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Special Issue: The Gut Microbiome and Aging

Gut Microbiota: From the Forgotten Organ to a Potential Key Player in the Pathology of Alzheimer's Disease

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Abstract

More than 300 years ago, Antony van Leewenhoeck first described observing single-celled microorganisms, which he termed "*animalcules*," examining his saliva under a microscope. Although the idea of the coexistence of microorganisms in our body is not new, we have only recently been able to investigate their ecological relationship to our body, with the development of high-throughput molecular techniques. The diverse microorganism communities residing in our guts are established and maintained by complex interactions among microorganisms and their host. Notably, their alteration has been implicated in influencing various diseases including neurological diseases. Alzheimer's disease (AD) is the most common cause of dementia characterized by a progressive decline in memory and thinking severe enough to interfere with daily life. Despite the great progress in linking genetic risk factors with AD pathogenesis, treatments targeted at AD pathology and its modifiers have not yet resulted in a disease-modifying therapy. There is mounting evidence that the gut microbiota interacts with AD pathogenesis by disrupting neuroinflammation and metabolic homeostasis—the gut microbiota has gone from being the forgotten organ to a potential key player in the AD pathology.

Keywords: Microbiome, Bacteria, Tau, Amyloid, Neurodegeneration

The resident microorganisms in our body, including bacteria, viruses, archaea, and fungi, outnumber eukaryotic cells by as many as ten to one and their collective genome is estimated at 150 times larger than the human gene complement (1,2). However, because of their remarkably small size—typically 0.5–5.0 μm, which is about one tenth the size of eukaryotic cells, the microorganisms make up a small portion of human body mass (0.3%–3% of total body mass). Nevertheless, while establishing their colonies, these microbes perform various vital functions in our body, including releasing biochemical byproducts (3).

Microbes live and act in ecological communities. Each will communicate with other microbes by exchanging genetic and molecular material, altering their collective behavior on a population-wide level in response to surrounding species or stimuli (eg, temperature and pH), and thereby remodeling the microbial composition of the community (4–6). Furthermore, community building within the host generates myriad products that modulate host physiology: extracellular enzymes, toxins, antimicrobial compounds, inflammatory cytokines, and metabolites (7,8). Through dynamic microbe-microbe and microbe-host interactions, the diverse community of microorganisms—collectively called microbiota or microbiome with their genomic content—is not only established and maintained, but also contributes beneficial or pathological influences to host health.

Despite the remarkable richness and diversity of human microbiota and their close physiological association to the human host, their significance to human health and disease has long been overlooked due to inadequate analytical methods. With recent genetic and metagenomic analysis tool development, more sophisticated microbiota profiling techniques facilitated the characterization of the structure of the microbiome and a better understanding of its contribution to human health. Recent advances in microbiology have characterized the functional interactions between microbiota and host. This "forgotten organ" is now considered not only as a key player in human homeostasis but also as direct/indirect causal agent in influencing various diseases such as allergy, irritable bowel syndrome, type 2 diabetes mellitus, obesity, and cancer (9).

Recently, there has been immense excitement on the potential contribution of the microbiota in our gastrointestinal (GI) system to central nervous system (CNS) disease. The CNS and enteric nervous system of the GI tract are tightly connected by hormones, neuromodulators, and neurotransmitters related to efferent/ afferent nerves including the vagus nerve. As most (>95%) of the microbiota in our body are GI residents, these microbes are perfectly situated to react to and influence neuronal, humoral, metabolic, or immune signaling underlying the gut-brain relationship. On the one hand, as an example, elevated levels of noradrenaline in the gut lumen (eg, by stress) can influence gene expression or abundance of some bacteria (10,11). On the other hand, mice grown in a germ-free (GF) environment demonstrate exaggerated stress responses and anxiety behaviors related to changes in the hypothalamic-pituitary-adrenal axis (12). Similarly, disruption of the maternal gut microbiome perturbs neurodevelopment of their offspring, which show autism-like behaviors that can be rescued by introducing the commensal bacteria species *Lactobacillus reuteri* (13,14). These studies emphasize this bidirectional relationship and support disruption of gut homeostasis as a potential risk factor in psychiatric or neurological disorders.

Considering that gut microbiota has immune regulatory functions, it is not surprising that there have been attempts to find a link between gut microbiota and diseases classically classified as immune-mediated neurodegenerative disorders, such as multiple sclerosis. For example, the induction of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis, causes a less severe phenotype in GF mice compared to that of conventionally raised mice, perhaps by decreasing the pathogenic inflammatory response, such as IL-17A-producing T cell activation (15,16). Recently, neuropathological relevance of the gut microbiota has been extended to progressive neurological disorders such as Parkinson's disease, characterized histologically by loss of dopaminergic neurons in the substantia nigra and phenotypically by motor dysfunction such as slowness of movement, tremor, and stiffness. Sampson and colleagues used an α-synuclein over-expressing mouse model of Parkinson's disease to study the relationship between parkinsonian motor dysfunction with gut microbiota. Notably, model mice treated with an antibiotic drug cocktail or raised in GF conditions had less severe motor deficits and brain pathology than untreated/normally raised mice (17).

Alzheimer's disease (AD) is the most common neurodegenerative disorder and is primarily marked by global decline in cognitive function, including in episodic memory, executive functioning, and reasoning. Recently, there is growing interest in investigating the role of the microbiota in AD pathogenesis (Table 1). However, most studies investigating the contribution of the microbiota in AD pathogenesis are correlational. The microbiota-host dynamic is immensely complex and is heavily influenced by various endogenous and exogenous factors. These have hindered investigation on precise biological causal pathways from specific microbes to AD pathology and neurodegeneration.

In this review, we present an outline of the gut microbiota-brain axis and summarize recent findings from both animals and humans on the potential involvement of the gut microbiota in AD pathogenesis. We hypothesize that an altered/unhealthy gut microbial community (*dysbiosis* by several factors; eg, genetic, diet, stress, age) can induce a peripheral inflammatory response that drives an altered neuroinflammatory response in the brain (Figure 1). Over decades, various factors may disrupt gut permeability and blood–brain barrier (BBB) integrity that accelerate entry of circulating inflammatory agents and pathogens into the brain driving excessive activation of the brain's innate cells (microglia). AD-related genetic risk factors may also contribute to the construction of a disease-associated microbiota (eg, the presence of ApoE4 alleles accompany reduced host-beneficial bacterial communities). Studies cited in this review are mostly based on bacterial 16S rRNA gene sequencing techniques. Therefore, from here, the terms "microbiota" or "microbiome" are referring to the bacterial community. Studies involving microbiota in other locations, such as nasal, skin, respiratory, are not discussed due to the lack of published data. There may be bidirectional communications between gut microbiota and AD pathogenesis, but we will focus on the contribution of gut microbiota to AD pathology (ie, less known if the hallmarks of AD pathologies modulate the gut microbiota communities). Finally, we will discuss current gaps in knowledge as well as future directions of microbiota research in AD.

Gut Microbiome: Fundamentals and Functions

Most microorganisms in the gut are bacteria (18). In the general public view, bacteria have been perceived as harmful to our health, but modern bacteriology argues there exists a spectrum of "good" to "bad" bacterial types depending on their influence on the host. In fact, most gut microbiota are completely harmless (commensal) or beneficial (symbiont), and relatively few types of bacteria pose a modest degree of risk or disease (pathobiont) (19,20). Most of the time, as in the case of the diverse gut bacteria, "harmful" bacteria are suppressed, and we live in harmony and balance with our numerous gut bacteria (21,22). However, when the gut ecosystem undergoes abnormal changes, the pathobionts become overpopulated, and the gut bacterial composition becomes unbalanced (*dysbiosis*). Then, this imbalance may grow to create a disease-related microbiota community and coordinate inflammatory reactions or toxin release.

It is estimated that around 1,000 different bacterial species exist in the human gut, and at least the same 10% of species are present in every individual (1,23). The human microbiota composition is established in early development and is stably maintained over time in healthy individuals. Eighty percent of our gut microbiota are made up of the phyla *Bacteroidetes* and *Firmicutes* (24). Several other phyla are represented: including the *Actinobacteria, Fusobacteria,* and *Verrucomicrobia*. However, these phyla make up a small proportion of the gut microbiota. Classically pathogenic species, such as *Campylobacter jejuni*, *Salmonella enterica*, *Vibrio cholera* and *Escherichia coli*, and *Bacteroides fragilis*, exist in low prevalence (~0.1%) (18,25). *Firmicutes* is the largest bacterial phylum in the gut microbiota and includes 200 genera: *Ruminococcus, Clostridium, Eubacterium, Lactobacillus, Faecalibacterium, Roseburia*, and *Mycoplasma*. Most of these bacteria have a Gram-positive cell wall structure with a thick peptidoglycan layer. On the other hand, *Bacteroidetes have* a Gram-negative cell wall structure. These bacteria include the genera *Bacteroides, Prevotella*, and *Xylanibacter* (26). Different from Gram-positive cell wall structure, Gram-negative cell structure consists of an extra outer membrane outside the thin peptidoglycan layer, which makes bacteria less susceptible to antibiotics. Also, the outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS), which elicits a variety of immune responses in the host animal in which the bacteria reside. The proportion of the two major phyla, *Firmicutes* and *Bacteroidetes*, are inversely related. It has been reported that the *Firmicutes* to *Bacteroidetes* ratio is correlated with obesity and other diseases, although it is debatable if the ratio is a good indicator associated with

Table 1. Evidence Supporting the Association of Gut Microbiota with AD Pathogenesis

Subject	Method	Main findings	Reference
AD Human patients	Specific microbial DNA qPCR Assay using fecal smaples.	*Escherichia/Shigella* (related to proinflammatory)↑; *Eubacterium rectale* (related to anti-inflammatory)↓	(62)
AD Human patients	16S rRNA gene sequencing using fecal samples.	*Firmicutes*↓; *Bacteroidetes*↑; *Bifidobacterium*↓	(61)
AD Human patients	16S rRNA sequencing using AD brain tissues	Bacterial population in the brain ↑	(98)
AD Human patients	Probiotic supplement	Kynurenine:tryptophan ratio↑	(97)
AD Human patients	16S rRNA gene sequencing using fecal samples.	*Bacteroides*↓, *Actinobacteria*↑, *Ruminococcus*↑, *Lachnospiraceae*↓	(60)
APP/PS1 mice	Life-long antibiotic treatment	Aβ plaque↓; plaque-localized glial reactivity ↓	(69)
APP/PS1 mice	Early postnatal antibiotic treatment	Aβ plaque↓; plaque-localized glial reactivity ↓	(68)
APP/PS1 mice	16S rRNA sequencing using fecal samples (8–12 mo of age)	SCFA ↓; Microbiota composition and diversity were perturbed (*eg, richness*↑ at ~6 month; *diversity*↓ at ~12 month; *Proteobacteria* and *Verrucomicrobia* ↑)	(63)
APP/PS1 mice	16S rRNA sequencing using fecal samples (6 mo of age)	Microbiota diversity↓; Spatial memory ↓; *Odoribacter* and *Helicobacter*↑ *(at the genus)*	(64)
APP/PS1 mice	Raising subjects in germ-free condition (8 mo of age)	Brain Aβ↓	(70)
APP/PS1 mice	16S rRNA sequencing using fecal samples (3–24 mo of age)	*Firmicutes*: *Bacteroidetes ratio*↑ with age; *Proteobacteria*↑ (genus *Shutterella*↑); *Erysipelotrichaceae*↑	(99)
3xTG-AD mice	Oral treatment with SLAB51 probiotic formulation at 8 wk for 4 mo. (Behavioral test at 24-mo-old of age)	Aβ deposition↓ Cognitive decline↓	(96)
5xFAD mice	16S rRNA sequencing using fecal samples (6–18 wk of age)	Locomotion (=); food consumption (=); Aβ was found in the gut; trypsin ↓; *Firmicutes*: *Bacteroidetes ratio*↑; *Clostridium leptum* ↑	(65)
5xFAD mice	GV-971 and Antibiotic treatment; Co-housing;	*Firmicutes*: *Bacteroidetes ratio*↑; Infiltrating Th1 cells & microglia activity ↑; Microglia activity ↓ with antibiotic drugs & GV-971	(78)
P301L mice	16S rRNA sequencing using fecal samples (1,3,6, and 10 mo of age)	*Firmicutes*: *Bacteroidetes ratio* ↓;	(67)
Rat	*Helicobacter pylori* filtrate i.p. injection	Aβ↑; Spatial memory ↓	(72)
Drosophila expressing Aβ42	Oral infection with *Ecc15*	Vacuolar degeneration ↑; Immune hemocyte in the brain ↑	(77)

Note: AD = Alzheimer's disease; APP = Amyloid precursor protein; APP/PS1 mice = Double transgenic mice expressing mutations in APP and PS1 genes; Aβ = Amyloid beta; Ecc15 = *Erwinia carotovora carotovora* 15; GV-971 = a seaweed-derived oligosaccharide; i.p. = Intraperitoneal; 3xTG-AD mice = Triple transgenic mice displaying both Aβ plaques and tau-containing neurofibrillary tangles; 5xFAD mice = Mice carrying five familial AD mutations in APP and PS1 transgenes; P301L = Transgenic mice expressing a mutation in human tau that causes a form of tau-related frontotemporal dementia that develop neurofibrillary tangles; PS1 = Presenilin-1; SLAB51 = a probiotic formulation made of multiple live bacterial strains.

disease state because there is significant variability among healthy individuals (23,27–29).

The commensal gut microbiota has many diverse roles in regulating host function. Extensive research has focused on the immunoregulatory effect of the commensal gut microbiota on both the innate and adaptive immune systems (30). The commensal bacteria prevent the invasion of pathogenic bacteria or the overgrowth of pathobionts in the residing bacteria community. Commensals have been reported to release antibacterial peptides (eg, cathelicidin and C-type lectin) or lactic acid to regulate the gut pH level (3,31). Recent studies also support the idea that commensal gut microbiota contribute to maintaining the structure of the gut barrier. For example, commensals express the small proline-rich protein 2A (sprp 2A) and angiogenin3, which are associated with cell-to-cell adhesion processes and vasculature development (25,32,33). The immunoregulatory effects are not limited to local infection sites: diffusion of microbial products or inflammasomes may lead to systemic distribution (34). The structural development of the immune system is also dependent on commensal bacteria. For example, mice raised in germ-free conditions have poorly developed intestinal lymphoid tissues (35). Also, in a separate study, GF mice had impairments in eliciting appropriate cytokine production accompanied by changes in various immune features: CD4+ T helper cells, FoxP3+, Tregs, B cells, Th17 cells, IgA, antimicrobial peptides, and MHC class II (16).

The diversity and composition of bacterial communities are important factors in maintaining immune homeostasis (34). For example, as pathogens invade tissues in the GI tract, they may activate the IL-23 – IL-17 inflammatory cytokine axis to induce T-helper 17 (Th17) cells, resulting in tissue inflammation and destruction during defense (36). However, an exaggerated immune response can induce Th17 cell over-proliferation that may promote autoimmune diseases, such as multiple sclerosis, arthritis, or inflammatory bowel disease. The IL-23 – IL-17 axis including Th17 cell activation can be suppressed by IL-10 and IL-25 signaling. Some of these anti-inflammatory cytokines are produced by species-specific bacterial signals (eg, polysaccharide A from *B. fragilis*), highlighting the importance of a diverse bacterial community in immune homeostasis (37).

Another critical function of the gut microbiota is assisting nutrient metabolic processes. Indigestible complex carbohydrates, such as cellulose fibers, are broken down into oligosaccharides by the bacteria in the GI tract. These are then converted to short-chain fatty acids (eg, butyrate, propionate, and acetate) that human cells can

absorb and use as an energy source (8). The gut bacteria are also involved in lipid/protein metabolic processes and in synthesizing vitamin K and vitamin B (25). In addition, recent studies have shown that the gut microbial enzymes are involved in bile acid and polyphenol related processes. We will further discuss the role of the gut microbiota in inflammation and metabolic homeostasis with AD pathology later in this review.

AD: Background and Key Risk Factors

AD is characterized by two distinct neuropathological hallmarks: extracellular deposition of amyloid plaques and intracellular neurofibrillary tangle accumulation (38). Amyloid plaques are mainly composed of Aβ peptides that are derived from the single-pass transmembrane amyloid precursor protein (APP) after its sequential cleavage by β- and γ- secretases. Under normal conditions, after Aβ is generated and released to the extracellular space, it is monomeric and soluble with a half-life of hours. However, during aging and in particularly those who go on to develop AD, Aβ aggregates into different structures including oligomers, protofibrils, and fibrils that have a β-sheet structure and are associated with local toxicity. As Aβ deposits in amyloid plaques, cerebrospinal fluid $A\beta_{42}$ levels decrease likely due to sequestration of Aβ in amyloid plaques (39,40). Aβ deposits may disrupt synaptic and neuronal activity as well as cause focal cell damage associated with microglial and astrocytic activation, thereby resulting in localized oxidative stress and mitochondrial dysfunction (38,41). Importantly, through unclear mechanisms, Aβ deposition appears to drive further aggregation and spreading of tau pathology.

Neurofibrillary tangles are the other histopathological hallmark of AD. Neurofibrillary tangles are composed of hyperphosphorylated, aggregated forms of the tau protein. Tau is present predominantly in neurons and contributes to the formation and stabilization of microtubules. Its excessive phosphorylation over time as well as other factors, leads to the detachment of tau from microtubules. In this state, it can aggregate, resulting in the formation of filamentous neurofibrillary tangles inside neurons. Aggregated forms of tau are associated with interference in synaptic communication as well as neuronal death (42,43).

AD is classically divided into two categories. Early-onset familial Alzheimer's disease (FAD) is inherited in an autosomal dominant fashion and manifests with cognitive decline beginning most commonly between age 30 and 60 depending on the gene mutation and family. FAD is caused by mutations in one of 3 genes *APP, PSEN1,* and *PSEN2* genes (encoding amyloid precursor protein, presenilin 1, and presenilin 2, respectively). Most of the mutations result in an increase in the relative level of more amyloidogenic species of Aβ such as $A\beta_{42}$ and $A\beta_{43}$. Mutations in these genes account for less than 1% of AD cases. Most AD cases are sporadic with relatively late onset typically after age 65, known as late-onset Alzheimer's disease (LOAD). The largest known risk factors for LOAD are age and genetic factors, though it is likely there are several environmental and other risks (44). The strongest genetic risk factor is the apolipoprotein E (*APOE*) gene, which has 3 common variants denoted ε2, 3, and 4 (APOE2/3/4) (45,46). Compared to the common ε3 variant, the presence of an APOE4 allele increases and APOE2 decreases AD risk. One copy of *APOE4* increases a person's risk of developing the disease approximately 4-fold, and two copies of APOE4 increase the risk approximately 12-fold relative to individuals who are APOE3/E3. One copy of APOE2 decreases risk by ~0.6 relative to individuals who are APOE3/E3 (47). APOE is the most abundant apolipoprotein produced in the brain, mainly expressed in astrocytes but also in microglia under inflammatory conditions. Neurons produce little APOE but express receptors for ApoE, such as LDLR and LRP1 (48).

The involvement of innate immunity in AD is supported by the fact that other risk factors for LOAD associate with genes expressed exclusively or at high levels in microglia (eg, TREM2, CD33, other) (49,50). Also, a recent epigenomic analysis showed that AD genome-wide association study (*GWAS*) loci are preferentially enriched in enhancer sequences associated with innate immune processes (51). Rare variants in the triggering receptor expressed on myeloid cells 2 (TREM2) increase the risk of developing AD by two- to threefold (47). TREM2 is a cell-surface receptor that is expressed in mononuclear phagocytes and microglia in the brain. Several lipids and lipid-associated ligands can activate TREM2. One such ligand is APOE, but whether APOE represents an important TREM2 ligand in vivo is not yet clear (52–54). Emerging evidence however suggests that APOE and TREM2 somehow influence microglial activation under different conditions but how this occurs is not yet clear. Inflammatory responses mediated by the APOE-TREM2 pathway will be key to understanding different aspects of the brain's innate immune response to AD pathology.

Whether innate immune activation in AD represents a neuroprotective or neurotoxic function is currently not clear. In fact, accumulating evidence argues that the innate immune response may differentially affect AD pathogenesis depending on disease stage (53). For example, microglial activation may be protective against AD pathology in early stages characterized by Aβ pathology via decreasing/clearing plaques and reducing neuritic dystrophy. On the other hand, in later stages of AD pathology with abundant tau pathology, a disease-associated microglial response may be deleterious by directly targeting injured neurons and by activating a toxic astrocyte response. In mouse models with Aβ deposition, genetic activation of microglia *via TREM2* signaling resulted in more compact Aβ plaques and a reduction in plaque-associated dystrophic neurites (55,56). In contrast, genetic ablation of APOE in a mouse model of tauopathy reduced glial activation which was associated with markedly decreased neurodegeneration (45). Despite progress in identifying substantial genetic risk factors and their functional link to neuroinflammation in AD pathology and progression, these risk-factor genes do not completely explain the etiology of LOAD. Specifically, inheriting these risk-factor genes does not definitely predict the development of AD (ie, some people who carry one or two APOE ε4 alleles never develop AD, and others can develop AD without APOE ε4 alleles). This suggests that other factors are involved that also disrupt the homeostatic orchestration of brain immunity and metabolism.

Although a vast literature in the field has focused on the role of CNS neuroinflammation and innate immunity in AD pathology, there is mounting interest in the contribution of the peripheral immune system. Some studies have demonstrated the presence of blood-derived leukocytes (eg, lymphocytes, monocytes, and neutrophils) in the brains of AD patients or animal models, suggesting possible involvement of the adaptive immunity in AD pathogenesis (57–59). Baruch and colleagues showed that, in a mouse model with Aβ deposition (5xFAD), transient suppression of regulatory T (Treg) cells using pharmacogenetic techniques led to a decrease in Aβ deposition (59). In a separate study, genetic ablation of adaptive immune cells, including T cells and B cells, in a mouse model with Aβ deposition, significantly accelerated amyloid pathogenesis. Interestingly, in the study, the authors observed that microglial morphology and brain

cytokine profiles were altered in the adaptive immune system deficient mice with amyloid burden, suggesting that increased amyloid burden is related to the abnormal microglial activity. Taken together, these studies support a modulatory role for the peripheral adaptive immune system in influencing the CNS innate immune system and consequently AD pathogenesis (58). Given the intimate relationship between the immune system and gut microbes, we might suspect that the peripheral immune system may bridge the gut flora to AD pathogenesis.

Experimental Evidences in the Contribution of Gut Microbiota to AD

A limited, but growing, body of evidence from AD patients and mouse models suggest that gut microbiota perturbation occurs with AD and may influence certain aspects of AD pathology (Table 1). Analysis of the gut microbiota profile in AD patients shown a stark contrast with controls (60–62). For example, one study examining AD patients showed decreased microbiota richness with a decrease in the ratio of *Firmicutes* to *Bacteroidetes* (61). Another study reported an increase in the ratio of *Firmicutes* to *Bacteroidetes* accompanied by decreased *Bacteroidetes* and similar *Firmicutes* abundance. Although these two studies support the interaction between gut microbiota and AD pathogenesis, the reason for the discrepancy in the changed composition of gut microbiota between two studies is not clear. It might be due to the differences in methodology (eg, sample size, RNA sequencing area) and/or subjects (eg, lifestyle, dietary habits). Notably, the increased ratio of *Firmicutes:Bacteroidetes* has previously been observed in patients with type 2 diabetes or obesity, implying gut dysbiosis as a common thread between pathology in other diseases (28). Proinflammatory related bacteria including *Escherichia/Shigella* are increased in AD cases, whereas anti-inflammatory related bacteria such as *Eubacterium rectale* are decreased (62) suggesting a possible link between the gut microbiome and neuroinflammation.

In a mouse model with Aβ deposition (APP/PS1), the microbiota richness was increased early in life, while the diversity was decreased with age (6–9 months) (63). In a separate study using the same animal model, the diversity of microbiota was also decreased with age (3–8 months) in the AD model, but not in wild-type mice (64). Moreover, reduced diversity was correlated with greater Aβ deposition in the hippocampus and a decline in hippocampal-dependent spatial memory. Notably, the authors highlighted that the reduction of overall diversity was accompanied by elevations in certain bacterial populations, such as *Odoribacter* and *Helicobacter*. Another study using a different Aβ deposition mouse model (5xFAD) reported an increased *Firmicutes*:*Bacteroidetes* ratio compared to wild-type mice at 9 weeks of age (65). Interestingly, in this study, Aβ peptide was found in gut tissue sections of 5xFAD mice, suggesting the possibility that, in this model, Aβ may also directly influence microbiota balance.

Altered microbiota composition is not always consistent among animal and human studies (eg, increased *Firmicutes*:*Bacteroidetes* ratio in 5xFAD vs decreased *Firmicutes*:*Bacteroidetes* ratio in AD patients). Perhaps, this is due to substantial differences in normative resident bacterial communities in each species, and/or divergence in genetic backgrounds inducing AD-like pathology in transgenic model systems (eg, differences in target promoter or differences in inducing different stages of AD stages, that is, tauopathy) (66). Until now, most animal-based studies in this field have focused on using amyloid-induced model systems. However, a recent study examined

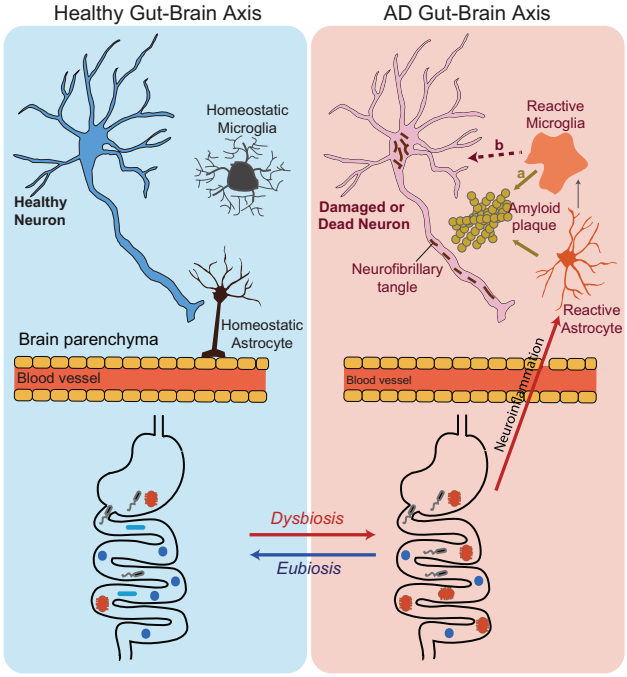

Figure 1. Hypothetical model illustrating impact of an altered gut microbiota on AD pathogenesis. A balanced composition of gut microbiota with an abundance of commensal bacteria is essential for healthy brain function (left). In the lower half of each figure, blue color represents symbiont; black color represents harmless commensal microbe; and red color represents pathobiont. In the healthy status of the gut-brain axis, homeostatic astrocytes maintain their extracellular environment and support the integrity of the brain blood barrier protecting the CNS from exposure to peripheral agents/molecules. Pathogens which do enter the CNS are scavenged by microglia. However, as pathobionts become dominant in the gut (dysbiosis), excessive proinflammatory cytokines and neurotoxic bacterial metabolites (eg, lipopolysaccharide) lead to disruption of gut permeability and blood–brain barrier integrity. This then accelerates entry of circulating inflammatory molecules and pathogens into the brain, resulting in excessive activation of innate immunity. In turn, inappropriate glial activity may worsen processes such as Aβ seeding and clearance or local effects of Aβ (**a**), or exacerbate tau-mediated neurodegeneration in later stages (**b**). AD = Alzheimer's disease; CNS = Central nervous system.

the diversity and composition of the gut microbiota in a tauopathy animal model (P301L). In the study, P301L mice had a decreased *Firmicutes*:*Bacteroidetes* ratio with age compared to wild-type controls, the pattern of which is opposite to the finding from some studies using amyloid-induced models (67). Despite this inconsistency, findings from these observational studies in AD-like animal models and AD patients strongly support gut dysbiosis influencing the pathogenesis of AD.

Several studies have attempted to demonstrate a causal link between microbiota perturbation on AD pathology using different manipulation strategies (eg, treating with antibiotics, germ-free conditions, rederiving normal microbiota, or introducing a specific pathogen). In one such study, Minter and colleagues treated an amyloid mouse model (APP/PS1 mice) with a cocktail of antibiotics during the early postnatal period. This resulted in long-term alterations of gut microbiota composition as well as reduced Aβ deposition in the hippocampus and in multiple cortical areas in APP/PS1 mice. The authors also observed that glial reactivity surrounding Aβ deposition was reduced with the early postnatal antibiotic treatment (68,69). Consistently, APP/PS1 mice raised in germ-free conditions

showed reduced Aβ deposition in the cortex and hippocampus and attenuated microglial activation compared to control mice. This result was reversed when the germ-free mice were recolonized with microbiota from conventionally raised animals (70). Taken together, these animal findings using germ-free conditions or antibiotic treatment support the notion that altering the gut microbiota in AD animal models induced changes in neuroinflammation and Aβ deposition.

At present, it remains unclear whether any single pathogen or assembly of pathogens is specifically involved in the microbiota influence on AD pathology. Some studies have tied cognitive decline and AD risk with exposure to common pathogens including *Cytomegalovirus*, *Herpes simplex virus type 1*, *Chlamydophila pneumonia*, and *Helicobacter pylori* (71). Others have even probed whether a specific microbe can induce or accelerate AD pathology. For example, recent observational studies suggest that *H. pylori* are more abundant in AD patients or animal models of aspects of AD than in controls. This has been tested experimentally: Wang and colleagues injected rats with *H. pylori* filtrate, which exacerbated $A\beta_{42}$ production in the hippocampus and spatial memory impairment (72). In addition, increased levels of *H. pylori* antibodies have been detected both in plasma and cerebrospinal fluid of AD patients (64,73).

Linking Gut Microbiota and AD Pathogenesis

As we discussed above, accumulating data support the potential contribution of the gut microbiome to AD pathology, but there are still many unanswered questions: what microbiological pathways are actually involved in pathogenesis? How is the gut microbiome able to modulate AD pathogenesis in the brain that is located distally and protected by the BBB? These are fundamentally challenging questions to address because genetic and environmental risk factors can modulate various steps in microbiota–gut–brain communication, and vice versa (Figure 2). Therefore, it is conceivable that such complex interplay may give rise to numerous putative pathways, for which several hypotheses have emerged over the years.

Various lines of reasoning argue that the immune system is the most likely bridge between the microbial community and AD pathogenesis. Microbes of the gut have considerable influence on the peripheral immune system, making it an attractive putative bridge to AD pathogenesis. Cell components and metabolites from an altered/unhealthy gut microbial community (dysbiosis caused by several factors; eg, genetic, diet, stress, age) can modulate innate and adaptive immunity in the periphery and thereby influence CNS neuroinflammatory activity (34). For example, pathogen-associated substances in the GI tract may activate mast cells that travel to other tissues and release inflammatory mediators such as cytokines and chemokines, and reactive oxygen species (74). While this process may protect against pathogens, it may also influence cells in the CNS including astrocytes, microglia, and blood vessels in the brain. Chronic mast cell activation can cause excessive neuroinflammation and contribute to neurodegeneration. In addition, BBB permeability is sensitive to proinflammatory mediators and regulation by innate immune cells including mast cells and microglia. Importantly, increased BBB permeability may facilitate brain infiltration of immune cells or mediators and thereby accelerate neuroinflammation (53,58,59,75). Postmortem analyses of AD brains have consistently demonstrated BBB damage, and cases carrying the ApoE4 variant have marked pericyte injury. In mice, ApoE deficiency causes BBB leakage through a pathway connecting the lipoprotein receptor

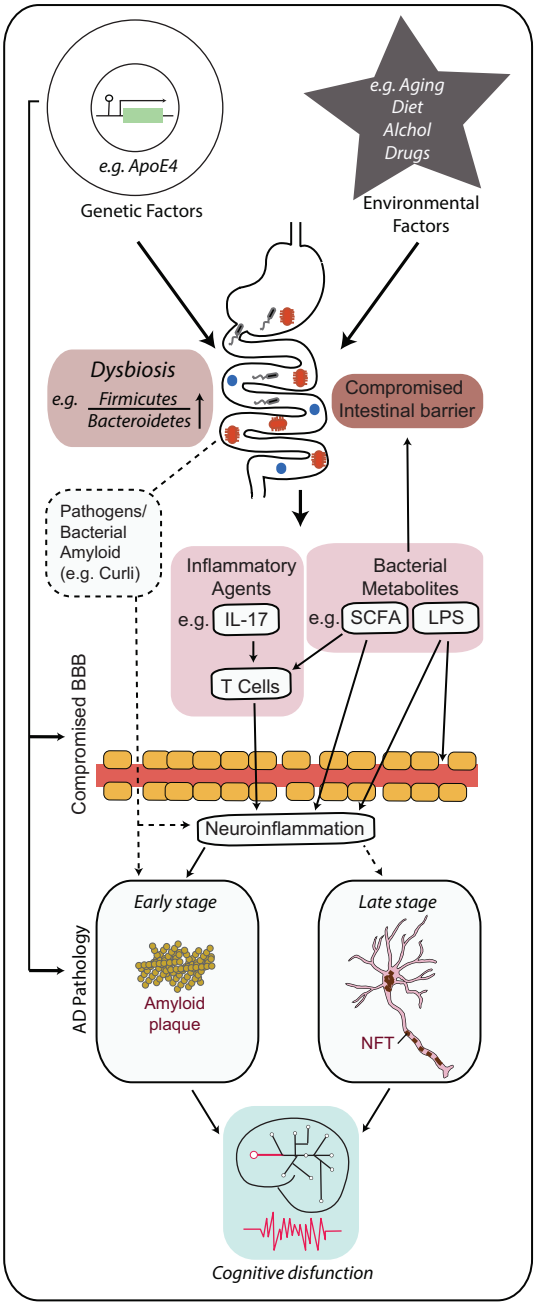

Figure 2. Schematic diagram of dysregulated gut-brain axis and its interaction with AD pathology. Arrows indicate the direction of the effect. Arrows with dashed lines indicate that no studies have explored this putative relationship yet in the AD—gut microbiome field. Multiple risk factors (top), such as genetic variants (eg, ApoE4 allele, which also can directly affect AD pathology and BBB permeability) and environmental factors (eg, aging, alcohol consumption, antibiotic drug treatment) lead to unbalanced gut microbiota composition (dysbiosis). This gut dysbiosis contributes to AD-pathology progression by generating inflammatory agents and bacterial metabolites that associate with increased intestinal barrier and BBB dysfunction. Some cytokines (eg, IL-17) and metabolites (eg, SCFA) can amplify the abundance of plasma T helper type 1 (Th1) cells, which invade the brain parenchyma. These promote neuroinflammation (ie, increase proinflammatory microglial abundance) and contribute to AD pathogenesis: amyloid-β (Aβ) deposition and neurofibrillary tangles (not investigated yet). Hypothetically the CNS-invading pathogens or toxic bacterial metabolites may directly cause or facilitate AD pathology. AD = Alzheimer's disease; BBB = Blood–brain barrier; CNS = Central nervous system; LPS = Lipopolysaccharide; SCFA = Short-chain fatty acids.

LRP1 with the CypA–nuclear factor-kB in pericytes. Notably, this signaling is suppressed in ApoE3, but not ApoE4 expressing mice (76).

It is known that peripheral infection (eg, respiratory infection by *Bordetella pertussis*) can promote T cell infiltration and stimulate neuroinflammation or Aβ deposition (71). Several recent studies have extended this role for infiltrating peripheral immune cells to bridge gut dysbiosis with Aβ-related pathogenesis. Wu and colleagues showed that, using a *Drosophila* model system of AD, oral infection with a nonpathogenic enterobacteria (*Ecc15*) resulted in a significant increase of vacuolar degeneration compared to noninfected controls. They further demonstrated that immune hemocyte recruitment into the brain may exacerbate this neurodegeneration (77). In an elegant study, Wang and colleagues used the 5xFAD model to report that shifts in gut microbiota track closely with changes in Aβ-related pathogenesis and increases of proinflammatory types of microglia. Intriguingly, these shifts were also tightly associated with the number and pattern of infiltrating proinflammatory T helper type 1 (Th1) cells in the brain (78). However, when the gut microbiota of 5xFAD mice was perturbed with antibiotic drugs, they exhibited fewer activated microglia and infiltrating Th1 cells. Collectively, these findings support the hypothesis that gut dysbiosis may stimulate the infiltration of peripheral immune cells into the brain and thereby contribute to Aβ-related pathogenesis through enhancing neuroinflammation (79).

A compromised BBB also opens the possibility for peripheral pathogens from multiple different organs to enter directly into the brain parenchyma and influence AD pathogenesis including Aβ formation. Tying Aβ directly to infectious disease, recent studies offer a putative role for Aβ in protecting the CNS from invading microorganisms. In an animal model with Aβ deposition, viral infection in the brain by *Herpesviridae* dramatically accelerated Aβ deposition which decreased viral spread (80). Similarly, it has been demonstrated that CNS-invading fungi *Candida albicans* can increase Aβ deposition as well as attracting microglia to the site of infection, thereby promoting antimicrobial activity (81). Additionally, bacterial products such as LPS, derived from the outer membrane of Gram-negative bacteria, have been detected in brains of AD patients and appear to amplify Aβ deposition. For example, intraperitoneal LPS injection, but not saline injection, elevated $A\beta_{42}$ levels in the brain of wild-type mice (82,83). Studies in AD cases or some animal models of AD show that the abundance of the phylum *Bacteroidetes*, which mostly consists of Gram-negative bacteria, increases alongside pathological insult. Collectively, such studies suggest a potentially interesting relationship tying neuroinflammation or AD pathology with elevations in *Bacteroidetes* abundance and associated LPS. In addition, a leaky gut (ie, increased intestinal permeability) may contribute to dysbiosis-related AD pathogenesis via increasing the translocation and systemic distribution of bacteria or bacterial-derived endotoxins. Examination of gut communities in AD subjects reveal reduced representation of the *Bifidobacterium* genus, which has a well-supported role in maintaining gut mucosal barrier properties. At present, studies directly examining the intestinal permeability in AD patients or model systems of AD are lacking. Similarly, the field would greatly benefit from studies assessing whether any correlation exists between intestinal permeability and genetic risk factors for AD including ApoE4 expression.

Further inspiration for putative pathways comes from the strong correlation between AD and metabolic disorders (eg, 80% of AD patients show impairment in glucose tolerance or have diabetes) (84). Specifically, certain bacterial metabolites may modulate or drive metabolic alterations in the host and could potentially influence AD pathogenesis. For example, the microbial-derived metabolite trimethylamine N-oxide (TMAO) can enter the brain and has been connected with AD. A recent study has reported that individuals with AD dementia showed elevated TMAO levels in their CSF, and interestingly the CSF TMAO was more associated with CSF p-tau, but not Aβ levels (85). It has been reported that bacterially produced bile acids, which are associated with cholesterol metabolism and clearance, are dysregulated in the serum of AD patients. Bile acids are primarily synthesized in the liver, but secondary synthesis by bacteria was elevated in AD patients beyond that of controls (86). However, it is not clear how or whether these metabolites directly influence AD pathology or neurodegeneration. Perhaps, this secondary bile acid promotes BBB permeability or influences brain metabolism. Elevated TMAO levels have been also implicated in diabetes and insulin resistance, which are known to be risk factors for developing dementia (85,87).

Short-chain fatty acids (SCFAs) have well-characterized roles in lipid/protein metabolic process and appear to have a protective role against AD. This protection may occur by conferring energy to the brain or regulating microglial maturation and function (8,88). Recent studies have examined the gut microbiota composition of humans or animal model systems carrying different ApoE alleles. Interestingly, analysis of the gut microbiota of ApoE2, E3, or E4 carriers revealed a lower abundance of the SCFA-producing *Ruminococcaceae* bacteria in ApoE4 carriers (89). SCFAs (ie, butyrate) play also plays an important role in orchestrating the integrity of intestinal barrier. It is possible that ApoE4 carriers lacking SCFA-producing bacteria face greater vulnerability to intestinal barrier loss or intestinal permeability. In addition, recent studies have elucidated the role of SCFAs in the modulation of the T cell fate through G-coupled protein receptor signaling (GPR41/GPR43) and epigenetic modifications, suggesting another possibility that microbial-SCFAs influence AD pathology through modulating peripheral inflammation (90,91).

The contribution of bacteria-derived amyloids to AD pathogenesis represents an interesting but still under-explored line of research. It has been reported that *Escherichia coli* produce extracellular amyloid fibers called curli, which also adopt a beta-sheet structure (92,93). The relationship between curli and brain Aβ amyloidosis and deposition is not clear. It is conceivable that amyloids like curli may propagate to the CNS via peripheral nerves (eg, vagus nerve), as recent studies demonstrated that α-synuclein protein, which also forms an "amyloid" in Parkinson's disease, travels along the vagus nerve from the gut to the brain where it can seed CNS synuclein and spread in the brain (94,95). Notably, a common pathway between CsgA (a major structural subunit of curli), LPS, and Aβ is the activation of toll-like receptors that are expressed in microglial cells. Given that the digestive track is a robust source of bacterial products, chronic exposure to bacterial products could over-stimulate both the peripheral and central nervous system. Increased inflammation by other factors such as aging and stress could also potentially stimulate permeability of both the GI tract and the BBB. Together, these may facilitate greater access of inflammatory agents and pathogens to the brain, altering the immunological balance (Figure 2).

Conclusion and Future Perspectives

In summary, the gut microbiota is actively involved in various aspects of human physiology, and its malfunction is closely associated with human disease including AD. Myriad factors maintain and modulate a healthy gut microbiota throughout life, including aging processes,

dietary changes, and drug/alcohol consumption, among innumerable others. Excessive, chronic, or acute shifts in critical factors may drive a proinflammatory state to influence the CNS. Additionally, genetic risk factors (eg, the presence of an ApoE4 allele) may contribute or influence the presence of more disease-associated microbiota. Finally, pathogenic agents may achieve direct access to the brain through a compromised BBB.

Progress has been made in establishing a causal relationship between alterations of the gut microbiota and AD pathogenesis. However, current studies in animal model systems have largely focused on how alterations in the microbiota affect Aβ pathology. It is hypothesized that the innate immunity of the brain has varying influences and roles in AD pathogenesis depending on early versus late phases; likewise, the relationship between AD pathogenesis and the gut microbiota likely depends on disease stage. Therefore, further systematic investigations are necessary to characterize the contribution of gut microbiota to more diverse aspects of AD pathology including tauopathy and neurodegeneration, which highly impacts cognitive function (Figure 2). In addition, links between known AD key genetic risk factors (eg, APOE and TREM2) and gut microbiota will extend our understanding on how gut microbiota contribute to AD pathology. The gut microbiota likely contributes to AD risk via influencing numerous avenues: aging, diabetes, and sleep or circadian rhythm dysfunction. For this reason, there will remain an immense number of putative pathways by which the microbiota might directly or indirectly affect AD pathogenesis.

With the increased understanding of the relationship between microbiome disruption and AD pathogenesis, shifting the gut microbiota balance towards a state of *eubiosis*, a healthy and balanced state of gut microbiota composition, will be an interesting future direction. A general concept in this field is "a diverse microbiota is a healthy microbiota." However, as we discussed above, this is not always true. For example, some studies showed increased bacterial richness in AD animal models or reduced amyloidosis alongside microbiota perturbation with antibiotic drugs (Table 1). A challenging but clinically significant barrier is the need to reduce pathological bacteria and increase beneficial bacteria, and recent testing in AD patients or animal models supports probiotics as a potential therapy. With the advent of new therapeutic tools, harnessing and manipulating the gut microbiota will represent an attractive and innovative strategy to counteract AD pathogenesis (96,97).

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Conflicts of Interest

D.M.H. is an inventor on a patent licensed by Washington University to C2N Diagnostics on the therapeutic use of anti-tau antibodies. DMH cofounded and is on the scientific advisory board of C2N Diagnostics, LLC. C2N Diagnostics, LLC has licensed certain anti-tau antibodies to AbbVie for therapeutic development. DMH is on the scientific advisory board of Denali and consults for Genentech and Idorsia.

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