function. A significant drop in TEER values was seen in wells tested with the concentrations of unconjugated *P. gingivalis* LPS (0.3, 10, 100 μg/ml) (Fig. 4A) and the recovery of TEER in these wells were also significantly less compared to controls (in 100 μg/ml highly significant, $p < 0.01$) (Fig. 4B). Furthermore, a significant drop in TEER

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was observed after application of *P. gingivalis* OMVs (0.3 and 50 μg/ml) ($p < 0.05$) which were highly significant after application of 0.1 and 100 μg/ml LPS ($p < 0.01$) (Fig. 8A). The lack of TEER recovery in the BBB treated with 50 and 100 μg/ml LPS was highly significant ($p < 0.001$) compared to the controls (Fig. 8B), indicating that these virulence factors affected the *in vitro* BBB model in a prolonged and negative fashion. However, at lower concentrations (0.1, 0.3, and 1 μg/ml), some of the TEER value reductions were temporary followed by partial or complete recovery (Fig. 8B). The authors suggest this may have arisen as a result of an initial apoptosis event subsequently overcome by the surviving neighboring cells expanding to repair the damaged area or an initial disruption of the tight junction complexes followed by a reparatory event. These observations are important as they indicate the cells or tight-junctions of the BBB model have an ability to recover if the endotoxin is applied at a low concentration. Applied to a human clinical scenario, this could mean that after a low level, low frequency endotoxin contact with the BBB, the NVU cells appear to retain the ability to preserve the BBB integrity. Clinically this could correlate with the adoption of an improvement in oral hygiene or if systemic risk factors for PD, such as diabetes, were eliminated or reduced.

The BBB test wells that demonstrated reduced ability to recover TEER values and hence barrier integrity, could indicate that the cells in those wells were unable to survive or expand, or the LPS and OMV could have influenced the continuity of the cell layer either by causing pyroptosis [41], apoptosis, or irreversible tight junction disruption [46, 64]. There were reductions in TEER readings in the BBB treated with FITC *P. gingivalis* LPS conjugate in media compared to controls, and these TEER levels also did not recover as well, though none of these TEER value differences were statistically significant compared to the controls (Fig. 6A, B).

The appearance of FITC dextran in the basolateral compartment gave an indication of disruption to the barrier integrity after unconjugated *P. gingivalis* LPS and OMV application at various concentrations. Average Papp values were then calculated at 60 min, 120 min, and 240 min and were found to be 1.04×10^{-8}cm/s, 8.7×10^{-8} cm/s, and 4.8×10^{-8} cm/s. In permeability assays for drug transportation poor permeability is indicated by Papp values of 0–1.4×10^{-6} cm/s and high permeability by values in the range of $5 \times 10^{-5} - 9 \times 10^{-5}$ cm/s. The low calculated Papp values in our study suggest that the Papp for the tracer compound FITC-dextran was low, indicating the BBB model retained its overall barrier function for the first 2 h, though allowing enough permeation to measure a difference between test wells (Fig. 5). The most significant effect in the BBB models TEER measurements were observed with concentrations of unconjugated LPS and OMV at 0.3 μg/ml, 10 μg/ml, 50 μg/ml, and 100 μg/ml. Contextualized to a theoretical physiological condition, the lowest of these values, 0.3 μg/ mL is most clinically applicable [7, 63].

The changes in the BBB model cells after application of the FITC-*P. gingivalis* LPS in conjunction with 10 μg/ml of *P. gingivalis* OMV showed a significant difference in the magnitude of change in the TEER values and a significant deficit in recovery of the values (highly significant in 100 μg/ml FITC-*P. gingivalis* LPS) which, highlighted the potency of the OMVs containing gingipains (Fig. 10).

The TEER measurements of an *in vitro* BBB model reflect the ionic conduction paracellularly in the cell layers, whereas the percentage appearance of a tracer compound in the basolateral compartment represents paracellular waterflow associated with increased pore size at the tight junctions [55]. Transcellular ion transport function and paracellular permeability of solute transport are differentially regulated [55]. The factors affecting perfusion of a molecule across the BBB are size, shape, and lipophilicity. TEER is a valuable assessment of the *in vitro* BBB integrity as it is easy to quantify and if carried out with care, non-invasive. It is, however, important to be aware of the limitations of TEER measurements, where variations can occur due to factors such as medium content, temperature, and the cell line passage number at the time of measurements [55]. As the protocols in this study were performed under the same conditions using the same equipment and cell passage numbers, some of the potential variables could be excluded. It is most likely that the recovery of the BBB observed by return of TEER to near baseline levels, throughout our experiments, could reflect dynamic cytoskeletal alterations in tight junction regulation or an increased number of cells in the BBB. There were no other significant increases in permeability recorded for the remainder of the test period when further endotoxin was applied, or when the concentrations of LPS added were not high enough to induce measurable changes to the percentage appearance in the basolateral compartment (Figs. 5 and 7).

Previous validation studies of *in vitro* BBB models have shown that a TEER measurement in the range

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of 120–130 ohms/cm^2 is sufficient for transport studies [65]. For the study reported here, a TEER (blank) $\geq$260 ohms/cm^2 [65], was achieved in all the protocols prior to experimentation.

A tracer compound used in an *in vitro* BBB model to study permeability could potentially interfere with the virulence test reagents and affect the integrity of the barrier [55]. Control wells containing BBB treated with FITC-dextran only were included throughout the study to enable any changes in test wells to be measured relative to this control. A limitation is that fluorophore and dye tracer compounds are not always sensitive enough to show subtle changes in barrier model permeability [55]. The FITC dextran molecule used here has been shown to cross the BBB model via intercellular diffusion [66], therefore any increase in intercellular diffusion due to loss of barrier function would be measured by an increase in appearance of FITC dextran in the basolateral compartment. This study demonstrated that the appearance of FITC dextran into the basolateral compartment occurred early (between 1 and 4 h) after the initial application of unconjugated *P. gingivalis* LPS, particularly with the testing of the higher concentrations of LPS. Subsequent applications of LPS failed to demonstrate any clear correlation between the concentration of applied endotoxin and the percentage appearance measured in the basolateral compartment. This implies that the higher concentrations of LPS were able to induce an increase in paracellular flow, possibly by increasing paracellular gaps, at initial application compared to controls (Fig. 5). However, further increases were not demonstrated by additional applications implying a finite capacity for paracellular flow increase. These findings were supported by the Papp calculations of the FITC dextran and the percentage appearance values which also remained low after application of unconjugated LPS. The levels of FITC dextran appearance seen throughout the unconjugated LPS experiments were persistently low with the maximum appearance at 0.7% (Fig. 5) which is encouraging in terms of demonstrating the quality of the barrier model [53]. In comparison, the percentage appearance of FITC dextran seen in the OMV alone experiment were higher with the maximum percentage appearance being 15-fold higher than the LPS alone (Fig. 9). The maximum percentage appearance of the FITC LPS conjugate in the basolateral compartment was 5% during the experiments (Fig. 7) and a small increase to 8% maximum percentage appearance was observed when 10 μg/ml was added to the FITC-LPS conjugate (Fig. 11). The increased percentage appearance in the OMV alone (Fig. 9) could be due to the presence of the proteolytic enzymes or gingipains within the OMVs which could also explain the increased permeability of the FITC *P. gingivalis* LPS in the presence of 10 μg/ml of OMV (Fig. 11). The enzymes could create greater gaps between the barriers cell layers allowing greater perfusion of the molecules to the basolateral compartment. The percentage appearance of FITC *P. gingivalis* LPS and the FITC dextran molecules in the basolateral compartment (Figs. 5 and 7), was approximately 5-fold larger compared to treatment with unconjugated LPS. This could be explained as a potential difference in the virulence between the two compounds (conjugated and unconjugated LPS) inflicting different effects on the barrier, maybe due to altered size, shape, or lipophlicity. The *P. gingivalis* LPS could also affect the cells of the BBB model differently at receptor level after being conjugated, even though the LPS product used for both reagents originated from the same source.

The inflammatory response initiated by application of the *P. gingivalis* LPS, *P. gingivalis* LPS FITC conjugate, and *P. gingivalis* OMV was examined by an IL-6 ELISA after application for 4 h to HBPC. IL6 is a proinflammatory cytokine and the induction of this cytokine would give an indication of the biological activity of the virulence factors utilized in this study, as the presence of LPS would induce TNF-α pathways in the HBPC to release IL6 [67]. The unconjugated *P. gingivalis* LPS used in this study was the standard preparation (defined by supplier) which has been noted to induce a stronger inflammatory response than the pure version (reduced impurity LPS level) (both supplied by Invivogen) possibly as a result of impurities of lipoproteins in the standard preparation, activating TLR2 as well as TLR4 [68]. Furthermore, the standard *P. gingivalis* LPS has also been found to show a stronger inflammatory response in human periodontal stem cells after 24 h than the pure version [68]. In our BBB protocols, we saw an increased response at 24 h with both *P. gingivalis* LPS and OMVs which indicates that the induced response could be more complex than a simple apoptosis of the cells and involve inflammatory pathways affecting the integrity of the barrier. The OMVs applied in this study were extracted from a culture of the laboratory strain *P. gingivalis* FDC 381 which is classed as a less virulent *P. gingivalis* strain but has a high ability to be internalized in human cells [69]. This non-capsular strain has been shown to be a strong immune stimulant (even activating TLR2), a property attributed to

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an intact fimB allele but with a less gingipain activity [70]. It is possible that the difference in appearance of the *P. gingivalis* LPS FITC conjugate on the CNS side of the model with and without the presence of the OMVs would have been more significant if a less virulent *P. gingivalis* LPS product had been used such as the purified product mentioned previously.

The *in vitro* BBB model has limitations such as the delicate nature of working with primary-derived short-term cell lines and the measurements derived from this study do not divulge much information about molecular changes at the cellular level, although there is potential for exploring this in future studies. There is an increased consensus that to gain more accurate knowledge of AD, microbial pathology human models need to be developed, as pathological and inflammatory pathways in rodents significantly differ from humans [6, 34] especially in relation to molecules such as LPS [41]. The human BBB model cells used here is more biologically relevant than the *in vivo* murine model. The role of microglial cells in neurodegeneration is undisputed and expansion of this model to include human microglial cells could broaden the applications for this type of protocol. In addition, brain organoids could potentially be applied to this type of study [71].

There is evidence to suggest that *P. gingivalis* may not need to enter the brain to cause neuroinflammation [41]. Healthy humans have been shown to have low levels of LPS in the blood [72], but this was elevated in AD and PD patients [73–75]. AD patients have been found to have 2×3 times as much LPS in the brains as healthy individuals [76]. LPS is released from the bacteria either when it is degraded or when outer membrane vesicles are released [41] and the GI microbiome has been shown to be the main contributor to a systemic presence. LPS has been suggested as an intermediary between bacteria and the CNS at low levels (physiological) conditions [77] in rodents, and a lipo-protein transport mechanism to the CNS has been suggested, but it is not yet known exactly how LPS enters the brain in humans. It is possible that transport mechanisms are responsible for the appearance of the LPS on the basolateral compartment (CNS) side of the model used in this study and this topic warrants further investigation, OMVs are known to have the ability to be internalized by human cells which is another avenue to explore.

This study, however, has demonstrated that the integrity of a BBB model is reduced by the presence of *P. gingivalis* virulence factors seen by a measurable reduction in TEER levels and making the barrier more permeable and the subsequent increased appearance of LPS in the basolateral compartment. Bacterial LPS has been found to change the permeability of the BBB at high doses [78] as seen in sepsis causing significant CNS disability. *P. gingivalis* LPS has the potential to cause neuroinflammation via the blood directly acting at the BBB, by inducing pro-inflammatory cytokines, initiating pro inflammatory pathways in the tissues of the NVU and activating microglial cells without entering the brain [41]. In addition, this study has indicated that *P. gingivalis* LPS also has the potential to cross the BBB as seen in the FITC *P. gingivalis* LPS experiments, potentially explaining how systemic circulating LPS could induce neuroinflammation. The subsequent immunological response to LPS is well documented (for a review, see [47]), and the toxicity of an endotoxin is determined by how the host reacts to it [79]. Both immunological activation and tolerance [80, 81] could explain how chronic exposure to even medium and low levels of *P. gingivalis* LPS could lead to neurodegeneration by induction of pro-inflammatory pathways and activation of microglia and it is plausible that a low concentration of *P. gingivalis* virulence factors can induce damage to the NVU cells [82, 83]. Here we have shown, in a BBB model, that whole bacteria do not need to be present as *P. gingivalis* LPS and OMVs have the armory to induce alteration of the barrier integrity providing access to the CNS tissues. A question remains about how much damage, over what period of time is required, before the balance is tipped towards a path where the BBB cells cannot recover?

The aim of applying *P. gingivalis* LPS and OMVs to a HBMEC monolayer was to investigate whether the changes observed in the BBB model protocols could be seen at a cellular level and to determine how these changes occurred. The negative control cells (no virulence factors applied) showed the expected position of the ZO-1 protein at the cell-cell junctions [84] with the signal of the ZO-1 protein appearing well organized (Fig. 12B, C). The HBMEC monolayer with application of *P. gingivalis* LPS showed no noticeable effect on the ZO-1 signal (Fig. 12 D-F) (only 0.3 μg/ml shown) and similar observations were made in the wells with 0.1 μg/ml *P. gingivalis* OMV application (Fig. 12 G-I) compared to the untreated controls (media only). The wells with application of 0.3 μg/ml *P. gingivalis* OMVs showed a more diffused signal from the ZO-1 proteins compared to untreated controls, which could appear as a reduction in the signal (Fig. 12 J-L). All the test wells and controls were imaged using the same exposure

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times and all post-exposure modifications were carried out to the same level with the Zen software. Though a change in the Cy5 signal after the application of 0.3 μg/ml *P. gingivalis* OMVs were seen in all 3 repeat experiments compared to untreated controls, it was not clear if the change was a displacement of the ZO-1 protein or reduced numbers as the experiment here did not quantify the protein. ZO-1 is a tight junction adaptor protein connecting the actin skeleton to tight junctions such as claudin and the binding of ZO-1 to actin is essential for regulation of permeability in epithelial cells [85]. Tornavaca et al. (2015) [86] showed that in primary endothelial cells ZO-1 is a central regulator of tight junctions depending on the strictly endothelial specific adhesion molecule vascular endothelial-cadherin. These endothelial junctions were found to influence the spatial actomyosin organization, cell–cell tension and migration across the endothelium, but also angiogenesis and barrier formation. This study used human dermal microvascular endothelial cells (HDMEC-c) and not HBMECs, but this places ZO-1 central to the development, integrity, and maintenance of the BBB and if *P. gingivalis* OMVs are able to disrupt the functionality of ZO-1 this could have devastating consequences to the integrity of the blood brain interface. ZO-1 is a large phosphoprotein and post-translational alterations such as phosphorylation would lead to ZO-1 dissociation from the tight junction complex [87]. This could potentially be how *P. gingivalis* virulence factors affect this protein and explain the TEER and diffusion changes observed in our study, where notably the application of *P. gingivalis* OMVs had the greatest effect on both measurements.

Multiple studies have investigated the effect of *P. gingivalis* LPS and OMVs on cells, both human and animal, but not on cells of the human BBB. It was clear from our study that the *P. gingivalis* LPS both unconjugated and FITC conjugated and OMVs did affect the cells of this *in vitro* BBB model and caused barrier integrity changes. There is potential to investigate more nuanced changes in the cells by using the protocol described here to reveal changes at the lower concentration of LPS level, which the TEER and percent appearance methods did not have the sensitivity to reveal.

Conclusion

The two virulence factors of *P. gingivalis* (LPS and OMVs) were seen to induce changes in the human *in vitro* BBB model cells. Unconjugated *P. gingivalis* LPS, FITC conjugated *P. gingivalis* LPS with 10 μg/ml of OMV, and OMVs alone had a significant effect on the integrity of the *in vitro* BBB model which were measurable by TEER showing a significantly greater magnitude of change after application and a significant deficit in recovery of the models TEER values. These changes were also observed after application of virulence factors at a physiological relevant level (0.3 μg/ml). The application of *P. gingivalis* OMVs resulted in the most significant magnitude of change in the TEER values of the barrier and the most significant deficit of recovery. These experiments also confirmed that endotoxin from *P. gingivalis* conjugated with FITC was able to cross the barrier model and that the percentage of LPS conjugate appearing on the CNS side of the model were increased by the presence of *P. gingivalis* OMVs. The ZO-1 proteins in a HBMEC monolayer model showed disruption after contact with *P. gingivalis* OMVs compared to controls.

Further investigations at cellular level are warranted to contribute to the knowledge of how endotoxin from periodontal disease could have an influence on neuroinflammatory states and potentially contribute to or exacerbate neurodegeneration. If these observations are applied to the human BBB, then infection with *P. gingivalis* and inflammagens LPS and OMVs could cause damage to an otherwise healthy and non-predisposed individual. It is tempting to attribute the link between sporadic AD and periodontal disease to the effect of this multi-talented pathogen, when the chronicity of the inflammatory state seen in established periodontal disease could be the main culprit. This highlights not only the need for good oral hygiene, but also the importance of diagnosis and optimal management of dental patients presenting with unstable periodontal disease. Until there is a therapeutic remedy which can protect the BBB from chronic inflammation, prevention remains the key.

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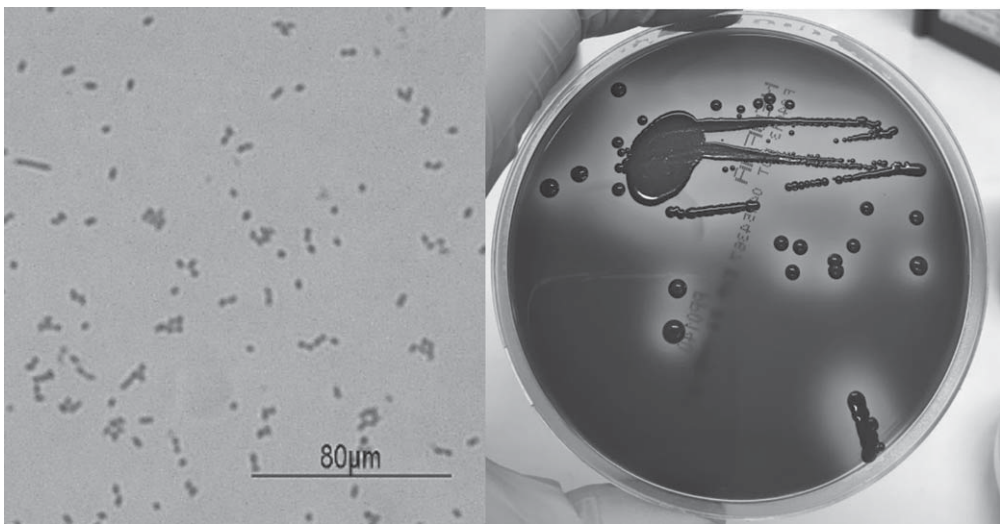
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SUPPLEMENTARY MATERIAL

An Investigation into the Effects of Outer Membrane Vesicles and Lipopolysaccharide of *Porphyromonas gingivalis* on Blood-Brain Barrier Integrity, Permeability, and Disruption of Scaffolding Proteins in a Human *in vitro* Model



Supplementary Figure 1. *P. gingivalis* FDC 381 Gram stain and on FAA Neomycin (E&O, UK) shows coccobacillus at x100 and Black stain colonies of the strain with hemolytic halos on NEOFAA agar.

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Handbook of Microbiome and Gut-Brain-Axis in Alzheimer's Disease
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