NEURODEGENERATIVE DISEASES: FROM GUT-BRAIN AXIS TO BRAIN MICROBIOME, 2nd Edition

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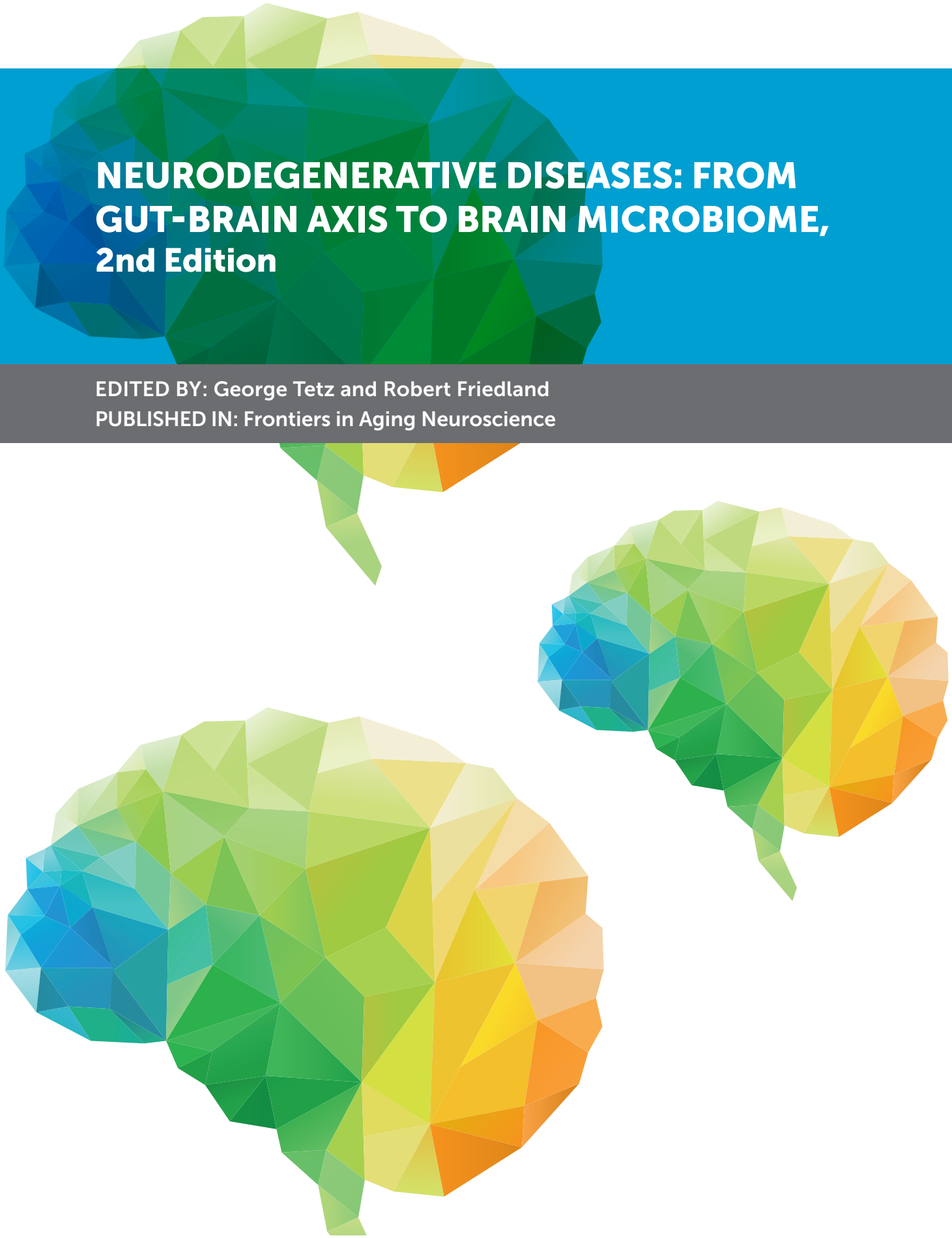

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NEURODEGENERATIVE DISEASES: FROM GUT-BRAIN AXIS TO BRAIN MICROBIOME,
2nd Edition

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Editorial: Neurodegenerative diseases: From gut-brain axis to brain microbiome

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Editorial on the Research Topic
Neurodegenerative diseases: From gut-brain axis to brain microbiome

This Research Topic was designed to explore the role of microbiota in neurodegenerative diseases. This topic is critical since it aims to shed light on the very first stages of neurodegenerative diseases. Understanding the multifaceted roles of microbiota in the development of these diseases enables the discovery of novel therapeutic targets. There are two major reasons why so many drugs for the treatment of neurodegenerative diseases have failed in the last few years (Yiannopoulou et al., 2019; Imbimbo and Watling, 2021). First, despite decades of research, the exact cause and trigger factors of these diseases have not been discovered, and neurodegenerative conditions are diagnosed when they are significantly advanced, yet the critical and irreversible pathogenic steps begins decades prior to the first clinical manifestations (Imbimbo and Watling, 2021; Sirkis et al., 2022). Second, it is impossible to develop a relevant animal model without knowledge of the exact pathogenesis. And without a clinically relevant animal model that can recapture not only familial, but also sporadic form of neurodegenerative diseases you cannot succed in clinical trials (Bjorkli et al., 2020). Therefore, understanding the triggering factors and protein misfolding in neurodegenerative diseases is the key to achieving a breakthrough in the successful prevention and treatment of neurodegenerative diseases.

Recently, microbiome-related triggering factors, such as bacterial extracellular nucleic acids and deoxyribonucleic acid (DNA) in particular, as well as extracellular DNA- and RNA-based bacterial TezR receptors or lipopolysaccharides (LPS), have been highlighted as novel and highly specific triggering factors for beta-amyloid and Tau prionogenic aggregation (Tetz et al., 2020; Tetz and Tetz, 2021, 2022a,b; Zhan et al.). The uniqueness of bacterial extracellular DNA and LPS is that they can reach the central nervous system (CNS) either through systemic circulation if the blood-brain barrier is impaired or can be released by microorganisms directly located inside the brain (bacterial presence within CNS is a benchmark of neurodegenerative diseases) (Zhan et al., 2016; Bennett et al., 2019; Dominy et al., 2019; Senejani et al., 2022). For example, high specificity in the DNA of a particular bacterial strain triggered the misfolding of proteins, while DNA of other strains did not. Such specificity could explain the recent

failure of a pivotal trial by Cortexyme Inc.; this trial was the first to use brain-localized bacteria as a therapeutic target to treat Alzheimer's disease but failed, possibly due to the overseeing the role of bacterial extracellular DNA as true triggering factor of protein misfolding in this condition (Imbimbo and Watling, 2021).

Another part of the microbiome-based research in neurodegenerative diseases is dedicated to studying the link between the gut microbiota and CNS through the modulation of the enteric nervous system. A review article published in this Research Topic by Geng et al., Shen et al., and Trejo-Castro et al. provided a comprehensive overview of the gut-brain axis in Alzheimer's and Parkinson's diseases, covering the landmark papers of the past decade. Another review in this Research Topic by Li et al. summarized the role of age-related changes in human gut microbiota and neurodegenerative diseases.

Since microbiota plays a critical role in gut-brain axis, a few papers in this Research Topic highlighted the use of different microbiota-targeting products to treat neurodegenerative diseases through the regulation of gut microbiota. Among them, Chung et al. provided an overview of the role of resveratrol in neurodegenerative diseases through the gut-brain axis.

Within the current Research Topic several research articles studying the role of microbiota and neurodegenerative diseases were published. The article published by Aimee Parker et al. reported that normal microbiota prevents dissemination of fungi to the brain in aging animals. Using germ-free mice, the authors have shown that without normal microbiome fungal gut commensals, Candida albicans, an opportunistic pathogen in humans, can traverse the gastrointestinal barrier and disseminate to brain tissue.

Liu et al. published a research article, showing how the interplay of gut microbiota and autophagy participate in the pathogenesis of Parkinson's disease. Another interesting article showed that the microbiota from subjects with neurodegenerative diseases when transferred to animals without these diseases affected the animal brains. Therefore, the transplantation of fecal microbiota from APP/PS1 mice and patients with Alzheimer's to non-affected animals enhanced endoplasmic reticulum stress in the cerebral cortex of wild-type mice (Wang et al.).

Overall, the articles published in this current issue cover the critical topic for the role of microbiota in neurodegenerative diseases. Together with previous data, they pave the way for using brain-localized bacteria and fungi, as well as those located in the gut, as novel potential therapeutic targets for the treatment of these devastating disorders.

Author contributions

GT analyzed and wrote the manuscript.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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REVIEW
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Resveratrol Modulates the Gut-Brain Axis: Focus on Glucagon-Like Peptide-1, 5-HT, and Gut Microbiota

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Resveratrol is a natural polyphenol that has anti-aging and anti-inflammatory properties against stress condition. It is reported that resveratrol has beneficial functions in various metabolic and central nervous system (CNS) diseases, such as obesity, diabetes, depression, and dementia. Recently, many researchers have emphasized the connection between the brain and gut, called the gut–brain axis, for treating both CNS neuropathologies and gastrointestinal diseases. Based on previous findings, resveratrol is involved in glucagon-like peptide 1 (GLP-1) secreted by intestine L cells, the patterns of microbiome in the intestine, the 5-hydroxytryptamine (5-HT) level, and CNS inflammation. Here, we review recent evidences concerning the relevance and regulatory function of resveratrol in the gut–brain axis from various perspectives. Here, we highlight the necessity for further study on resveratrol's specific mechanism in the gut–brain axis. We present the potential of resveratrol as a natural therapeutic substance for treating both neuropathology and gastrointestinal dysfunction.

Keywords: resveratrol, gut–brain axis, glucagon-like peptide-1 (GLP-1), 5-hydroxytryptamine (5-HT), gut microbiota

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INTRODUCTION

Resveratrol is a polyphenol that is secreted by grapes and berries (Wang et al., 2013) and could regulate insulin action, lipid metabolism, and glucose homeostasis (Chen et al., 2017). Resveratrol has been reported to have an anti-aging effect and to regulate inflammation in various organs (Buhrmann et al., 2017; Malaguarnera, 2019).

Recent researchers have highlighted the connection between the gut and brain, called the "gut–brain axis," owing to the proven linkage between many factors related with the brain and the intestine (Louwies et al., 2020; Parker C. G. et al., 2020). In addition, researchers have discussed the connection between the gut and brain as key to finding therapeutic treatments for both neurological dysfunction such as cognitive decline and impaired gastrointestinal homeostasis (Rhee et al., 2009). Many cell types associated with the enteric nervous system including enteric epithelial cells, cells of Cajal, and enterochromaffin cells are influenced by the gut–brain axis (Mayer et al., 2014b).

Numerous researchers have highlighted resveratrol as a multiple regulator in various organs including the pancreas, liver, brain, and gut (Kumar et al., 2013; Movahed et al., 2013; Caron et al., 2014). Based on previous studies, we assume that there is considerable potential for resveratrol to regulate the gut–brain axis. Here, we review the significant evidences related to resveratrol's beneficial roles in the gut and brain.

RESVERATROL

Resveratrol, a natural polyphenol, is secreted by specific plants such as grapes and berries in response to stress conditions including infection, sunlight, and climate (Singh et al., 2015; De Sa Coutinho et al., 2018).

Resveratrol could boost glucose uptake in the absence of insulin (Zhao et al., 2019). It also exerts an anti-diabetic property via enhancing mitochondrial function and an anti-aging property via promoting energy expenditure (Ren et al., 2017; Zou et al., 2017). Resveratrol induces the expression of adiponectin (one of the adipokines) and improves insulin resistance in adipocytes and inhibits the inflammatory response (Sadruddin and Arora, 2009; Timmers et al., 2011). Resveratrol is specifically known to activate mammalian nicotinamide adenosine dinucleotide-dependent deacetylase SIRT1, which is involved in regulating glucose homeostasis, lipid metabolism, and the activation of mitochondrial function (Baur, 2010; Zhou et al., 2018). SIRT1 is a NAD+-dependent protein deacetylase that is a critical regulator of energy homeostasis-dependent nutrient metabolism (Vassilopoulos et al., 2011; Aguilar-Arnal et al., 2016).

Resveratrol activates mitochondrial function and enhances insulin secretion by activating SIRT1 (Ahuja et al., 2007; Ma et al., 2017). Resveratrol could also activate SIRT2, which subsequently mimics calorie restriction and expands lifespan (Smith et al., 2009; Gambini et al., 2015).

Several other studies demonstrated that resveratrol could alleviate hyperglycemia in a diabetic mouse model and obese mouse model (Ramadori et al., 2009; Rehman et al., 2018).

In the obese mouse model, some studies demonstrated that resveratrol had improved motor dysfunction, reduced fat mass, and induced positive changes in lipid profiles (Shang et al., 2008; Rivera et al., 2009; Haley et al., 2017).

Clinically, resveratrol has been reported to improve pathologies in type 2 diabetes, cardiovascular disease, and cognitive dysfunction (Novelle et al., 2015). Moreover, resveratrol could reduce fasting blood glucose and the level of HbA1c under diabetic conditions (Bhatt et al., 2012; Movahed et al., 2013).

Mechanistically, resveratrol induces the secretion of insulin through sulfonylurea receptors mediated by the adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway (Hubbard et al., 2013) and peroxisomal proliferator-activated receptor α (PPARα) (Caron et al., 2014). Furthermore, resveratrol-induced SIRT1 activation attenuates inflammatory responses and pro-inflammatory cytokine secretion mainly through NF-kB- and AP-1-dependent signal pathways (Deng et al., 2008; Dao et al., 2011; Xu L. et al., 2018) (**Figure 1**).

In the CNS, resveratrol protects neurons which were damaged under oxidative conditions and contributed to low levels of antioxidant defense enzymes, which ultimately enhanced memory function (Kumar et al., 2013).

Similarly, another study showed that resveratrol has a neuroprotective effect via its anti-inflammatory action by regulating various neurotransmitters such as brain-derived neurotrophic factor (BDNF) and phosphodiesterases (PDEs) (Chung, 2012) (**Figure 1**).

Additionally, resveratrol activates the *SIRT1* gene, considered an anti-aging related gene, in the duodenum and also rescues insulin resistance and improves neuronal networks in the brain (Cote et al., 2015).

Considering these findings, resveratrol has beneficial effects against stress conditions such as inflammation, oxidative stress, hyperglycemia, and dyslipidemia. Further, resveratrol influences various organs including the brain and intestine through blood and may act as a crucial mediator in the gut–brain axis.

RESVERATROL AND THE GUT–BRAIN AXIS

Resveratrol Contributes to the Gut–Brain Axis by Regulating the Expression of GLP-1

Lately, the relationship between the gut and brain has emerged as a critical issue for treatment of neuronal disorders, such as depression and dementia, as well as gastrointestinal diseases, such as diarrhea and irritable bowel syndrome (Haj Kheder et al., 2018; Simren et al., 2018, 2019).

Based on recent researches, the pathogenesis of gastrointestinal diseases is related to the connection between the neuroendocrine network and gastrointestinal function (Koloski et al., 2012; Browning and Travagli, 2014; Yarandi et al., 2016).

Glucagon-like peptide-1 (GLP-1), an incretin hormone and a major hormone of the gut–brain axis, is linked to the control of energy homeostasis and the development of obesity (Salehi and Purnell, 2019).

GLP-1 is produced from intestinal L cells and stimulates the secretion of insulin. It enhances impaired glucose and lipid metabolism and also inhibits inflammation (Liu et al., 2013; Shah et al., 2013; Mulvihill, 2018). The major role of GLP-1 is to stimulate insulin secretion by inducing pancreatic beta cell proliferation (Morris, 2017). GLP-1 crosses the blood–brain barrier (BBB) and influences the brain as well as diverse organs (Hunter and Holscher, 2012).

In the brain, GLP-1 is synthesized by specific neurons within the nucleus of the solitary tract (Tauchi et al., 2008; Card et al., 2018). Subsequently, these GLP-1 producing neurons project to wide brain areas including the hypothalamus and cortex (Llewellyn-Smith et al., 2011; Ghosal et al., 2013).

One study demonstrated that central administration of GLP-1 leads to marked improvement of neuronal function in several brain regions such as the paraventricular nucleus, area postrema, supraoptic nucleus, arcuate nucleus, and nucleus tractus solitarius (Tauchi et al., 2008).

Another study showed that GLP-1R agonist exendin-4 could increase c-fos expression on neurons in various brain regions including nucleus tractus solitarius (Sarkar et al., 2003; Baggio et al., 2008).

GLP-1 could rapidly control glucose homeostasis after food intake, because GLP-1 receptors are located in the intestine, portal vein, pancreas, and brain, and also GLP-1 induces vagal afferent neurons innervated into gut (Iwasaki et al., 2018).

One study showed that GLP-1R antagonist's administration damages glucose tolerance and aggravates insulin resistance (Vahl et al., 2007).

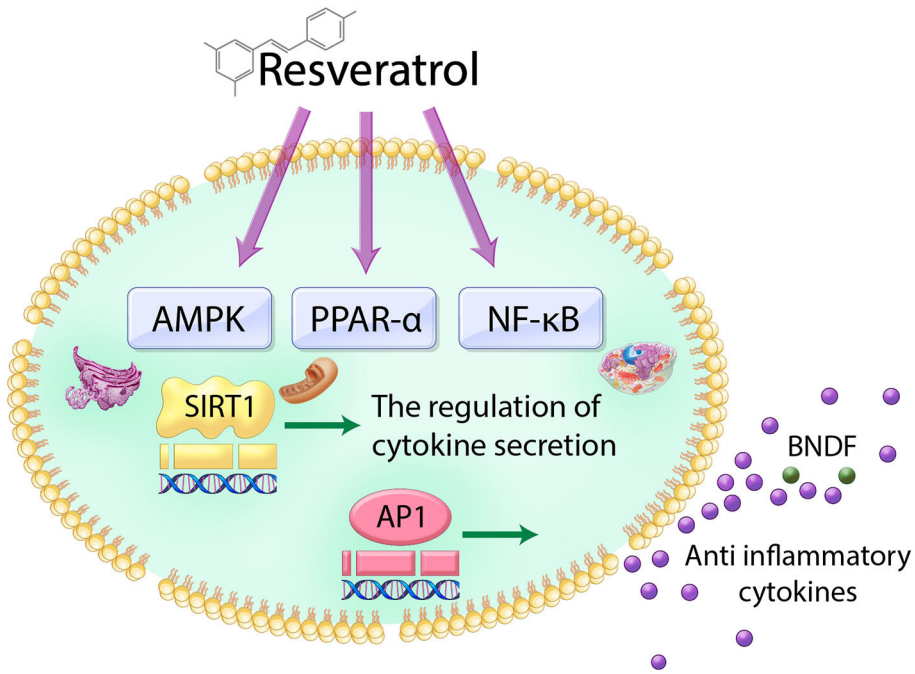

FIGURE 1 | Schematic image of the action of resveratrol in cell. Resveratrol triggers the NF-κB, AMPK, and PPAR-α and subsequently regulates AP-1 and SIRT1 genes. Therefore, the action of resveratrol in the cell leads to the increase of antioxidant cytokine secretion and neurotrophic factor BDNF.

In addition, another study demonstrated that the inhibition of GLP-1R in brain aggravates glucose homeostasis and insulin sensitivity (Knauf et al., 2008). Taken together, GLP-1 and GLP-1R agonist act as the important regulators of glucose homeostasis and insulin action both in systemic circulation and in CNS.

Some studies have suggested that GLP-1 has neuroprotective roles (Martin et al., 2009; Wang et al., 2018) in neurodegenerative diseases, such as Alzheimer's disease (Holscher, 2014), Parkinson's disease (Li et al., 2009), and stroke (Darsalia et al., 2012, 2018).

One study demonstrated that GLP-1 receptor signaling is considerably related to the connection between diabetes and the brain (Duarte et al., 2013). Other studies have reported that GLP-1 and its analogs including exendin-4 could protect neurons against oxidative stress in brain with dementia and dementia mimic *in vitro* cell models (An et al., 2015; Chen et al., 2016; Wang et al., 2018). Based on these findings, GLP-1 is a major gut hormone that circulates in the body and influences brain function.

One study also reported that treatment with GLP-1 receptor agonist could enhance both glycemic control and memory function (Berezin, 2016).

The positive correlation between impaired glycemic control like hyperglycemia and memory loss caused by neuronal cell damage have been proved as previous significant data (Biessels and Gispen, 2005; Carvalho et al., 2012).

Previous study mentioned that GLP-1 could be used as a controller for diabetes and ultimately GLP-1 mimic and GLP-1R agonists such as liraglutide clinically could be used for patients with diabetes in the present time (Vilsboll and Holst, 2004; Amori et al., 2007; Campbell and White, 2008; Gallwitz, 2011; Duarte et al., 2013).

Furthermore, GLP-1 may contribute to memory loss as well as improved systemic body function in diabetes by enhancing glucose tolerance and insulin resistance, suggesting that impaired glucose metabolism and poor insulin sensitivity aggravates memory loss and neuroinflammation (Rom et al., 2019).

A recent study mentioned that GLP-1R agonists improve synaptic dysfunction, strengthen long-term potentiation (LTP), and finally lead to improved cognitive function (Isacson et al., 2011).

Several studies reported that GLP-1R agonists and GLP-1 analogs could promote learning and memory function (During et al., 2003; Abbas et al., 2009), attenuate neuroinflammation against brain damage (Hattori et al., 2010), and promote neurite outgrowth, leading to stable neural connectivity (Perry et al., 2002).

One study demonstrated that GLP-1 receptor agonist exenatide could ameliorate cell stress response through SIRT1 (Xu et al., 2014).

These functions of GLP-1 receptor agonist are strongly linked to the activation of SIRT1, which could be promoted by resveratrol (Samson and Bajaj, 2013; Xu et al., 2014).

Another *in vitro* study reported that GLP-1 protects cellular apoptosis through the activation of SIRT1 in *in vitro* system (Shi and Huang, 2018).

Several studies have reported that GLP-1 and the GLP-1 receptor stimulate the activity of FoxO1 that plays a crucial

role in cellular metabolism through SIRT-1-dependent FoxO1 deacetylation and Akt-dependent FoxO1 phosphorylation (Bastien-Dionne et al., 2011; Daitoku et al., 2011; Lee et al., 2012). Other studies demonstrated that SIRT1 activation by the GLP-1 agonist exendin-4 treatment protects mice under a high fat diet condition (Lee et al., 2012) and attenuates palmitate-induced ER stress and mitochondrial dysfunction (Lee et al., 2014). Resveratrol increases the release of GLP-1 in a high-fat-fed diabetes mouse model (Dao et al., 2011) and improves the epithelial cells of the intestine (Zhuang et al., 2019).

Another study demonstrated resveratrol does not directly affect the release of GLP-1 (Knop et al., 2013; Thazhath et al., 2016). Thus, the mechanism of resveratrol on the release of GLP-1 is controversial until now; we need to investigate more directly to understand the mechanism of resveratrol about the release of GLP-1.

Based on these previous studies, we assume that resveratrol could promote the effect of GLP-1 in the intestine and CNS through the activation of diverse genes such as SIRT1 and Foxo genes. Further studies on the genetic and cellular mechanisms elicited by resveratrol via GLP-1 may be helpful to understand the correlation between resveratrol and the gut–brain axis.

Resveratrol Contributes to the Gut–Brain Axis by Involving 5-HT

Resveratrol has regulatory functions in the gut–brain axis through another hormone pathway as well as the GLP-1 pathway. Serotonin 5-hydroxytryptamine (5-HT) is expressed in both the CNS and gastrointestinal tracts, and currently 5-HT has been considered as an important target in the gut–brain axis.

5-HT is a growth factor, a paracrine factor, and an enteric neurotransmitter (Gershon and Tack, 2007), which is mainly found in the gut enterochromaffin cells. It is directly linked to depressive behavior, sleep pattern, food appetite, sexual behavior, or the control of temperature (Li et al., 2011; Yohn et al., 2017). 5-HT influences maintenance of the gastrointestinal mucosa and modulates the enteric nervous system (Gross et al., 2012).

Enterochromaffine cells in gut produce intrinsic afferent neurons of myenteric plexus in gut and are influenced by 5-HT_3 antagonist and 5-HT_4 agonist's inhibition (Bertrand et al., 2000; Bertrand and Bertrand, 2010; Hoffman et al., 2012). Previous studies mentioned that 5-HT produced from enterochromaffin cells in gut could promote sensory nerve activation and finally contribute to neuronal electrical activity evoked in CNS (Johanson, 2004; Chey and Cash, 2005).

Furthermore, 5-HT derived from gut protects gastrointestinal cells against neuroinflammation (Linden et al., 2005; Spohn et al., 2016).

A previous study demonstrated that the 5-HT neurotransmitter derived from brain promotes cyclic AMP (cAMP) synthesis through 5-HT receptors (Prasad et al., 2019). The change in cAMP signaling could affect both the neuropathology of major depressive disorder in CNS and gastrointestinal epithelial dysfunction in gut (Reierson et al., 2011; Cheung et al., 2019). Thus, the regulation of cAMP signaling by 5-HT should be studied further because cAMP signaling in gut and in brain contributes to various neuronal functions. Moreover, an impaired 5-HT system in gut triggers irritable bowel syndrome, and the gastrointestinal motility is increased (Grenham et al., 2011). In addition, the receptors of 5-HT have been reported to be directly involved in depression (Celada et al., 2004), anxiety, and stress-induced dyspeptic ulcers (O'mahony et al., 2006).

Considering previous data, 5-HT derived from gut and brain contributes to nervous systems globally, and the circulation of 5-HT in the body mediates the gut–brain axis (Yano et al., 2015).

A current study proved that resveratrol regulates the gut–brain axis by controlling the 5-HT-dependent pathway in an irritable bowel syndrome rat model and specifically that resveratrol influences various organs including brain hippocampus, ileum, and colon through 5-HT axis (Yu et al., 2019). One recent study highlighted that resveratrol contributes to many pathological responses through 5-HT_{2C} receptor-dependent signaling (Peng et al., 2018).

Another recent study demonstrated that resveratrol could increase the expression of 5-HT, leading to the improvement of brain function (Nabavi et al., 2017). Furthermore, the neuroprotective function of resveratrol in the depressive brain hippocampus was proved to be exerted via 5-HT (Xu et al., 2010). Most of the released 5-HT is stored in enteroendocrine cells in the intestine, and therefore gut homeostasis is important to maintain the 5-HT level in the body (Enck et al., 2016).

Several studies have mentioned the neurological role of resveratrol in depression and anxiety (Yu et al., 2013; Li et al., 2017) and the gut homeostasis-related role of resveratrol in stress-induced irritable bowel syndrome (Xu Y. et al., 2018).

One current study reported that the inhibition of 5-HT release attenuates the activation of GLP-1 receptor signaling and highlighted the relationship between GLP-1 and 5-HT serotonin system (Anderberg et al., 2017).

Another study mentioned that GLP-1 receptor agonist liraglutide could reduce the expression of 5-HT2A receptor and subsequently reduces body weight and inhibits serotonin synthesis in mice model (Nonogaki and Kaji, 2018).

TABLE 1 | The relationship between resveratrol and gut–brain axis.

Relevance between resveratrol and gut-brain axis	References
Resveratrol and gut–brain axis	
Resveratrol and GLP-1	Bastien-Dionne et al., 2011; Daitoku et al., 2011; Dao et al., 2011; Lee et al., 2012, 2014; Samson and Bajaj, 2013; Xu et al., 2014; Shi and Huang, 2018; Zhuang et al., 2019
Resveratrol and 5-HT	Xu et al., 2010; Yu et al., 2013, 2019; Enck et al., 2016; Li et al., 2017; Nabavi et al., 2017; Peng et al., 2018; Xu L. et al., 2018
Resveratrol and gut microbiota	Dao et al., 2011; Wu et al., 2011; Amri et al., 2012; Rotches-Ribalta et al., 2012; Bode et al., 2013; Qiao et al., 2014; Hsieh et al., 2015; Basholli-Salihu et al., 2016; Ling et al., 2016; Bird et al., 2017; Carrera-Quintanar et al., 2018; Hu et al., 2019

Ripken et al. suggested that serotonin treatment could boost GLP-1 release, and the blocking of 5-HT receptor could affect the production of GLP-1 (Ripken et al., 2016).

A recent study proved that 5-HT enterochrnomaffin cells in gut regulates gut microbial metabolism and homeostasis and is affected by the activation of GLP-1 (Lund et al., 2018).

Further, ghrelin, known as a hormone for regulation of motivation and reward system among brain function, has been interacted with GLP-1 and the monoamine transmitter 5-HT (Currie et al., 2010; Abtahi et al., 2019).

GLP-1 derived from brain mainly is produced by the nucleus tractus solitarius in brain (Alhadeff et al., 2012). GLP-1 receptors are expressed in various brain areas including the hypothalamus, and GLP-1 projects to neurons in the ventral tegmental area, nucleus accumbens, and 5-HT-producing neurons in the dorsal raphe (Anderberg et al., 2017). Based on previous studies, the activation of GLP-1 leads to the release of 5-HT in brain, which is related with neurological behavior pattern.

Given previous evidences, resveratrol can control 5-HT and its receptor and also modulate release of 5-HT through GLP-1 regulation. Ultimately, resveratrol could control the neuropathology of neurological diseases such as depression and stress-induced anxiety. Also, resveratrol can regulate gut dysfunction in irritable bowel syndrome via 5-HT. Thus, we emphasize the necessity for further study of the specific mechanism and cellular pathways regulated by resveratrol and mediated by 5-HT to fully understand the gut–brain axis.

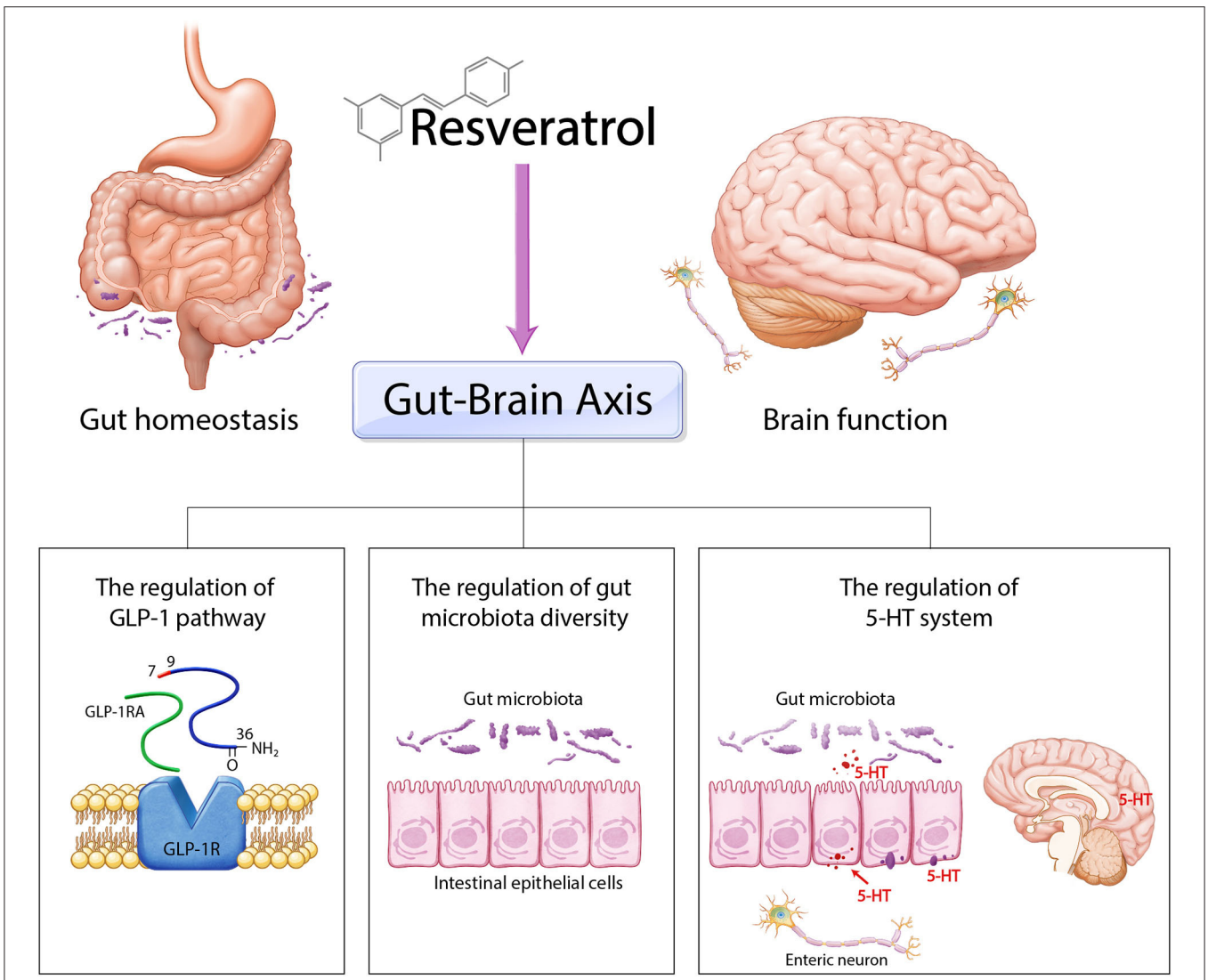

FIGURE 2 | Schematic image of the role of resveratrol in gut–brain axis. Resveratrol could control gut–brain axis through three ways. Firstly, resveratrol regulates gut homeostasis and brain homeostasis by GLP-1 pathway. Secondly, resveratrol modulates gut microbiota diversity and subsequently is involved in gut–brain axis. Finally, resveratrol is related with the 5-HT system and ultimately contributes to the regulation between gut homeostasis and brain function.

Resveratrol Modulates the Gut–Brain Axis by Involving Gut Microbiota

Resveratrol is involved in the gut–brain axis through another mode in addition to the GLP-1 pathway and 5-HT system.

Recently, gut microbiota is emerging as an important node in the gut–brain axis (Louwies et al., 2020). A previous study suggested that there is an interaction between intestinal microbes and the brain and proved that intestinal microbes could dramatically enhance encephalopathy (Parker A. et al., 2020). Significant data from other studies support the function of microbiota in neurological disorders such as anxiety, autism, and depression (Mayer et al., 2014a; Sharon et al., 2019; Sun et al., 2019; Du et al., 2020).

Resveratrol administration could be metabolized by the liver, intestinal tract, and gut microbiota (Walle, 2011). A recent study demonstrated that gut microbiota contributes to metabolization of resveratrol precursors to resveratrol and also could increase resveratrol's bioavailability (Rotches-Ribalta et al., 2012; Basholli-Salihu et al., 2016). Dihydroresveratrol, 3,4′-dihydroxybibenzyl, and 3,4′-dihydroxy-*trans*-stilbene have been reported to be the major microbiota-derived metabolites made from resveratrol (Juan et al., 2010; Bird et al., 2017; Brandt et al., 2018).

Specifically, dihydroresveratrol as a metabolite of resveratrol is produced in the intestines such as the cecum, colon, and rectum through fermentation by the gut microbiota (Amri et al., 2012; Hu et al., 2019). Moreover, resveratrol was also glycosylated in the intestine to produce piceid (Rotches-Ribalta et al., 2012). Given that resveratrol was metabolized by gut microbiota (Bode et al., 2013; Carrera-Quintanar et al., 2018), resveratrol could influence the composition and diversity of gut bacteria (Carrera-Quintanar et al., 2018). Likewise, resveratrol and gut microbiota could influence each other. Specifically, *Bifidobacteria infantis* and *Lactobacillus acidophilus* are strongly linked to piceid production from resveratrol (Basholli-Salihu et al., 2016). Interestingly, a study demonstrated that resveratrol promotes gut microbiota diversity by suppressing the growth of *Enterococcus faecalis* and increasing the *Lactobacillus* and *Bifidobacterium* populations (Qiao et al., 2014).

Recently, resveratrol has been reported to improve gut microbiota in bowel diseases under harsh oxidative stress (Hu et al., 2019). One study suggested that resveratrol attenuated inflammation and improved effects of GLP-1 such as the secretion of insulin and ultimately induced a prebiotic effect to control gut microbiota in a diabetic mouse model (Dao et al., 2011).

A clinical study has reported that resveratrol treatment exerts cardiovascular and anti-obesity effects by ameliorating gut microbiota diversity (Bird et al., 2017). Resveratrol enhances the improvement of gut permeability and the integrity of intestinal tight junction proteins by controlling gut microbiota diversity (Hsieh et al., 2015; Ling et al., 2016). It has also been reported that resveratrol influences the glucuronidation and sulfation reactions in the duodenum (Wu et al., 2011).

These previous findings demonstrate that resveratrol and gut microbiota influence each other. Furthermore, resveratrol could enhance the gut microbiota diversity and the gut barrier's homeostasis. These effects of resveratrol should be investigated further to determine the specific gut bacteria that affect the gut–brain axis.

CONCLUSIONS

Here, we reviewed previous significant evidence of the effect of resveratrol on the gut–brain axis (**Table 1**). We summarized three regulatory nodes of resveratrol in the gut–brain axis including the regulation of GLP-1, the involvement of the 5-HT system, and the control of gut microbiota diversity (**Figure 2**). Resveratrol modulates various cellular responses such as lipid droplet accumulation and insulin resistance and regulates diverse cellular signalings including AMPK, cAMP, and NF-κB signaling and also controls the balance of neurotransmitters such as BDNF and 5-HT, involved in both the progression of neuropathology and gut homeostasis. Hence, we emphasize the necessity for further experimental study about the specific mechanism of resveratrol in gut and brain. Taken together, we suggest that the application of resveratrol as a natural polyphenol for treatment of both neurological disorders and intestinal dysfunction may be a safe and effective therapeutic solution for CNS and intestinal diseases simultaneously.

AUTHOR CONTRIBUTIONS

JC, J-HJ, and JS contributed to the writing of the text. JC and J-HJ made and revised all figures. JS wrote and finalized the revised manuscript. All authors contributed to the article and approved the submitted version.

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The Association Between the Gut Microbiota and Parkinson's Disease, a Meta-Analysis

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Patients with Parkinson's disease (PD) were often observed with gastrointestinal symptoms, which preceded the onset of motor symptoms. Neuropathology of PD has also been found in the enteric nervous system (ENS). Many studies have reported significant PD-related alterations of gut microbiota. This meta-analysis was performed to evaluate the differences of gut microbiota between patients with PD and healthy controls (HCs) across different geographical regions. We conducted a systematic online search for case-control studies detecting gut microbiota in patients with PD and HCs. Mean difference (MD) and 95% confidence interval (CI) were calculated to access alterations in the abundance of certain microbiota families in PD. Fifteen case-control studies were included in this meta-analysis study. Our results showed significant lower abundance levels of *Prevotellaceae* (MD = −0.37, 95% CI = −0.62 to −0.11), *Faecalibacterium* (MD = −0.41, 95% CI: −0.57 to −0.24), and *Lachnospiraceae* (MD = −0.34, 95% CI = −0.59 to −0.09) in patients with PD compared to HCs. Significant higher abundance level of *Bifidobacteriaceae* (MD = 0.38, 95%; CI = 0.12 to 0.63), *Ruminococcaceae* (MD = 0.58, 95% CI = 0.07 to 1.10), *Verrucomicrobiaceae* (MD = 0.45, 95% CI = 0.21 to 0.69), and *Christensenellaceae* (MD = 0.20, 95% CI = 0.07 to 0.34) was also found in patients with PD. Thus, shared alterations of certain gut microbiota were detected in patients with PD across different geographical regions. These PD-related gut microbiota dysbiosis might lead to the impairment of short-chain fatty acids (SCFAs) producing process, lipid metabolism, immunoregulatory function, and intestinal permeability, which contribute to the pathogenesis of PD.

Keywords: Parkinson's disease, non-motor symptoms, gut-brain axis, gut microbiota, meta-analysis

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INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive, multisystem neurodegenerative movement disorder (Poewe et al., 2017). Patients with PD suffer from characteristic motor symptoms including resting tremor, bradykinesia, rigidity, and gait abnormalities, as well as non-motor symptoms such as hyposmia, sleep disorders, depression, and gastrointestinal (GI) symptoms

(Kalia and Lang, 2015). Up to 80% of patients with PD are observed with constipation, the most common GI symptom in PD and are often preceded by the onset of motor symptoms by years (Su et al., 2017). Thus, the constipation symptom is regarded as a clinical biomarker for diagnosing prodromal PD (Berg et al., 2015). The main neuropathological characteristics of PD are loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies (LBs) or Lewy neurites, which consist of the abnormal α-synuclein aggregates (Abeliovich and Gitler, 2016). Braak staging traced the course of pathology, stating that PD started when a pathogen enters the body *via* the nose or the GI system (Braak et al., 2003), leading to the formation of LBs and spreading from the enteric nervous system (ENS) to the central nervous system (CNS) through the vagus nerve (Rietdijk et al., 2017). Therefore, the role of the "gut-brain axis" started drawing more attention in investigating the pathogenic mechanism of PD.

Higher susceptibility to PD was observed when intestinal infection existed (Huang et al., 2018; Brudek, 2019), which might trigger PD-like symptoms (Matheoud et al., 2019). PD-derived gut microbiota could enhance α-synuclein-mediated motor deficits and brain pathology in a mouse model, while germ-free mouse PD model showed milder α-synuclein pathology (Sampson et al., 2016). Thus, intestinal microbiota disturbance could be considered as a potential risk factor for PD. Gut microbiota is a complex system, producing all sorts of protective compounds and acting as a barrier against pathogens (Nair et al., 2018). Growing evidence has indicated that the abnormality of gut microbiota and its metabolic products may be triggers for the formation of LBs in the ENS. The *Hepatitis C virus* infection was thought to be dopaminergic toxic, which was similar to the effect of 1-methyl-4-phenylpyridinium (MPP+; Wu et al., 2015). The *helicobacter pylori* infection was observed to be associated with an increased risk of PD and worse PD motor severity (Shen et al., 2017). However, although fungal DNA and proteins were detected in post-mortem PD brains, there was no compelling evidence of gut microbiome contribution to PD pathophysiology (Cirstea et al., 2020), which still needs further investigation.

Recently, studies mainly focused on the bacterial component of microbiota in fecal samples. And PD-related alterations of abundance and equilibrium of gut microbiota were reported (Keshavarzian et al., 2015; Hill-Burns et al., 2017; Mertsalmi et al., 2017; Heintz-Buschart et al., 2018; Tetz et al., 2018; Aho et al., 2019; Barichella et al., 2019; Li C. et al., 2019; Li F. et al., 2019; Ren et al., 2020). Significant reduction of several gut microbiota's metabolic products were found in patients with PD, which may contribute to constipation in patients with PD (Unger et al., 2016). Functional differences in β-glucuronate and tryptophan degrading pathways were revealed in patients with PD compared to healthy controls (HCs) (Bedarf et al., 2017). Putative neuroprotective bioactive molecules such as short-chain fatty acids (SCFAs), ubiquinones, and salicylate, as well as neurodegeneration related compounds such as ceramides, sphingosine, and trimethylamine N-oxide, were altered in PD (Tan et al., 2020). Several gut microbiota were also found to be correlated with the clinical characteristics of PD, including disease duration, motor symptom severity, and non-motor symptoms (Qian et al., 2018). In addition, metabolome compositional differences such as the lower SCFAs were associated with poorer cognition, and lower butyrate levels were correlated with worse postural instability gait disorder scores in PD (Tan et al., 2020). A 2-year follow-up study indicated that the total counts of gut microbiota decreased during the course of PD progression and differed between deteriorating and stable PD groups, which may be used as a diagnostic tool for monitoring the progression of PD (Minato et al., 2017). An index was calculated based on 25 gene markers from the gut microbiota that were significantly changed in PD, and this potential diagnostic biomarker had the power to distinguish patients with PD from multiple system atrophy patients (Qian et al., 2020). Furthermore, alterations in the gut microbial activities could possibly lead to heterogeneous responses to levodopa observed among patients with PD, including decreased efficacy and harmful side effects (Maini Rekdal et al., 2019). Therefore, the pathogenesis and clinical manifestations of PD may be related to the dysfunction of the "gut microbiota-gut-brain axis."

However, the gut microbiota structure varied across different geographical regions, which might lead to inconsistent results. Therefore, in order to verify the shared variations of certain gut microbiota, which presented relatively stable in patients with PD, we performed a meta-analysis to review the alterations of gut microbiota in patients with PD compared to HCs around the world and discussed its possible role in PD.

METHODS

This meta-analysis was performed in accordance with the guidelines of the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group on meta-analyses of observational studies (Stroup et al., 2000), and also referenced the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (Liberati et al., 2009).

Literature Search

In order to identify relevant studies of gut microbiota analysis in PD for this meta-analysis, a systematic literature search was conducted using the following English and Chinese databases (up to August 2020): PubMed, Web of Science, Chinese National Knowledge Infrastructure (CNKI) databases, and Wanfang database. The search strategy to identify all potential studies involved using combinations of the following terms: (Parkinson's disease OR Parkinson disease OR Parkinsonism) AND (microbes OR microbiome OR microbiota OR bacteria) in Title/Abstract. We also manually searched the references cited in the selected articles or reviews to identify additional relevant studies.

Study Selection

The inclusion criteria for this meta-analysis were as follows: (1) gut microbiota studies comparing patients with PD with HCs; (2) fecal samples; (3) microbiota abundance being expressed as mean proportions of each microbiota; and (4) ability to obtain the mean difference (MD) with 95% confidence interval (CI) in these two groups or sufficient data to calculate these. Studies were excluded

if they were any of the following: (1) failure to finally obtain sufficient data; (2) duplicate data reported in other studies; and (3) case-only studies, family-based studies, intervention studies, and review articles. Two investigators (Shen and Yue) jointly screened for eligible studies by reading the titles and abstracts of identified studies, then carefully reviewed full articles of the rest studies, and excluded studies not meeting the inclusion criteria.

Data Extraction

Two investigators (Shen and Yue) independently extracted data from included studies including the following items: (1) general information (author, year of publication, and location); (2) patient characteristics (gender and age); (3) experimental methods (diagnostic criteria, sample size, and microbiology assessment technique); and (4) effect size of microbiota abundance (MD and 95% CI). Graphs and plots were also common forms of data reports. Because some articles showed relevant data indirectly, the software GetData Graph Digitizer 2.25 (http://getdata-graph-digitizer.com/) was applied to digitize and extract sufficient data (Fedorov, 2012; Liao et al., 2015; Tang et al., 2015).

Quality Assessment

Three investigators (Shen, Yue, and He) independently rated the quality of the included studies. The nine-star Newcastle-Ottawa Quality Assessment Scale (NOS) for case-control studies was used to assess the methodological quality of the included studies (Stang, 2010). Disagreements were solved through discussion or involvement of a third investigator, if necessary. The NOS scale includes three criteria: selection, comparability, and exposure. The selection criteria included four items: (1) adequate case definitions, (2) the representativeness of the cases, (3) the selection of controls, and (4) the definition of controls. The comparability criteria included one item: control for an important factor. The exposure criteria included three items: (1) the ascertainment of exposure, (2) the same method of ascertainment for cases and controls, and (3) the non-response rate.

Statistical Analysis

Statistical analysis was performed by Review Manager 5.3 software to compare the abundance level of some gut microbiota in patients with PD with HCs if the number of studies for a single bacterium was five or more. Included studies provided means for continuous variables, and we calculated mean difference (MD) and 95% confidence interval (CI) of microbiota abundance as summary statistics. The heterogeneity between studies was assessed using the I^2 statistics. An I^2 value closer to 0% was considered as low heterogeneity, $I^2 > 50\%$ was considered to indicate substantial heterogeneity, and I^2 value closer to 100% was considered as large heterogeneity. Data analysis was performed using the fixed-effect model with low significant heterogeneity or using a random-effect model with substantial and large heterogeneity ($I^2 > 50\%$). The level of significance was set at $p < 0.05$. A funnel plot was applied to estimate the potential publication bias. Asymmetry of funnel plots indicates significant heterogeneity between selected studies, which lead to publication bias.

RESULTS

Study Selection and Characteristics

The screening process is summarized in a flow diagram (**Figure 1**). The literature database searches yielded 922 potentially relevant records, of which, 192 duplicates were removed, and 696 obviously irrelevant publications were excluded after the review of titles and abstracts. After a further review of full texts, eight records were excluded due to lack of control group, incomplete data, or non-fecal samples. Of the 24 records that were included in the systematic review, 14 studies were included in this meta-analysis after removing nine studies that were not able to provide quantitative data about gut microbiota abundance at the family level and one study that had unmatched age between two groups. The main characteristics of these studies are summarized in **Table 1**. All the 14 studies were observational case-control studies and were considered relatively high quality according to the NOS scale (**Table 2**). These studies were conducted in the USA (Hill-Burns et al., 2017), Finland (Scheperjans et al., 2015; Aho et al., 2019), Germany (Unger et al., 2016; Bedarf et al., 2017; Hopfner et al., 2017; Heintz-Buschart et al., 2018), Russia (Petrov et al., 2017), China (Li et al., 2017; Lin et al., 2018; Li C. et al., 2019; Li F. et al., 2019; Ren et al., 2020), and Italy (Barichella et al., 2019). This meta-analysis involved 959 patients with PD and 744 HCs. The mean age of included patients ranged from 62.2 to 76.5 years old, and the number of female subjects ranged from 0.00 to 76.9%. All the 14 included studies obtained gut microbiota from a fecal sample, one of them evaluated microbiota abundance through quantitative polymerase chain reaction (qPCR), and the other 13 studies used the next-generation sequencing (NGS) technique.

Meta-Analysis of Standardized Mean Difference

We extracted continuous data from included studies to conduct the meta-analysis. We analyzed the alterations of the abundance of *Prevotellaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Faecalibacterium*, *Ruminococcaceae*, *Verrucomicrobiaceae*, *Enterobacteriaceae*, *Bacteroidaceae*, *Christensenellaceae*, and *Lachnospiraceae* in patients with PD compared to HCs in the present study. A fixed-effect model was used to evaluate the alterations of *Faecalibacterium*, *Bacteroidaceae*, and *Christensenellaceae* families due to low heterogeneity between studies, while a random-effect meta-analysis was performed in evaluating the abundance of *Prevotellaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Verrucomicrobiaceae*, *Enterobacteriaceae*, and *Lachnospiraceae* families due to substantial and large heterogeneity.

This meta-analysis showed significant lower abundance levels of *Prevotellaceae* (MD = −0.37, 95% CI = −0.62 to −0.11; $I^2 = 72\%$; $p = 0.005$; nine studies; **Figure 2A**), *Faecalibacterium* (MD = −0.41, 95% CI = −0.57 to −0.24; $I^2 = 52\%$; $p < 0.00001$; five studies; **Figure 2B**), and *Lachnospiraceae* (MD = −0.34, 95% CI = −0.59 to −0.09; $I^2 = 67\%$; $p = 0.009$;

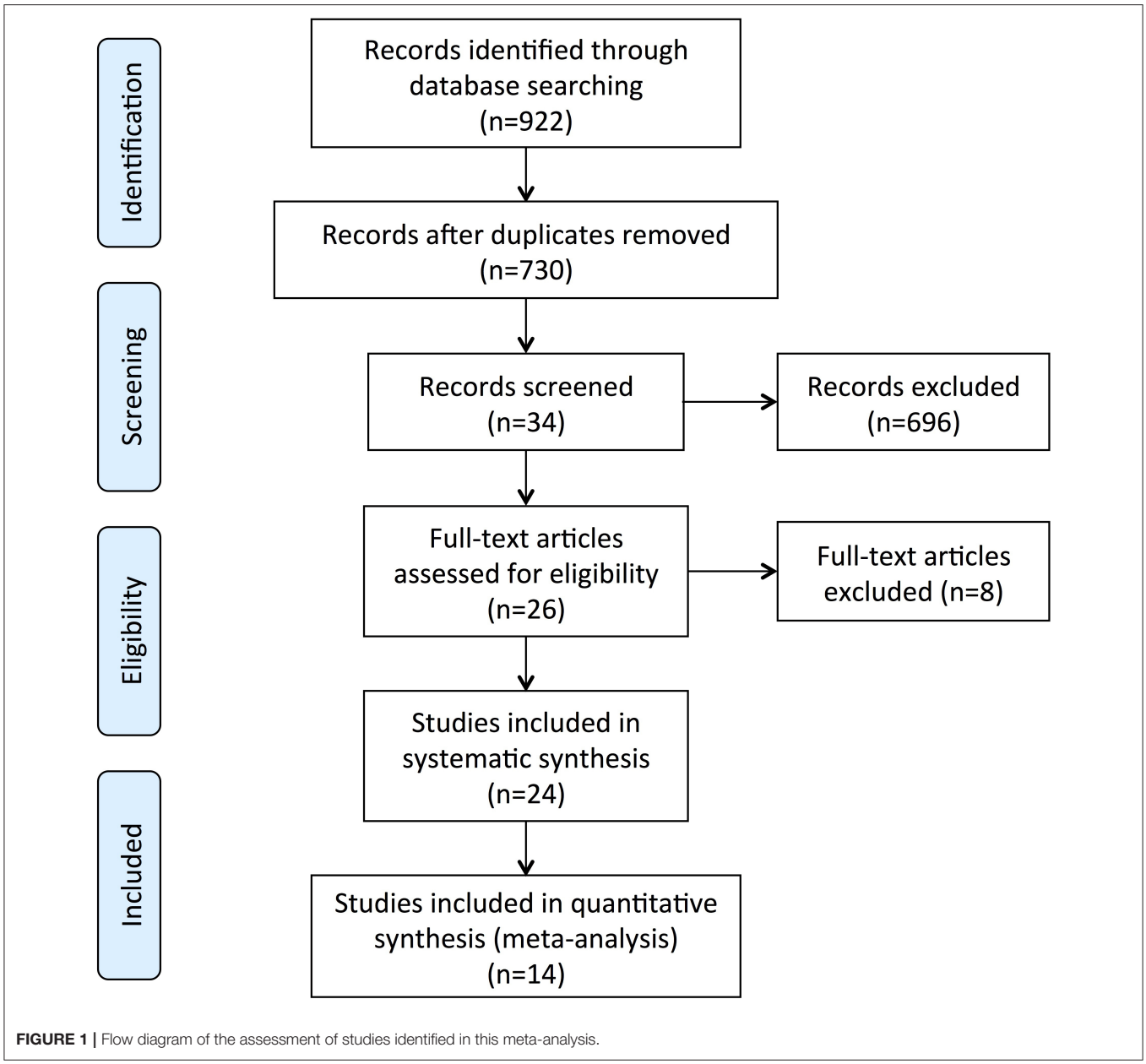

FIGURE 1 | Flow diagram of the assessment of studies identified in this meta-analysis.

seven studies; **Figure 2C**) in patients with PD compared to HCs. Significant higher abundance level of *Bifidobacteriaceae* (MD = 0.38, 95% CI = 0.12 to 0.63; I^2 = 72%; $p < 0.004$; seven studies; **Figure 2D**), and *Ruminococcaceae* (MD = 0.58, 95% CI = 0.07 to 1.10; I^2 = 91%; $p < 0.03$; nine studies; **Figure 2E**), *Verrucomicrobiaceae* (MD = 0.45, 95% CI = 0.21 to 0.69; I^2 = 68%; $p = 0.0003$; seven studies; **Figure 2F**), and *Christensenellaceae* (MD = 0.20, 95% CI = 0.07 to 0.34; I^2 = 0%; $p = 0.003$; seven studies; **Figure 2G**) was found in patients with PD. The differences of abundance of *Lactobacillaceae*, *Enterobacteriaceae*, and *Bacteroidaceae* between the two groups were not statistically significant ($p > 0.05$).

Analysis of Publication Bias

The publication biases were detected with funnel plots (**Figure 3**). The funnel plots of *Prevotellaceae* (**Figure 3A**), *Bifidobacteriaceae* (**Figure 3B**), *Lactobacillaceae* (**Figure 3C**), *Ruminococcaceae* (**Figure 3E**), *Verrucomicrobiaceae* (**Figure 3F**), *Enterobacteriaceae* (**Figure 3G**), and *Lachnospiraceae* (**Figure 3J**) suggested possible bias, which indicated significant heterogeneity between the selected studies. Besides, the shape of funnel plots of *Faecalibacterium* (**Figure 3D**), *Bacteroidaceae* (**Figure 3H**), and *Christensenellaceae* (**Figure 3I**) showed no obvious asymmetry, which indicated that there was no significant heterogeneity between these studies, and the pooled results were not influenced by publication bias. However, the result of publication bias

TABLE 1 | Characteristics of 14 studies included in the systematic review.

References	Location	PD case/HCs			Experimental Methods	
		Number	Mean Age ± SD	Female Ratio (%)	Sample	Technique
Scheperjans et al. (2015)	Finland	72/72	65.3 ± 5.5/64.5 ± 6.9	48.6/50.0	Feces	NGS
Unger et al. (2016)	Germany	34/34	67.7 ± 8.9/64.6 ± 6.6	29.4/47.1	Feces	qPCR
Hill-Burns et al. (2017)	USA	197/130	68.4 ± 9.2/70.3 ± 8.6	33.0/60.8	Feces	NGS
Hopfner et al. (2017)	Germany	29/29	69.2 ±6.5/69.4 ± 6.7	20.7/55.2	Feces	NGS
Bedarf et al. (2017)	Germany	31/28	64.8 ± 9.5/65.6 ± 10.4	0/0	Feces	NGS
Petrov et al. (2017)	Russia	89/66	67.4 ± 2.4/64.5 ± 3.0	-	Feces	NGS
Li et al. (2017)	China	24/14	73.8 ± 6.3/74.6 ± 5.6	33.3/57.1	Feces	NGS
Lin et al. (2018)	China	75/45	60.5 ± 10.7/63.2 ± 6.0	34.7/48.9	Feces	NGS
Heintz-Buschart et al. (2018)	Germany	76/78	68.0 ± 9.7/68.4 ± 6.7	34.0/41.0	Feces	NGS
Barichella et al. (2019)	Italy	193/113	67.6 ± 9.7/65.9 ± 9.9	40.4/58.4	Feces	NGS
Li C. et al. (2019)	China	51/48	62.4 ± 8.2/62.2 ± 9.2	37.3/60.4	Feces	NGS
Li F. et al. (2019)	China	10/10	76.5 ± 7.1/79.5 ± 7.6	30.0/50.0	Feces	NGS
Aho et al. (2019)	Finland	64/64	65.2 ± 5.5/64.5 ± 6.9	48.4/50.0	Feces	NGS
Ren et al. (2020)	China	14/13	60.0 ± 9.2/63.0 ± 8.8	76.9/28.6	Feces	NGS

PD, Parkinson's disease; HCs, healthy controls; SD, standard deviation; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction. "–" means not mentioned in that study.

TABLE 2 | Scores of the 14 studies included in this meta-analysis based on NOS.

References	Selection				Comparability	Exposure			Total Score
	Adequate Definition of Cases	Representativeness of Cases	Selection of Controls	Definition of Controls	Control for Important Factor*	Ascertainment of Exposure	Same Method to Ascertain for Cases and Controls	Non-response Rate	
Scheperjans et al. (2015)	☆	☆	–	☆	☆	☆	☆	☆	7
Unger et al. (2016)	☆	☆	–	☆	☆☆	☆	☆	–	7
Hill-Burns et al. (2017)	☆	☆	☆	☆	–	☆	☆	–	6
Hopfner et al. (2017)	☆	☆	–	☆	☆	☆	☆	–	6
Bedarf et al. (2017)	☆	☆	–	☆	☆☆	☆	☆	–	7
Petrov et al. (2017)	☆	☆	–	☆	☆☆	☆	☆	–	7
Li et al. (2017)	☆	☆	☆	☆	☆☆	☆	☆	–	8
Lin et al. (2018)	☆	☆	–	☆	☆	☆	☆	–	6
Heintz-Buschart et al. (2018)	☆	☆	☆	–	☆	☆	☆	☆	7
Barichella et al. (2019)	☆	☆	☆	☆	☆☆	☆	☆	–	8
Li C. et al. (2019)	☆	☆	☆	☆	☆☆	☆	☆	–	8
Li F. et al. (2019)	–	☆	–	☆	☆☆	☆	☆	–	6
Aho et al. (2019)	☆	☆	☆	☆	☆☆	☆	☆	☆	9
Ren et al. (2020)	☆	☆	–	☆	☆☆	☆	☆	–	7

**A maximum of two stars can be allotted in this category, one for Age and Sex, the other for other controlled factors.*

analysis might be not sufficiently reliable due to the limited number of included studies.

DISCUSSION

Recent studies have reported gut microbiota alterations and intestinal metabolism abnormality in patients with PD, which may affect brain activity through the microbiota-gut-brain axis (Mayer et al., 2015). However, there is a certain degree of difference between the different research results. Since it was not easy to obtain available original datasets of gene sequencing, one previous meta-analysis was conducted based on only five studies. The abundance of four genera including *Akkermansia*, *Roseburia*, *Faecalibacterium,* and *Lachnospiraceae ND3007 group*,

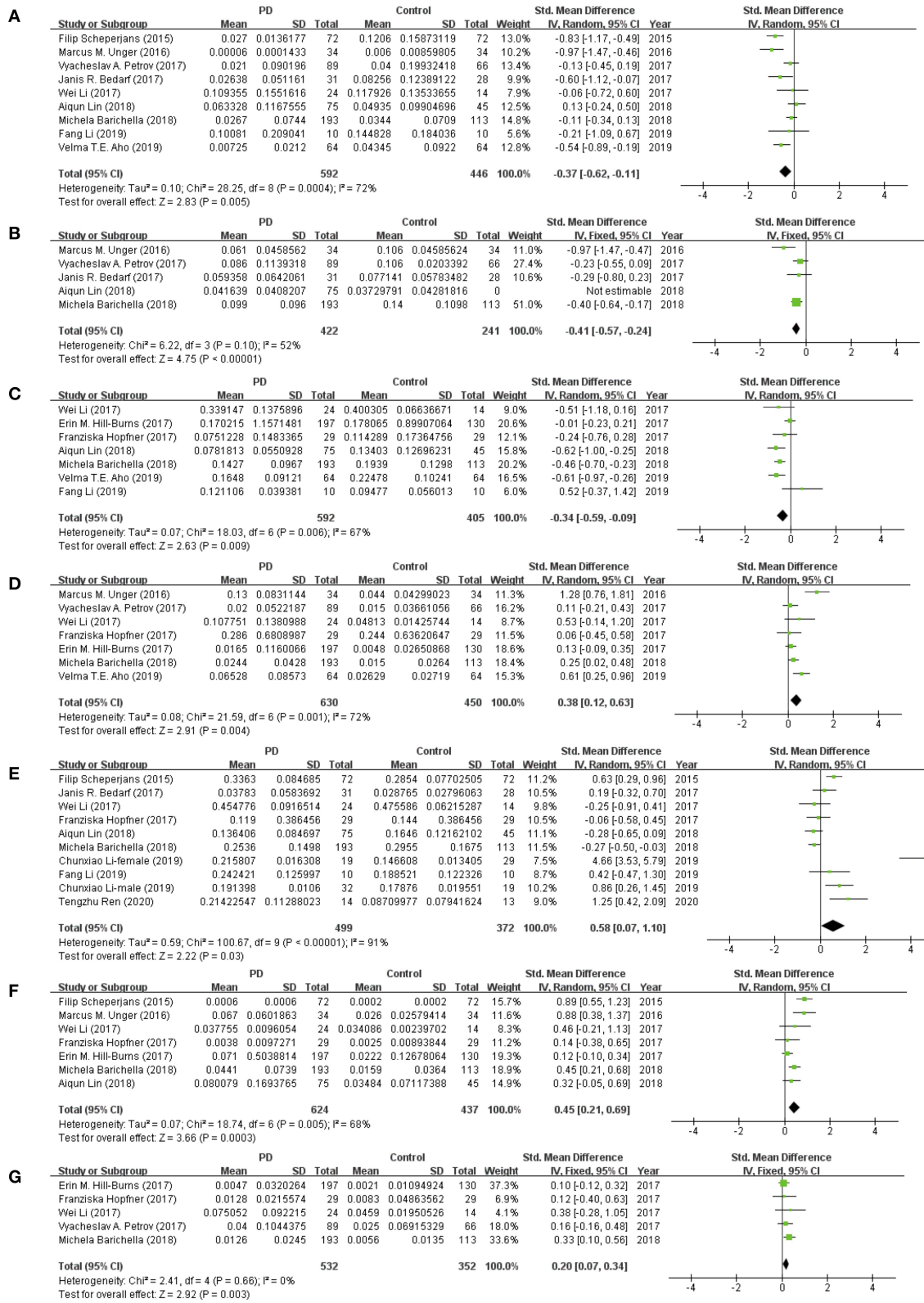

FIGURE 2 | Forest plots of alterations of gut microbiota in patients with Parkinson's disease (PD) vs. healthy controls (HCs): **(A)** *Prevotellaceae*, **(B)** *Faecalibacterium*, **(C)** *Lachnospiraceae*, **(D)** *Bifidobacteriaceae*, **(E)** *Ruminococcaceae*, **(F)** *Verrucomicrobiaceae*, **(G)** *Christensenellaceae*.

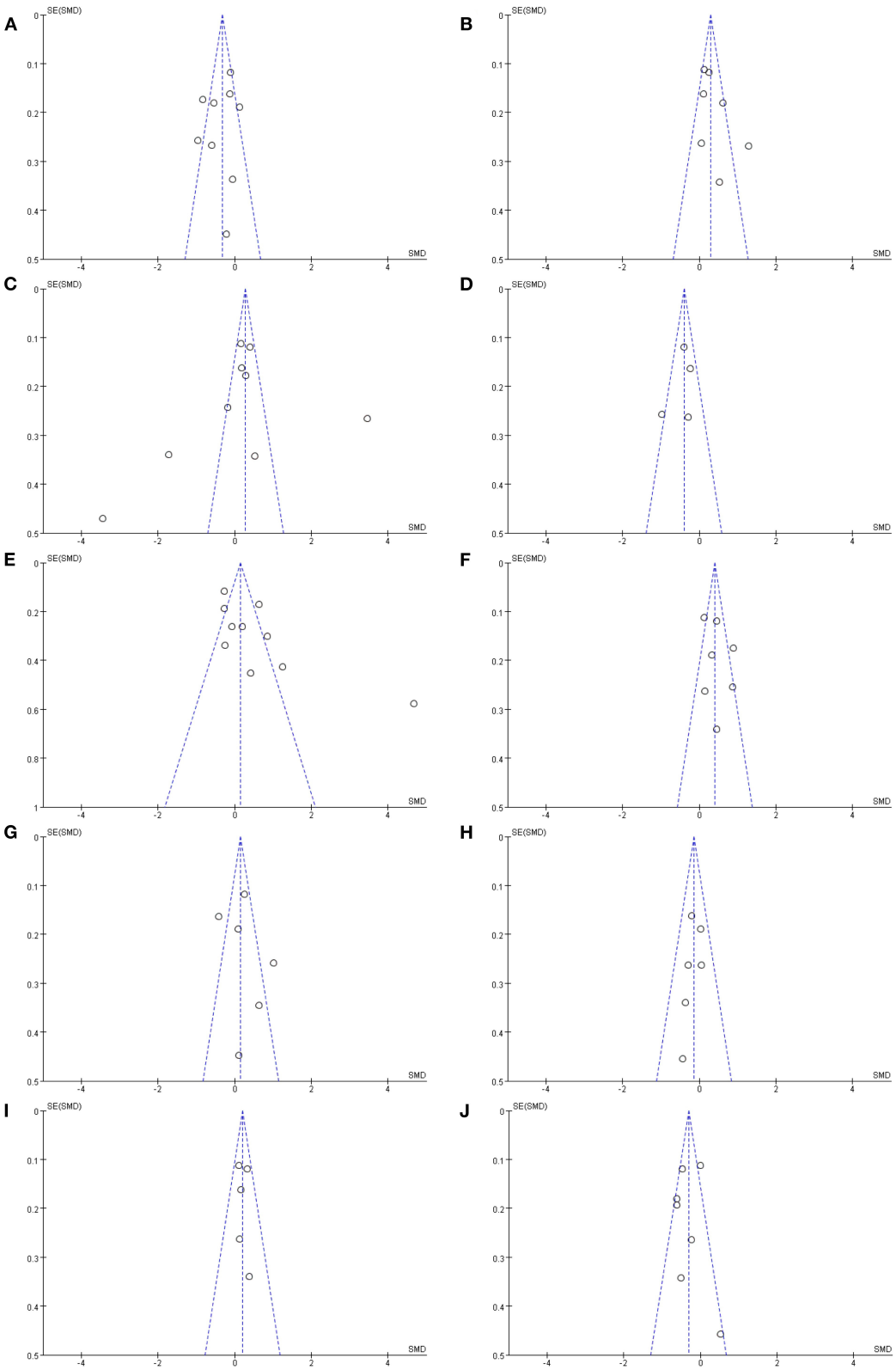

FIGURE 3 | Funnel plots of included studies analyzing alterations of gut microbiota in patients with PD vs. HCs: **(A)** *Prevotellaceae*, **(B)** *Bifidobacteriaceae*, **(C)** *Lactobacillaceae*, **(D)** *Faecalibacterium*, **(E)** *Ruminococcaceae*, **(F)** *Verrucomicrobiaceae*, **(G)** *Enterobacteriaceae*, **(H)** *Bacteroidaceae*, **(I)** *Christensenellaceae*, **(J)** *Lachnospiraceae*.

as well as one family *Akkermansiaceae,* was found to be changed in PD (Nishiwaki et al., 2020). We attempted to include as many studies as possible, thus we extracted analyzed results of microbiota abundance instead of obtaining the original datasets (Prosberg et al., 2016; Zhuang et al., 2017). This meta-analysis was performed to evaluate the differences of gut microbiota between patients with PD and HCs based on 14 case-control studies. We observed that significantly lower abundance levels of *Prevotellaceae*, *Faecalibacterium,* and *Lachnospiraceae* in patients with PD compared to HCs, therefore they might be potentially "beneficial" microbiota against PD. Moreover, the families of *Bifidobacteriaceae*, *Ruminococcaceae*, *Verrucomicrobiaceae,* and *Christensenellaceae* showed increased abundances in PD. Our results covered discoveries of the previous meta-analysis, and further reported a wider range of altered gut microbiota in patients with PD.

The family *Prevotellaceae* helps the breakdown of carbohydrates from dietary fiber and produces SCFAs, which modulates the activity of ENS and helps to maintain gut homeostasis (Unger et al., 2016; Bedarf et al., 2017; Nair et al., 2018). Decreased abundance of *Prevotellaceae* has been revealed to be associated with decreased levels of the gut hormone ghrelin (Queipo-Ortuno et al., 2013; Scheperjans et al., 2015), which participates in regulating dopaminergic neuron function in the substantia nigra pars compacta and may fight against neurodegeneration in PD (Bayliss et al., 2011; Scheperjans et al., 2015). It has been reported that *Prevotellaceae* was associated with the Unified Parkinson Disease Rating Scale (UPDRS)-III score evaluating the severity of PD (Scheperjans et al., 2015), and the abundance of *Prevotellaceae* obviously decreased along with the progress of PD (Minato et al., 2017). Therefore, the reduction of *Prevotellaceae* could be regarded as a biomarker for PD.

The family *Faecalibacterium* also displays an important role in producing SCFAs and anti-inflammatory metabolites that helps to maintain gut health (Ferreira-Halder et al., 2017). Reduction of *Faecalibacterium* might impair the gut-barrier function and make the ENS more susceptible to infection of enteric pathogens and increase the risk of α-synuclein formation in the ENS (Unger et al., 2016). The abundance of *Faecalibacterium* was negatively correlated with UPDRS score and PD duration, which indicated that it might be related to the development and neuropathology of PD (Li et al., 2017).

Similarly, the family *Lachnospiraceae* also participates in producing beneficial butyrate, which could help to maintain the gut epithelium (Hill-Burns et al., 2017; Lin et al., 2018). The lower abundance of *Lachnospiraceae* might lead to the aggravation of gut inflammation, increased production of toxic substances, and impairment of the gut epithelial barrier (Lin et al., 2018; Barichella et al., 2019). Especially in patients with PD, the reduced abundance of *Lachnospiraceae* was reported to correlate with longer PD disease duration (Keshavarzian et al., 2015; Barichella et al., 2019), cognitive decline, and worse motor symptoms (Barichella et al., 2019). And the use of catechol-O-methyltransferase (COMT) inhibitors might influence the level of *Lachnospiraceae* (Barichella et al., 2019). It is thus clear that the lack of these "beneficial" microbiota might contribute to the pathophysiology of PD. At the same time, anti-Parkinson medication might affect the gut epithelium.

In regard to the family *Bifidobacteriaceae*, a kind of important dominant probiotic participates in several physiological functions including the inhibition of the overgrowth of harmful gut bacteria, the improvement of gut ecological environment, and immune regulation (Hsieh et al., 2020). In PD mouse models, long-term administration of probiotics had neuroprotective effects on dopamine neurons in the substantia nigra and further attenuated motor impairments in gait pattern, balance function, and motor coordination (Hsieh et al., 2020). In patients with PD, probiotics administration also could reduce UPDRS score and improve motor function (Tamtaji et al., 2019). The *Bifidobacteriaceae* family should be "beneficial" microbiota. However, all seven studies reported consistent results that the abundance of *Bifidobacteriaceae* was increased in patients with PD, which might indicate potential compensatory regulation to reconstruct gut homeostasis (Wallen et al., 2020).

The family *Ruminococcaceae* is considered an important cellulose-degrading bacteria that also produce SCFAs. Findings for this gut microbiota in PD were mixed, which might be due to the disease duration. The increased abundance of *Ruminococcaceae* was found to correlate with longer PD disease duration (Hill-Burns et al., 2017). However, the abundance was only increased in patients who had PD for over 10 years, but not in the first 10 years of disease (Hill-Burns et al., 2017). And the use of COMT inhibitors might reduce the level of *Ruminococcaceae* (Barichella et al., 2019), which would also influence the structure of gut microbiota.

And the family *Verrucomicrobiaceae*, one of its main genera, the mucus-degrading bacterium *Akkermansia*, converts mucin to SCFAs that may mediate the immunoregulatory effects. Moreover, it is also involved in proinflammatory pathways, due to its mucus-degrading feature, which leads to the breakdown of the gut-barrier and increased exposure of resident immune cells to pathogens (Jangi et al., 2016; Fujio-Vejar et al., 2017), and thereby causes abnormal aggregation of α-synuclein formation in ENS. Furthermore, the increased level of *Akkermansia* might accelerate the progression of PD (Nishiwaki et al., 2020). In our meta-analysis, a significantly higher abundance level of *Verrucomicrobiaceae* was found in patients with PD, which might link to the development of PD. Similarly, a significant trend effect for disease duration on the increasing abundance of *Verrucomicrobiaceae* was also found in PD (Barichella et al., 2019).

The family *Christensenellaceae* may also play an important role in human health, and its abundance was inversely related to host body mass index (BMI) and visceral fat mass (Waters and Ley, 2019). Since previous studies found that patients with PD were three times more likely than HCs to have a low BMI (Suzuki et al., 2020), and progressive weight loss was commonly seen in PD, with greater loss of both visceral and subcutaneous fat (Yong et al., 2020). Accordingly, all five studies consistently reported a higher abundance of *Christensenellaceae* in patients with PD, indicating that *Christensenellaceae* might play a role in lipid metabolism and provided evidence that an increase of certain gut microbiota would lead to weight loss through

influencing lipid absorption. As previously reported, some of the non-motor manifestations in PD were caused by intestinal dysbiosis (Hasuike et al., 2020). Thus, the increased abundance of the family *Christensenellaceae* was also found to be correlated with worse non-motor symptoms (Barichella et al., 2019).

Gut microbiota structure varied within individuals due to a series of factors including the mode of delivery, infant feeding, dietary habits, culture, geographical region, age, gender, and so on (Zhuang et al., 2017). But even after taking into account these factors, we found shared changes of abundance levels of *Prevotellaceae*, *Faecalibacterium*, *Lachnospiraceae*, *Bifidobacteriaceae*, *Verrucomicrobiaceae*, and *Christensenellaceae* in patients with PD across different studies conducted in different geographical regions. Thus, these alterations of gut microbiota were mainly attributed to PD disease statues or might be promotion factors for the progression of PD. Inflammation has been proved to be linked to the development of PD, which activates microglia that play a cruel role in the damage of dopaminergic neurons and aggregation of α-synuclein (Baizabal-Carvallo and Alonso-Juarez, 2020). SCFAs have potential anti-inflammatory and anti-oxidant properties, which might help to regulate neuroinflammation and gut permeability and rescue neuronal damage (Bullich et al., 2019). Imbalance of SCFAs-producing bacteria may cause microglial activation and increased risk of α-synuclein deposition in PD. Regarding the lipid metabolism pathway, lipid dysregulation might be involved in promoting PD pathophysiological processes through oxidative stress and inflammation reaction (Hu et al., 2020). Lipids interact with α-synuclein and further affect the aggregation of α-synuclein and transport of synucleinopathy (Hu et al., 2020; Mori et al., 2020). Thus, altered gut microbiota that participates in lipid metabolism may also contribute to PD pathology. Based on this accumulated information, we suggested that these reported PD-related gut microbiota dysbiosis might

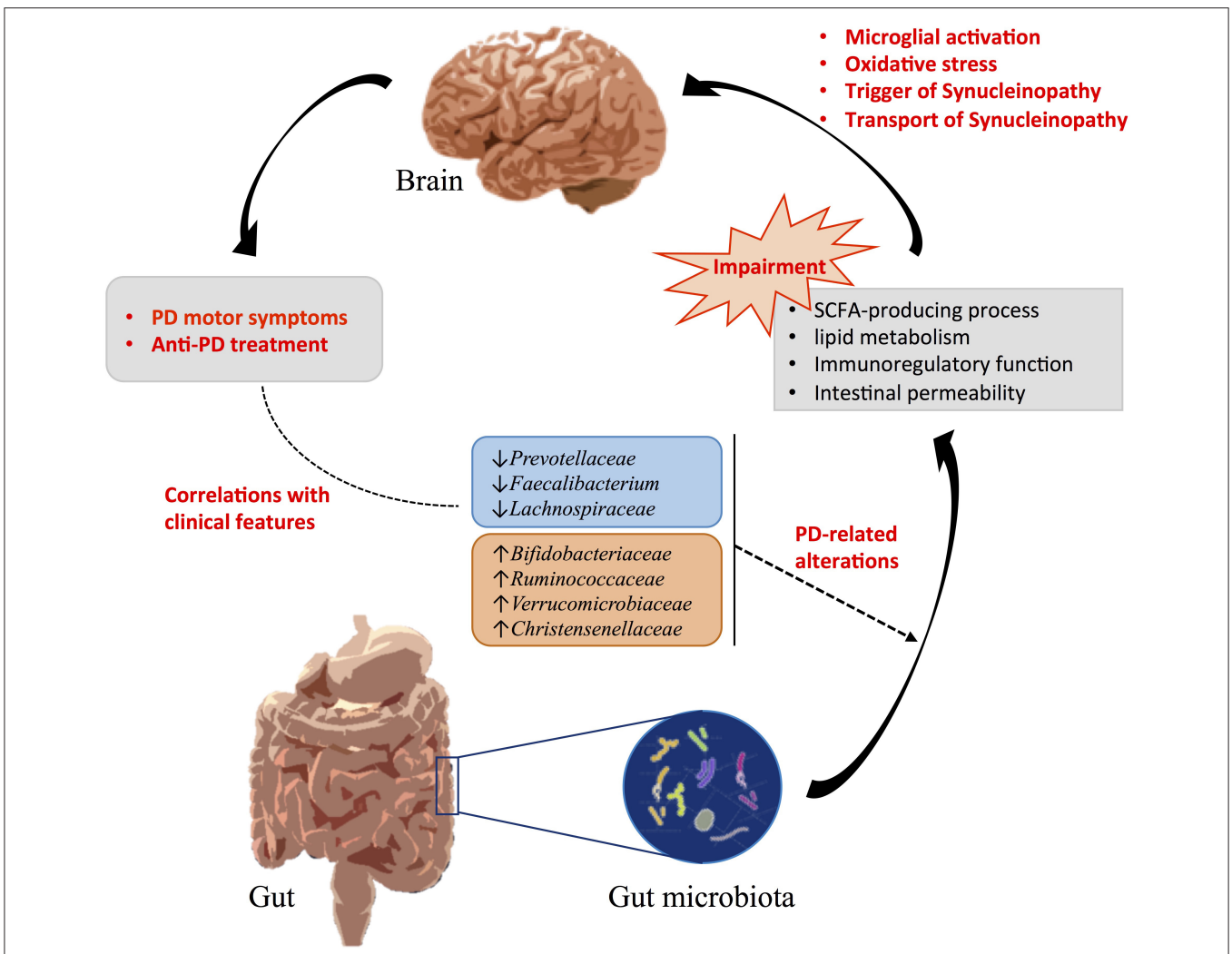

FIGURE 4 | Influences of gut microbiota alterations in PD. PD-related gut microbiota dysbiosis might be related to the clinical features and contribute to triggering synucleinopathy by impairing the "gut microbiota-gut-brain axis" in PD.

contribute to triggering synucleinopathy by impairing the "gut microbiota-gut-brain axis" in PD (**Figure 4**). Future researches could be further conducted to clarify the cause and effect between gut microbiota and brain pathology, the detailed roles in PD disease progression, and the potential therapeutic targets (Wallen et al., 2020).

However, there are still some limitations in our meta-analysis. Firstly, statistical heterogeneities existed among the included studies, which could be explained by the differences in sample size, geographical regions, study methodology, and criteria of PD. Secondly, it is difficult to obtain raw data from all the included studies, and we used the software GetData Graph Digitizer to digitize and extract sufficient data from graphs and plots of several studies, which might cause another outcome bias. In addition, we only discussed the structure and composition of gut microbiota, and not the transcriptomics and proteomics studies that would provide a deeper understanding of gut microbiota function. These all need to be improved in future studies.

CONCLUSION

We reported shared alterations of certain gut microbiota in patients with PD compared to HCs across different geographical regions. Significant lower abundance levels of *Prevotellaceae*, *Faecalibacterium,* and *Lachnospiraceae*, and higher abundance levels of *Bifidobacteriaceae*, *Ruminococcaceae*, *Verrucomicrobiaceae,* and *Christensenellaceae* in patients with PD were observed. The ecological imbalance of these gut microbiota might lead to the impairment of the SCFA-producing process, lipid metabolism, immunoregulatory function, intestinal permeability, etc. Thus, the alteration of the gut microbiota could be considered as an environmental trigger of the PD pathological process and contribute to the development of PD. In future work, a large sample study, as well as metagenomics and metabonomics techniques, are needed to further evaluate the effect of gut microbiota on the development of PD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

TS, YY, and H-YL conceived and designed the study. TS, YY, and TH contributed to data collection and analysis. TS and YY wrote the original draft of the manuscript. WL and BQ provided the technical support. CH and H-YL revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

BRIEF RESEARCH REPORT
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Colonic Dopaminergic Neurons Changed Reversely With Those in the Midbrain via Gut Microbiota-Mediated Autophagy in a Chronic Parkinson's Disease Mice Model

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The role of gut-brain axis in the pathogenesis of Parkinson's disease (PD) have become a research hotspot, appropriate animal model to study gut-brain axis in PD is yet to be confirmed. Our study employed a classical PD mice model achieved by chronic MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) injection to study concurrent changes of dopaminergic neurons in the midbrain and the colon of mice. Our results showed such a PD model exhibited apparent locomotor deficits but not gastrointestinal dysfunction. Tyrosine hydroxylase expressions and dopamine content reduced greatly in the substantia nigra pars compacta (SNpc) or striatum, but increased in the colon of PD mice. Mechanism investigation indicated autophagy activity and apoptosis were stimulated in the SNpc, but inhibited in the colon of PD mice. Interplay of gut microbiota (GM) and autophagy in response to chronic MPTP injection led to GM dysbiosis and defective autophagy in mice colon. Meanwhile, fecal short chain fatty acids (SCFAs), acetate and propionate in particular, declined greatly in PD mice, which could be attributed to the decreased bacteria abundance of phylum *Bacteroidetes*, but increased abundance of phylum *Firmicutes*. GM dysbiosis derived fecal SCFAs might be one of the mediators of downregulated autophagy in the colon of PD mice. In conclusion, colonic dopaminergic neurons changed in the opposition direction with those in the midbrain via GM dysbiosis-mediated autophagy inhibition followed by suppressed apoptosis in response to chronic MPTP injection. Such a chronic PD mice model might not be an ideal model to study role of gut-brain axis in PD progression.

Keywords: Parkinson's disease, dopamine, tyrosine hydroxylase, autophagy, apoptosis, short chain fatty acids, gut microbiota dysbiosis

INTRODUCTION

The pathological hallmarks of Parkinson's disease (PD) is the slow and progressive loss of dopaminergic neurons in the nigrostriatal pathway, which leads to classical motor symptoms like bradykinesia, rigidity, and resting tremor (Seppi et al., 2019). Meanwhile, impairment of dopaminergic neurons in the enteric nervous system (ENS) of the intestine was found to contribute to reduced intestinal motility in PD patients (Nadeau et al., 2019). Gastrointestinal (GI) dysfunction is the major non-motor manifestation at the early stage of PD, and it occurs long before the appearance of motor symptoms (Yang et al., 2019). The GI tract, ENS, gut microbiota (GM) and gut-brain crosstalk have recently become research hotspots. The pathological process of the gut-brain axis might spread from the gut to the brain and contribute to the pathogenesis of PD (Mulak and Bonaz, 2015), but the underlying mechanisms are not fully understood.

Apoptosis and autophagy are two major machineries of the degeneration of dopaminergic neurons in the pathogenesis of PD (Ghavami et al., 2014). The balance between apoptosis and autophagy is vital for maintaining normal cellular homeostasis, and their imbalance accelerates neurodegeneration and is closely related to the progression of PD (Liu et al., 2019). Excessive autophagy, which simultaneously accelerated apoptosis, leading to quick death and loss of dopaminergic neurons (Li et al., 2011). Recent studies demonstrated GM is closely linked to intestinal pathology and inflammation through its interplay with autophagy in mechanism studies of colon cancer and inflammatory bowel disease (IBD). GM dysbiosis could regulate autophagy (Kim et al., 2020) and autophagy has a role in the control of GM compositions (Larabi et al., 2020). Many clinical studies observed GM dysbiosis in PD patients, which plays key roles in aggravating PD through the promotion of inflammatory cascades or oxidative stress in the brain via short chain fatty acids (SCFA)-production or a lipopolysaccharide (LPS)-mediated mechanism (Breen et al., 2019; Roy Sarkar and Banerjee, 2019; Yang et al., 2019; Pascale et al., 2020). However, little researches have investigated the relationships of GM dysbiosis with autophagy and apoptosis in the intestine during PD pathogenesis.

Researchers are actively seeking for an appropriate animal model to study the gut-brain axis in PD progression. Although many classical PD mice or rats models which can mimic brain pathology of patients are applicable, it is still unknown if intestinal pathology in these models can mimic those occurred in PD patients and correspond to brain pathology. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a selective dopamine (DA) neuron toxin, is the most widely used toxin to generate PD animal models, and can represent the earliest phase of PD (Meredith and Rademacher, 2011). Different protocols to use MPTP are applied in making PD animal model, among which, acute (1 day), subacute (5 days), and chronic (5 weeks) intraperitoneal (i.p.) injection of MPTP to mice are three classical usages. These three classical MPTP mice models can show obvious behavioral deficits and dopaminergic neuronal loss in the midbrain. MPTP impairs dopaminergic neurons not only in the central nervous system (CNS), but also in the ENS. Recent studies (Ellett et al., 2016; Poirier et al., 2016; Lai et al., 2018; Sun et al., 2018; Zhou et al., 2019; Dong et al., 2020; Liao et al., 2020) have observed changes of GM composition, GM metabolites of short chain fatty acids (SCFAs), and/or enteric dopaminergic neurons in these three classical MPTP mice models. The detailed information is summarized and shown in **Supplementary Table 1**. It was found different MPTP injection protocols and even different collection time of animal samples after MPTP injection could lead to various alterations of GM compositions and SCFAs production (**Supplementary Table 1**). Two studies (Ellett et al., 2016; Poirier et al., 2016) discovered that acute injection of MPTP in 1 day led to loss of dopaminergic neurons in the ileum by immunohistochemistry staining, but no GM and SCFAs data were provided in their studies. Only one published study (Lai et al., 2018) using chronic MPTP injection protocol (twice a week for 5 weeks) reported significant changes of GM compositions and dopaminergic neurons in the ileum when animal tissue samples were collected on the 2nd day and 22nd day, but not at the end of experiment (classically 5 weeks). No published paper has observed simultaneous alterations of GM compositions, SCFAs together with enteric dopaminergic neurons and explored their relationships with autophagy and apoptosis in ENS in either MPTP mice model.

In this study, we chose a classical PD mice model made by chronic MPTP injection (twice a week for 5 weeks) and collected animal samples after 5 weeks, to study the concurrent changes of dopaminergic neurons in the midbrain and colon, followed by mechanism investigations to reveal the relationships of enteric dopaminergic neurons with autophagy, apoptosis and GM. Our study purpose is to explore if intestinal pathology in such a chronic MPTP model can mimic those occurred in PD patients and correspond to brain pathology, and this study will provide evidence to appraise if it could be an appropriate animal model to study the role of gut-brain axis in PD progression.

MATERIALS AND METHODS

Animal Experimental Design

Sixteen 8-week-old male C57BL/6J mice (23 ± 2 g) were purchased from Beijing Vital River Laboratory (Beijing, China) and housed in an air-conditioned room at 22 ± 2°C with 55% ± 5% relative humidity and a 12 h light/dark cycle. The experiment was conducted according to the guidelines approved by the Ethical Committee of Experimental Animal Care (approval no: 180703) at The Hong Kong Polytechnic University Shenzhen Institute and all efforts were made to minimize animal suffering or discomforts. The mice were given a standard laboratory rodent diet (AIN-93M) and provided free access to distilled water. Animal experiment started on Monday and the PD mice model was achieved by i.p. injection of MPTP (20 mg/kg) twice a week in the morning (Wednesday and Saturday) for 5 weeks (Model group); while normal mice were injected with the same volume of saline twice a week for 5 weeks (Normal group). All the mice had adapted to the environment for 1 week before the start of the experiment. The animal experimental timeline is shown in **Figure 1A**.

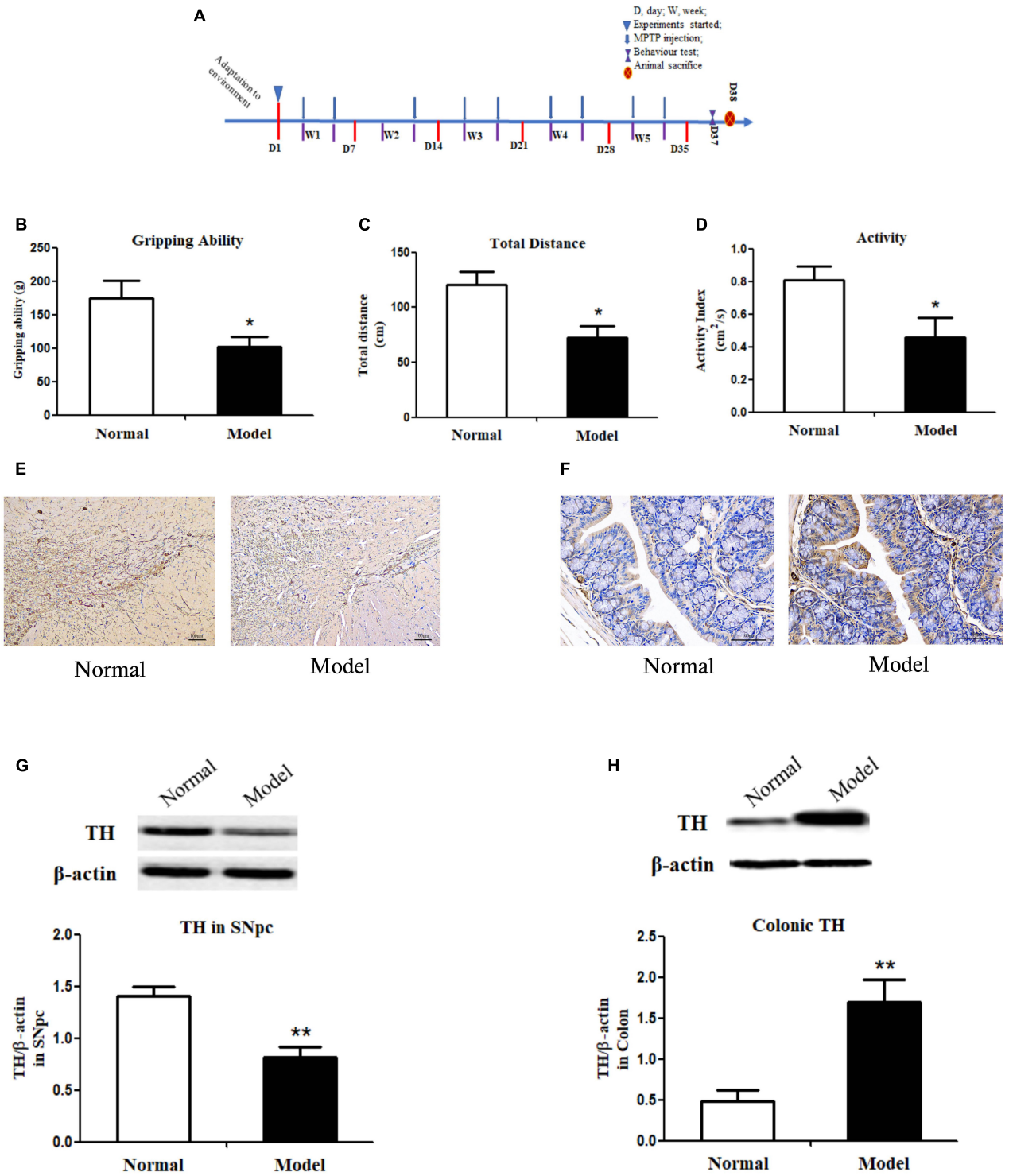

FIGURE 1 | Experimental timeline, behavior tests, immunochemistry staining of tyrosine hydroxylase and its protein expressions in both the substantia nigra pars compacta and colon of mice. **(A)** Timeline of animal experiments. **(B)** Grip strength test of mice indicated by gripping ability (g); Open field test of mice indicated by total distance traveled (cm) **(C)** and activity index (cm^2/s) **(D)** within a 5-min period (n = 8). Representative IHC staining of TH in sections of the SNpc **(E)** and the colon **(F)** in normal and PD mice. TH protein expressions in the SNpc **(G)** and the colon **(H)** of normal and PD model mice (One representative band for TH and endogenous β-actin from each group; and the following bar chart (n = 6–8) shows the ratio of TH/β-actin in the SNpc and colon of mice). Values are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ vs. Model group. IHC, immunochemistry; TH, tyrosine hydroxylase; SNpc, substantia nigra pars compacta; PD, Parkinson's disease.

Grip Strength Test

The grip strength of both the forelimb and hindlimb (4 paws) was measured using a grip strength meter (An Hui Zheng Hua Biologic Apparatus Facilities Ltd., Co., China). The grip strength of each mouse was measured 3 times with a 3 min interval between each measurement to prevent fatigue of animals. Individual muscular function was assessed by sensing the peak amount of force for each measurement.

Open Field Test

The open field test is another frequently used behavior test to assess spontaneous locomotor activity in PD models (Yan et al., 2018). The apparatus is a chamber consisting of a square arena with a surrounding wall; and it is connected to a video tracking system (SMART 3.0 Panlab Harvard Apparatus Instruments, Inc., United States). In the field (40 cm * 40 cm), an area is appointed to be a target area (20 cm * 20 cm) in the center of the field. Each mouse was placed in the center of the field, and their locomotor activity was recorded by the computer as total distance traveled (cm) and moving speed (cm/sec) in the target area within a 5-min period. Furthermore, an activity index (cm^2/s) was defined as the whole area crossed by mice in the 5-min period.

Sample Collection

After the behavioral tests, the mice were fasted overnight then sacrificed. Fecal samples (n = 8) for 16S rRNA gene sequencing were taken from the proximal colon, approximately one inch downstream of the cecum. Colon contents were collected by elevating one end of the large intestine and pushing contents into a sterile sample container. After rinsing with saline, colon tissues (n = 8) were collected and stored at −80°C. Brain tissues of the substantia nigra pars compacta (SNpc) and striatum (n = 6) were separated and stored at −80°C for further measurements. The whole brain (n = 2) was removed for immunohistochemistry staining.

Analysis of Fecal Short Chain Fatty Acids (SCFAs)

Mice fecal samples were thawed, homogenized, acidified and centrifuged. Fifty microliter internal standard (1% 2-ethyl butyrate acid solution) and 500 μl diethyl ether anhydrous were added into the supernatants and centrifuged again. One microliter of the upper diethyl ether layer was injected into a GC instrument (Agilent 7820A; Agilent Technologies, United States) for determination of SCFAs concentrations using a published method (David et al., 2014).

Western-Blot Analysis

SNpc and colon tissues were collected and homogenized. Lysates were centrifuged and total proteins were acquired and separated by SDS-PAGE in gels. After separation, the proteins were transferred to PVDF membrane. The membranes were blocked with 5% non-fat milk followed by incubation with primary antibody of mouse anti-TH (1:1,000, Millipore, United States), rabbit anti-LC3B (1:1,000, Novus, United States), mouse anti-p62 (1:1,000, Santa Cruz, United States), mouse anti-Bax (1:1,000, CST, United States), mouse anti-Bcl-2 (1:1,000, Santa Cruz, United States), mouse anti β-actin (1:1,000, Santa Cruz, United States) and secondary antibody of goat-anti-mouse IgG (1:1,000, Santa Cruz, United States) or goat-anti-rabbit IgG (1:1,000, Beyotime Biotechnology, China). Bands were visualized by using a chemiluminescence kit under the ECL system. Densitometry was performed by using Image J software.

Measurement of Striatal Dopamine (DA) Levels by LC-MS

0.1 g of each striatum sample was put into a centrifuge tube and diluted with 500 μl distilled and deionized water, followed by homogenization. Acetonitrile was added into the homogenate, and the mixture was centrifuged at 14,000 g/min for 10 min. Then, supernatants were collected, blow dried with nitrogen, and finally dissolved in mobile phase. An ultra-high performance liquid phase system (Waters, MA, United States) that included a binary pump, an online degasser, an autosampler system and a column oven was used in chromatographic analysis. Standards of DA were freshly prepared and injected to the column for calibration.

Immunohistochemistry (IHC) Staining of TH-Immunoreactive (TH-IR) Neurons

The brain and colon were post fixed in 4% paraformaldehyde at 4°C overnight and immersed in different concentrations of sucrose for dehydration. Frozen coronal sections (16 μm) were serially cut through the SNpc or the colon with a freezing cryostat microtome (CM1950, Leica, Germany). Sections were then attached to slides and treated with 1% bovine serum to block non-specific binding sites followed by incubation with mouse anti TH (1:1,000, Millipore, United States) and secondary antibodies (goat anti-mouse IgG, 1:1,000, Santa Cruz, United States). TH-IR neurons in the SNpc or the colon were visualized by 3′-diaminobenzidine (DAB) staining.

16S rRNA Gene Sequencing and Data Analysis

The proximal colon content DNA was extracted by QIAamp DNA stool kit (Qiagen, United States) with a previously described protocol (Lacombe et al., 2013). The extracted DNA from each sample was used as a template to amplify the V3-V4 region of 16S rRNA genes. PCR amplification, pyrosequencing of the PCR amplicons, and quality control of raw data were performed as described in previously published paper (Liu et al., 2017). The sequence data were preprocessed using MiSeq Control Software v2.4.1. 16S rRNA gene sequences were analyzed by QIIME pipeline (v1.8.0). GreenGene database (v201305) was used for sequence alignment and taxonomy assignment (greengenes.lbl.gov). All raw sequence data have been deposited to the China National GeneBank (CNGB) with project accession number: CNP0001553.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Subsequent statistical analyses were performed using Graphpad Prism version 5.0 (Graphpad, United States). Unpaired T-test was performed between two groups. Differences

in p-values of less than 0.05 were considered statistically significant. In addition, the linear discriminant analysis (LDA) effect size (LEFSe) method was used to test significant differences of microbiome features between two groups; and the cutoff value is the absolute $\log_{10}$ LDA score > 2.0.

RESULTS

Colonic Dopaminergic Neurons Changed Reversely With Those in the Midbrain of PD Mice

The pole test, open filed test and grip strength test were employed to evaluate the locomotor deficits of mice. Fecal discharge frequency test was performed to appraise gastrointestinal (GI) function. As expected, the muscle strength of PD mice declined greatly ($p < 0.05$ vs. Normal; **Figure 1B**), suggesting the destroyed motor control and coordination by chronic MPTP injection in mice. In the open field test, PD mice (vs. Normal) exhibited locomotor deficits with reduced walking distance ($p < 0.05$; **Figure 1C**) and activity ($p < 0.05$; **Figure 1D**). No statistically significant changes were found in PD mice in the pole test and GI function test (**Supplementary Figure 1**). Accordingly, TH (a marker of dopaminergic neurons) protein expressions (**Figure 1G** and **Supplementary Figure 2A**) and its immunohistochemistry (IHC) staining (**Figure 1E**) in the SNpc of PD mice declined greatly ($p < 0.01$ vs. Normal); and DA content in the striatum ($p < 0.05$ vs. Normal; **Table 1**) reduced significantly in such a chronic PD mice model. Conversely, our results demonstrated that TH protein expressions ($p < 0.01$; **Figure 1H** and **Supplementary Figure 2B**), IHC staining of TH (**Figure 1F**) and DA content ($p < 0.05$; **Table 1**) were highly elevated in the colon of PD mice (vs. Normal). Opposite changes of dopaminergic neurons were found in the midbrain and the colon of mice in response to chronic MPTP injection.

Autophagy and Apoptosis Changed Reversely in the Colon With the Midbrain in PD Mice

During autophagy, protein light chain 3 (LC3I) is conjugated to phosphatidylethanolamine to form LC3-II, which results in the recruitment of ubiquitinated p62/SQSTM1 (p62) attached to the cargo molecules destined for degradation (Yoshii and Mizushima, 2017). Higher turnover of LC3II to LC3I (LC3 conversion) accompanied by low levels of p62 in cells could reflect stimulated autophagic activity. Similarly, Bax protein acts as a pro-apoptotic member, but Bcl-2 protein is an anti-apoptotic member; Bax and Bcl-2 expressions and their ratios are markers of apoptosis (Antonsson, 2001).

As shown in **Figure 2A** and **Supplementary Figure 3A**, LC3II/LC3I ratio (LC3 conversion) was significantly enhanced ($p < 0.05$ vs. Normal), while p62 protein expression was seemingly downregulated in the SNpc of PD mice, indicating the stimulated autophagy in the SNpc of PD mice. Conversely, LC3II/LC3I ratio was apparently repressed ($p < 0.05$ vs. Normal), and p62 expression appeared to have increased in the colon of PD mice, suggesting inhibited autophagy in the colon of PD mice. **Figure 2B** and **Supplementary Figure 3B**, suggesting inhibited autophagy in the colon of PD mice.

Bax proteins were found to increase ($p < 0.05$), while Bcl-2 did not change much in the SNpc of PD mice; and Bax/Bcl-2 ratio increased significantly ($p < 0.05$) in the SNpc of PD mice (vs. Normal; **Figure 2A** and **Supplementary Figure 4A**). Colonic Bax proteins were not influenced while colonic Bcl-2 protein expression was significantly increased ($p < 0.001$) in response to chronic MPTP injection, the ratio of Bax/Bcl-2 in the colon was greatly lowered ($p < 0.01$) in PD mice (vs. Normal; **Figure 2B** and **Supplementary Figure 4B**). The results demonstrated autophagy and apoptosis were enhanced in the SNpc, but inhibited in the colon of PD mice.

Fecal SCFAs Production Declined in PD Mice

Chronic MPTP injection resulted in significant decline of the total fecal concentrations of SCFAs in mice ($p < 0.01$ vs. Normal; **Table 2**). PD mice had significantly lower concentrations of acetate (61% decline; $p < 0.001$) and propionate (50% decline; $p < 0.05$) in feces (vs. Normal), while fecal butyrate concentrations in PD and normal mice were equal.

Significant Changes of Microbial Compositions in PD Mice

Alpha diversity analysis (**Figure 3C**) showed that microbiota in PD model mice had higher values of Chao1 ($p < 0.05$), ACE ($p < 0.01$), and Shannon ($p < 0.05$), but lower values of Simpson ($p < 0.05$) (vs. Normal). In beta diversity analysis (**Figure 3D**), principal coordinates analysis (PCoA) derived

TABLE 1 | Striatal and colonic dopamine (DA) content and fecal short chain fatty acids (SCFAs) levels in normal and model mice of Parkinson's disease (PD)[a,b].

Groups	DA Content (ng/mg tissues)		Fecal SCFAs (nmol/g wet feces)			
	Striatum	Colon	Total SCFAs	Acetate	Propionate	Butyrate
Normal	6.26 ± 0.73	0.06 ± 0.02	5.85 ± 0.94	2.28 ± 0.28	2.06 ± 0.39	0.72 ± 0.11
Model	3.99 ± 0.52*	0.30 ± 0.11*	2.62 ± 0.37**	0.90 ± 0.17***	1.03 ± 0.10*	0.69 ± 0.10

[a] *Values are expressed as mean ± SEM, n = 6–8. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Normal group.*
[b] *DA, dopamine; SCFAs, short chain fatty acids; PD, Parkinson's disease.*

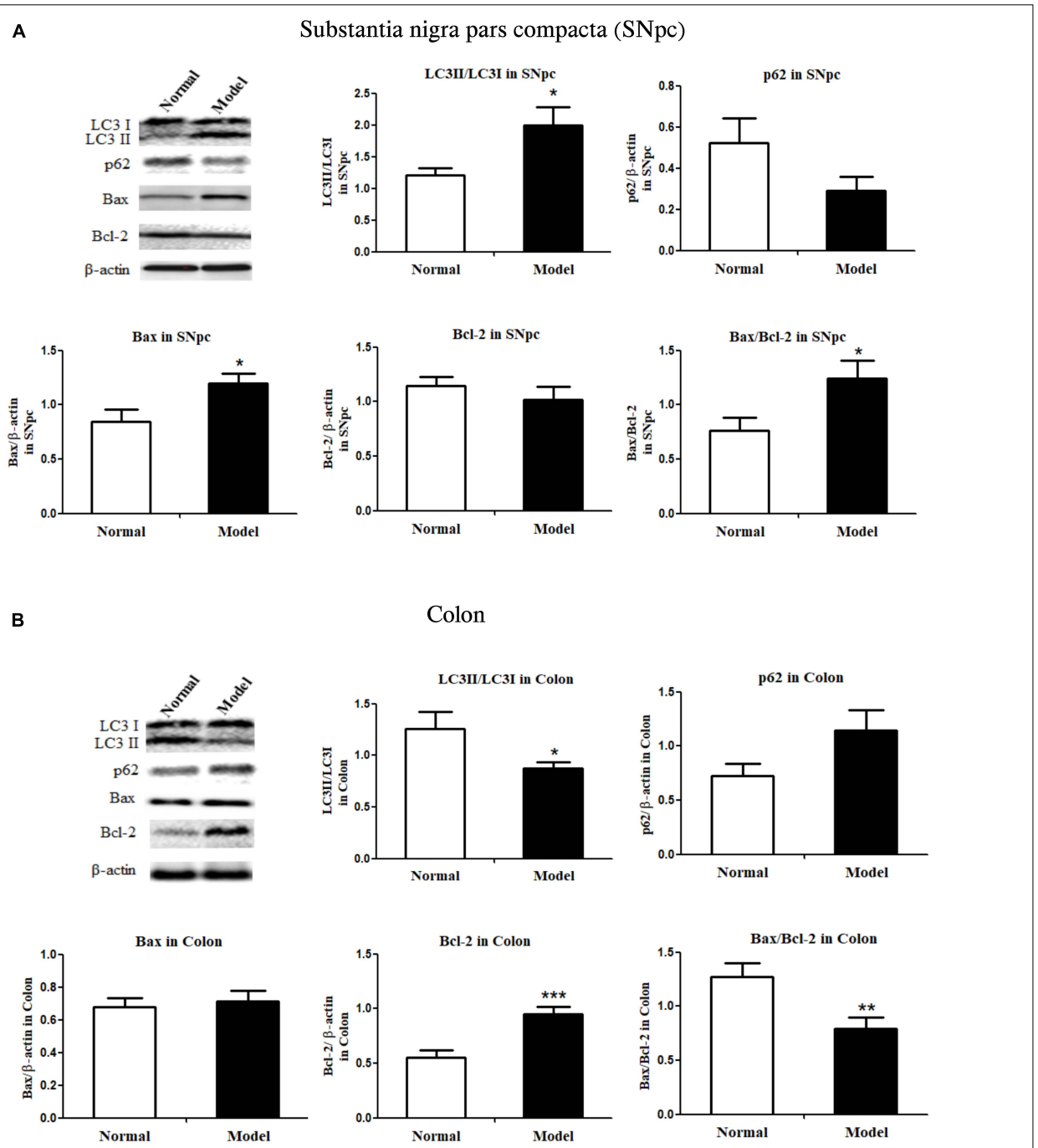

FIGURE 2 | Critical protein expressions in autophagy and apoptosis in both the substantia nigra pars compacta and colon of mice. Protein expressions of LC3I, LC3II, p62, Bax, Bcl-2 in SNpc **(A)** and colon **(B)** in normal and PD model mice (one representative band for target protein and endogenous β-actin from each group; and the following bar chart (n = 6–8) shows the ratio of target protein/β-actin or ratio of LC3II/LC3I and Bax/Bcl-2 in both SNpc and colon of mice). Values are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Model group. SNpc, substantia nigra pars compacta; PD, Parkinson's disease.

from Bray-Curtis distances, unweighted and weighted UniFrac revealed there were no significant differences between model and normal groups. These results suggested chronic MPTP injection resulted in higher microbial community richness and diversity in PD mice, but PD mice had no significant contrast of microbial communities compared with normal mice.

As shown in **Figures 3A,B** and **Table 2**, chronic MPTP injection significantly decreased the abundance of the phylum *Bacteroidetes* ($p < 0.05$), but increased the abundance of the phylum *Firmicutes* ($p < 0.05$). Accordingly, the abundance of *Bacteroidia* (Class) ($p < 0.05$), *Bacteroidales* (Order) ($p < 0.05$), *S24_7* (Family) ($p < 0.01$) under the phylum

TABLE 2 | Relative abundance of phylum, class, order, family, genus > 1% in normal and PD model mice[a].

Groups	Phylum		Class	
	Bacteroidetes	***Firmicutes***	***Bacteroidia***	***Erysipelotrichia***
Normal	70.35 ± 1.79	26.32 ± 1.66	70.35 ± 1.79	1.66 ± 0.42
Model	60.57 ± 3.21*	34.91 ± 2.76*	60.56 ± 3.21*	2.97 ± 0.52*

Groups	Order		Family			Genus
	Bacteroidales	***Erysipelotrichales***	***S24_7***	***Ruminococcaceae***	***Erysipelotrichaceae***	***Allobaculum***
Normal	70.35 ± 1.79	1.66 ± 0.42	58.62 ± 2.03	5.27 ± 0.45	1.60 ± 0.42	1.53 ± 0.41
Model	60.56 ± 3.21*	2.97 ± 0.52*	48.26 ± 3.07**	6.81 ± 0.53*	2.97 ± 0.52*	2.81 ± 0.52 *

[a]*Values are expressed as mean ± SEM, n = 8. *p < 0.05, **p < 0.01 vs. Normal group.*

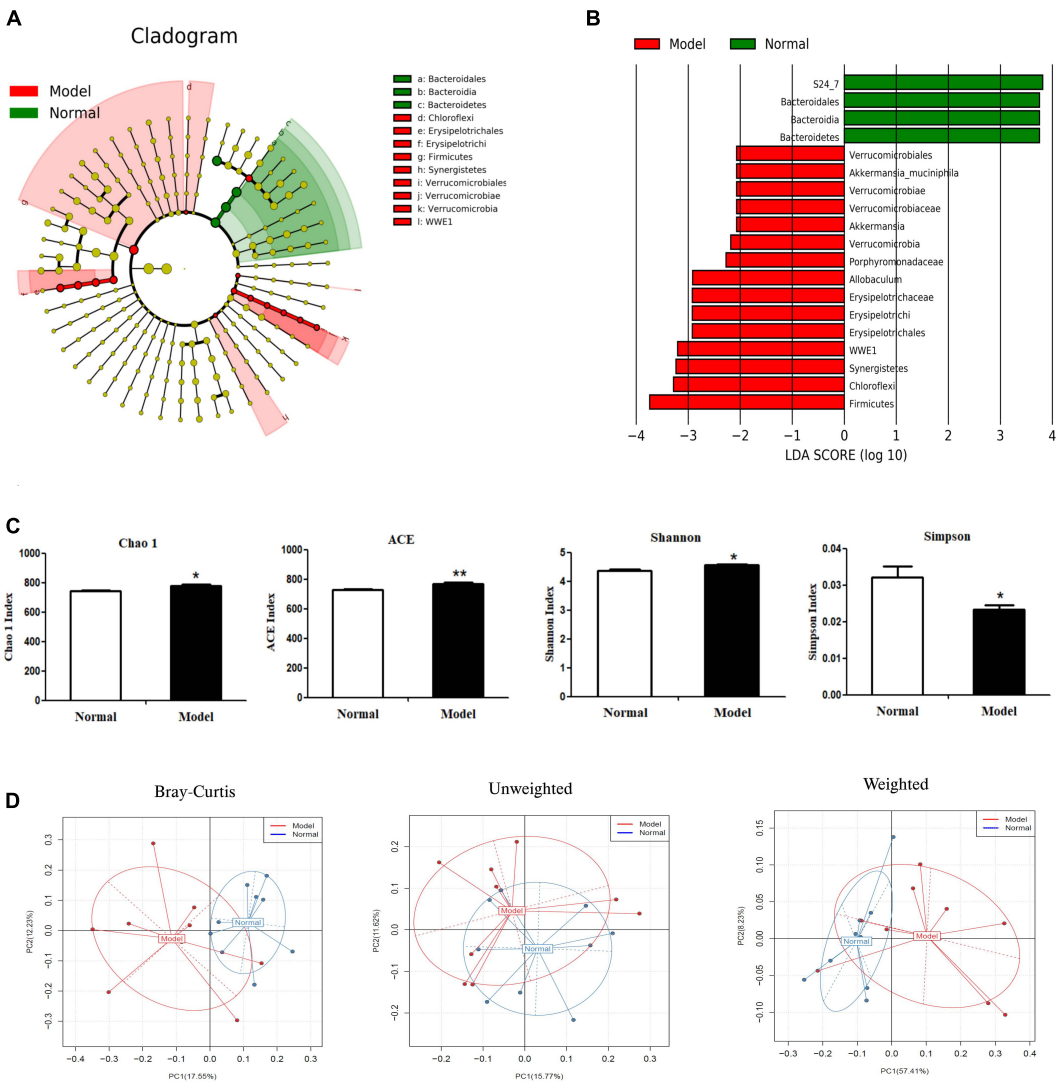

FIGURE 3 | Microbial composition analysis by 16S rRNA gene sequencing. **(A)** Cladograms displaying the taxa with significantly different abundance. Model vs. Normal group. Only the taxa with absolute $\log_{10}$ LDA scores > 2.0 are displayed. **(B)** Selected microbial taxa significantly impacted in PD mice. Only those taxa with the absolute $\log_{10}$ LDA scores > 2.0 are listed in the charts. **(C)** α-diversity as indicated by microbial diversity indices of Chao1, ACE, Shannon, Simpson. **(D)** β-diversity analysis: Principal coordinates analysis (PCoA) derived from Bray-Curtis distances, unweighted and weighted UniFrac among samples of two groups (Model vs. Normal). *$p < 0.05$, **$p < 0.01$ vs. Model group.

Bacteroidetes decreased greatly in PD mice (vs. Normal); while the abundance of *Erysipelotrichia* (Class) ($p < 0.05$), *Erysipelotrichales* (Order) ($p < 0.05$), *Erysipelotrichaceae* (Family) ($p < 0.05$), *Ruminococcaceae* (Family) ($p < 0.05$), *Allobaculum* (Genus) ($p < 0.05$) under the phylum *Firmicutes* increased significantly in PD mice (vs. Normal). Some other species also exhibited significantly higher abundance in PD mice compared with normal mice, including the phyla *Chloroflexi*, WWE1, and *Verrucomicrobia*, but their relative abundance was extremely low (**Figures 3A,B**). These results suggested chronic MPTP injection resulted in gut microbiota (GM) dysbiosis in PD mice.

DISCUSSION

The role that the gut-brain-microbiota axis plays in the pathogenesis of PD has become a research hotspot, but the detailed mechanisms are still unclear. Appropriate animal model to study gut-brain axis in PD were not confirmed. Our results demonstrated such a classical chronic PD model mice exhibited pathological hallmarks of PD in mice brain as indicated by apparent locomotor deficits and nigral dopaminergic neuronal loss. However, colonic dopaminergic neurons were increased and GI dysfunction test did not show any changes in PD mice compared with normal mice. Limited researches investigated effects of MPTP on dopaminergic neurons in ENS. Results from two animal studies demonstrated acute injection of MPTP in 1 day led to loss of dopaminergic neurons in mice ileum (Ellett et al., 2016; Poirier et al., 2016). One study by using chronic MPTP injection (same protocol as that in the present study) showed the increase of TH expressions in mice ileum which was contradictory with those in mice midbrain (Lai et al., 2018). Such result in this study is consistent with our finding, although the animal sample collection time and detected intestinal segment are different. Relevant information was all included in **Supplementary Table 1**, and we can see that different MPTP injection protocols might lead to different changes of enteric dopaminergic neurons.

Apoptosis and autophagy are two major machineries of the degeneration of dopaminergic neurons and their imbalance accelerates PD progression (Ghavami et al., 2014; Liu et al., 2019). MPTP was reported to induce excessive autophagy and apoptosis in the CNS (Li et al., 2011; Wang et al., 2020), but there weren't any reports on its actions on autophagy and apoptosis in the ENS. Our results showed autophagy activity (higher LC3 conversion) and apoptosis (higher Bax levels) were stimulated in mice SNpc, but inhibited (lower LC3 conversion and higher Bcl-2 levels) in mice colon in response to chronic MPTP injection for 5 weeks. These results implied opposite changes of autophagy and apoptosis in the SNpc and colon determined the reversed changes of dopaminergic neurons in the CNS and ENS in such a chronic MPTP model.

As reported, PD patients exhibited GM dysbiosis and lower production of fecal SCFAs (Unger et al., 2016). Our results indicated chronic MPTP injection resulted in GM dysbiosis in PD mice. Several clinical studies reported that the GM in PD patients displayed higher microbial community richness and diversity indicated by alpha diversity indexes in comparison with matched healthy controls (Keshavarzian et al., 2015; Qian et al., 2018; Lin et al., 2019). Consistently, in the present study, chronic MPTP injection resulted in higher values of alpha diversity indexes including Chao1, ACE and Shannon that were positively related to microbial richness and diversity, and lower values of Simpson that was inversely associated with microbial richness and evenness. However, the diverse results of the gut microbiome in PD patients may make it hard to compare the present results in mice with those in humans. And it was found different MPTP injection protocols and even different collection time of animal samples after MPTP injection could lead to various alterations of GM compositions and SCFAs production (**Supplementary Table 1**). The mechanism for MPTP to regulate GM compositions is yet to be elucidated. Recent studies presented bacterial phages (known as gut phagobiota) might also be implicated in regulating GM during neurodegenerative disease including PD (Tetz et al., 2018; Tetz and Tetz, 2018). GM and gut phagobiota interactively involved in PD pathogenesis, which might be one of the possible directions to disclose GM dysbiosis mechanisms in either PD patients or animal models.

The relationship of GM and autophagy is a novel topic in recent years. Although the detailed mechanism is not fully clarified, most relevant findings were disclosed in mechanism studies of IBD. Defective autophagy was suggested to have a strong impact on IBD pathogenesis, via disruption of intestinal barrier integrity, affecting GM composition and amplifying intestinal inflammation (Larabi et al., 2020). Furthermore, GM and autophagy influence each other and their interplay help to maintain intestinal homeostasis (Kim et al., 2020; Larabi et al., 2020). Until now, no study has reported the function of interplay of GM with autophagy during PD pathogenesis. In our present study, MPTP was found to influence both GM compositions and autophagy activity simultaneously, and it can be speculated their interplay finally resulted in GM dysbiosis and defective autophagy in the colon of PD mice.

Short chain fatty acids (SCFAs) (mainly including acetate, propionate and butyrate) are the main microbiota metabolites produced in the colon (Miller and Wolin, 1996). Acetate and propionate are mainly produced by *Bacteroidetes*, whereas *Firmicutes* are the primary contributors of butyrate (Feng et al., 2018), the significantly lower fecal acetate and propionate levels in our study might be attributed to the decreased abundance of *Bacteroidetes*; while no decrease in fecal butyrate concentration could be partially due to the simultaneously increased *Firmicutes* abundance in PD mice. Acetate (Xu et al., 2019) and butyrate (Zhou et al., 2020) were found to stimulate autophagy in normal intestinal epithelial cells. Propionate was reported to activate autophagy in colon cancer cells via decreased mTOR activity and enhanced AMP kinase activity (Tang et al., 2011). It suggested lower production of fecal SCFAs derived by GM dysbiosis, acetate, and propionate in particular, might be one of the mediators of the downregulated autophagy in the colon of PD mice. Accordingly, the dysfunctionally inhibited autophagy could explain the reduced apoptosis in the colon of PD mice.

The scope of the present study is limited to observe end-point changes of dopaminergic neurons in both the SNpc and the

colon from a classical PD mice model made by chronic MPTP injection for 5 weeks, followed by mechanism investigations to reveal the relationships of enteric dopaminergic neurons with autophagy, apoptosis and GM. Our findings were absent in the published information and would be helpful to fill a knowledge gap. However, our mechanism studies might not be very comprehensive, and be limited by the complexity of too many factors involved. Further studies are required to disclose how GM and autophagy interact in regulating enteric dopaminergic neurons; and how gut phagobiota interactively with GM involve in PD pathogenesis.

CONCLUSION

In conclusion, our results showed opposite changes of dopaminergic neurons in the colon and in the midbrain in a classical PD model established by chronic MPTP injection. PD mice exhibited apparent locomotor deficits and nigral dopaminergic neuronal loss in the CNS, but there were opposite changes of colonic dopaminergic neurons in the ENS. Mechanism investigation implied autophagy activity and apoptosis were stimulated in the SNpc, but inhibited in the colon of PD mice, which contributed to the opposite changes of dopaminergic neurons in CNS and colonic ENS. Chronic MPTP injection influenced GM compositions and autophagy activity simultaneously, and their interplay finally resulted in GM dysbiosis and defective autophagy in the colon of PD mice. Furthermore, GM dysbiosis derived fecal SCFAs, acetate and propionate in particular, might be one of the mediators of the downregulated autophagy in the colon of PD mice. Although alterations of GM composition and SCFAs production in such a chronic PD mice model may, to some extent, mimic those in PD patients, intestinal pathology found in this PD mice showed reversed changes with those in the brain. Such a chronic PD mice model might not be an ideal model to study role of gut-brain axis in PD progression. It is hope this study could provide more evidences to researchers in selection of animal models for PD studies.

DATA AVAILABILITY STATEMENT

All raw sequence data have been deposited to the China National GeneBank (CNGB) with project accession number: CNP0001553.

ETHICS STATEMENT

The experiment was conducted according to the guidelines approved by the Ethical Committee of Experimental Animal Care (approval no: 180703) at the Hong Kong Polytechnic University Shenzhen Institute.

AUTHOR CONTRIBUTIONS

XL and XW performed the animal experiments. Z-RD detected most of the parameters. K-HL and C-HC helped to reorganize data and revise the manuscript. FZ helped to conduct analysis for 16S rRNA gene sequencing. XC, QZ, and W-TW helped to revise the manuscript. K-HW supervised the study and revised the manuscript. X-LD designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.649627/full#supplementary-material

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REVIEW
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Gut Microbiota: Critical Controller and Intervention Target in Brain Aging and Cognitive Impairment

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The current trend for the rapid growth of the global aging population poses substantial challenges for society. The human aging process has been demonstrated to be closely associated with changes in gut microbiota composition, diversity, and functional features. During the first 2 years of life, the gut microbiota undergoes dramatic changes in composition and metabolic functions as it colonizes and develops in the body. Although the gut microbiota is nearly established by the age of three, it continues to mature until adulthood, when it comprises more stable and diverse microbial species. Meanwhile, as the physiological functions of the human body deteriorated with age, which may be a result of immunosenescence and "inflammaging," the guts of elderly people are generally characterized by an enrichment of pro-inflammatory microbes and a reduced abundance of beneficial species. The gut microbiota affects the development of the brain through a bidirectional communication system, called the brain-gut-microbiota (BGM) axis, and dysregulation of this communication is pivotal in aging-related cognitive impairment. Microbiota-targeted dietary interventions and the intake of probiotics/prebiotics can increase the abundance of beneficial species, boost host immunity, and prevent gut-related diseases. This review summarizes the age-related changes in the human gut microbiota based on recent research developments. Understanding these changes will likely facilitate the design of novel therapeutic strategies to achieve healthy aging.

Keywords: gut microbiota, brain aging, machine learning, cognitive impairment, Alzheimer's disease, diet, probiotics

Abbreviations: BGM, brain-gut-microbiota; SCFAs, short chain fatty acids; PERMANOVA, permutational multivariate analysis of variance; SPD, Sample Progression Discovery; GF, germ-free; IL-10, interleukin-10; TGF-β, transforming growth factor beta; Tregs, TGFβ-producing regulatory T cells; Th17, T helper 17; ILC2, type-2 lymphoid innate cells; IgA, immunoglobulin A; BMI, body mass index; CNS, central nervous system; ENS, enteric nervous system; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; BBB, blood–brain barrier; GPR, G protein-coupled receptor; 5-HT, 5-hydroxytryptamine; ECCs, enterochromaffin cells; SPF, specific-pathogen-free; MAMPs, microbial-associated molecular patterns; LPS, lipopolysaccharide; BLP, bacterial lipoprotein; CpG, cytosine-phosphate-guanosine; IL-1β, interleukin-1-β; IL-6, interleukin-6; TNFα, tumor necrosis factor-α; PAMPs, pathogen-associated molecular patterns; CSF, cerebrospinal fluid; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; AD, Alzheimer's disease; IBS, irritable bowel syndrome; PD, Parkinson's disease; Aβ, amyloid-β; NFTs, neurofibrillary tangles; MCI, mild cognitive impairment; TLR4, Toll-like receptors 4; TLR2, Toll-like receptors 2; SOD, superoxide dismutase; GSH, glutathione; GSK-3β, glycogen synthase kinase 3β; NMDA, N-methyl-D-aspartate glutamate receptor; NR2B, NMDA receptor subunit 2B; FOS, fructo-oligosaccharide; GOS, galactooligosaccharide; HTN, Hypertension; TMAO, trimethylamine-N-oxide; DM, Diabetes mellitus; T2DM, Type 2 diabetes mellitus; FODMAPs, low-fermentable, oligo-, di-, mono-saccharides and polyols; GFD, gluten-free diet; MOS, manno-oligosaccharides; XOS, xylo-oligosaccharide; COX-2, cyclooxygenase-2; iNOS, inducible Nitric Oxide Synthase; 6-OHDA, 6-hydroxydopamine; FMT, fecal bacteria transplantation; CDI, *Clostridium difficile* infection; SAMR1, senescence-accelerated mouse resistant 1; SAMP8, senescence accelerated mouse prone 8.

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INTRODUCTION

The average human lifespan expectancy in most countries is longer than ever before, benefiting from the achievements of modern medicine and lifestyle improvements. However, this has led to the rapid growth of the world's aging population (60 and older) (Riaz Rajoka et al., 2018). Aging is usually associated with various high-risk and long-lasting diseases, such as cancer, neurodegenerative disorders, diabetes, and metabolic syndrome, that seriously affect the life quality of the elderly (Dillin et al., 2014). The question of how to increase life expectancy while also reducing the duration and severity of morbidity poses a serious challenge for society.

Over 100 years ago, Elie Metchnikoff linked the gut microbiota with human health and aging for the first time, theorizing that health and lifespan could be enhanced and prolonged by regulating the gut microbiome *via* the consumption of host-friendly bacteria found in yogurt (Mackowiak, 2013). The gut microbial community, a "super organism" residing in the human intestinal tract, comprises $1 \times 10^{13-14}$ microorganisms, including approximately 500-1,000 bacterial species, that form a mutually beneficial symbiosis with their human hosts (Qin et al., 2010; Biagi et al., 2012). Besides aiding in digestive processes and food absorption, other functions of this super organism include synthesizing essential amino acids and vitamins, metabolizing fibers into short-chain fatty acids (SCFAs), maintaining the integrity of the intestinal mucosal barrier, regulating host immunity, and protecting the host from pathogen attack (Gill et al., 2006), all of which contribute to the maintenance of human health and well-being. Contrasting with this, gut dysbiosis can be a major contributor to physiological deterioration and the onset of geriatric diseases in humans (Kim and Jazwinski, 2018).

The application of 16s rRNA sequencing, based on second-generation, high-throughput technology, has greatly advanced the study of gut microbiota and opened the door for modern research into intestinal microecology (Dave et al., 2012; Bischoff, 2016). Additionally, the development of metagenomics has made it possible to define the gene content and encoded functional attributes of the gut microbiome in humans (Gill et al., 2006). Owing to the large amount of sequencing data generated in gut microbiota research, how to acquire useful information from the massive amounts of data has become an urgent task. The rise of machine learning provides new ideas for the study of gut microbiota. Various machine learning approaches, which can effectively identify taxonomic or functional signatures, have recently been applied to reveal the aging-related changes in the microbial community and identify the bacterial genera associated with these changes (Hopkins et al., 2001; Odamaki et al., 2016; Bian et al., 2017).

In this review, we summarize recent research findings on the role of gut microbiota in the aging process, and the application of machine learning for the analysis of age-related changes in gut microbe composition. We also highlight the link between bacterial communities and human health and the mechanisms underlying how the gut microbiota influence the development of age-related diseases, particularly cognitive impairment disorders, as well as the contribution of diet and probiotics/prebiotics to the maintenance of a beneficial gut microbiome to achieve healthy aging.

AGE-RELATED CHANGES IN THE HUMAN GUT MICROBIOTA

The presence of bacteria in the placenta, amniotic cavity, umbilical cord, and meconium suggests that the relationship between humans and microbes may begin in the uterus (Jimenez et al., 2005; Collado et al., 2016). The mode of delivery at birth directly influences the diversity of the microbiota in an infant (Rutayisire et al., 2016). Vaginally delivered infants have a more diverse and healthier bacterial community in their gut than those born by cesarean section (Jakobsson et al., 2014). In the first two to 3 years after birth, the gut microbiota of infants undergoes marked changes, influenced mainly by their feeding pattern (formula milk, breast milk, or solid food) (Cong et al., 2016; Kundu et al., 2017). Subsequently, the composition of the bacterial community remains relatively stable until adulthood, when it becomes fully established (Lynch et al., 2015). Generally, the most abundant phyla in a healthy adult gut are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*, with *Firmicutes* and *Bacteriodetes* accounting for almost 80% of this abundance (Eckburg et al., 2005). In adults, diet becomes the main influencing factor, i.e., the function of the gut microbiome changes from the lactate utilization seen in the infant to plant polysaccharide breakdown, xenobiotic degradation, and vitamin biosynthesis (Riaz Rajoka et al., 2018). With the deterioration of physiological functions (such as reduced intestinal functionality, chewing problems, and reduced immunity) with aging, the composition of the gut microbiota in the elderly changes markedly. Compared with healthy younger adults, the microbiota in the elderly gut is generally characterized by a reduced abundance of some beneficial genera, such as *Bifidobacterium* and *Lactobacillus*, and a marked increase in that of pro-inflammatory commensal microbes, such as Enterobacteriaceae and Clostridia (O'Toole and Jeffery, 2015; Odamaki et al., 2016; **Figure 1**). Aging is often accompanied by a decline in the diversity of the gut microbiota (Bischoff, 2016), which may be linked with the increased frailty index of the elderly (van Tongeren et al., 2005; Jackson et al., 2016). Cognitive impairment is a common disorder and is usually accompanied by changed microbiota in the elderly compared with that of a healthy elderly person. Clinical evidence has connected the gut microbiota dysbiosis with cognitive deficit in the elderly, because subjects with cognitive decline and brain amyloidosis had lower abundance of anti-inflammatory *E. rectale* and higher abundance of pro-inflammatory *Escherichia/Shigella* in their stools when compared with that of control subjects (Cattaneo et al., 2017). It was proved that the abundance of *Lactobacillales* members was positively while *Enterobacteriaceae* and *Porphyromonadaceae* were negatively associated with cognition in the elderly (Bajaj et al., 2016). This evidence have disclosed a direct link between microbes and cognitive conditions in the elderly.

Although the composition of the gut microbiome of longevous elderly individuals (mainly centenarians) also changes,

its diversity and beneficial species are retained (Ragonnaud and Biragyn, 2021), partially explaining how they maintain homeostasis and health, and, consequently, achieve their longevity. A recent study of centenarians in Italy revealed a cumulative decline in the abundance of a core microbiota with aging, but the age-related enrichment of subdominant taxa, including *Ruminococcaceae*, *Lachnospiraceae*, and *Bacteroidaceae* families, was boosted in longevity (Biagi et al., 2016). Similar studies in China have suggested that the gut microbiota of long-lived individuals (>90 years old) displays greater robustness and abundance than that of younger individuals (65-70 years old) as indicated by greater alpha-diversity (Kong et al., 2016; Wang N. et al., 2019). Eleven of the top 50 features were shared between these two independent studies, including community richness and significant changes in members of *Blautia*, *Clostridium* cluster XIVa, *Faecalibacterium*, *Escherichia*, and *Shigella*, and unclassified Lachnospiraceae, suggestive of a similar characteristic in the microbiota composition shared by longevous people from different regions. However, in most cases, the microbiota of humans display great inter-individual variations, as ethnicity, geography, and lifestyle also influence age-related changes in gut microbe composition (Ragonnaud and Biragyn, 2021). The typical diet in the United States is rich in protein, whereas in Malawi and among Amerindian populations the diet is dominated by corn and cassava. Accordingly, the microbes in the guts of inhabitants of metropolitan areas in the United States were significantly different from those of residents of rural Malawian communities and Amerindians from the Amazonas of Venezuela (Yatsunenko et al., 2012). Japanese adults (21–69 years old) have a greater abundance of *Blautia* and *Bifidobacterium* and a relatively lower abundance of *Bacteroidetes*-related genera compared with adults of other nations (Odamaki et al., 2016), including the United States, Colombia, South Korea, and China (Nam et al., 2011; Escobar et al., 2014). The abundance of *Clostridium* cluster XIVa was lower in Japanese, Italian, and Finnish elderly (Hayashi et al., 2003; Mueller et al., 2006; Makivuokko et al., 2010), but was higher in elderly Germans (Mueller et al., 2006). Meanwhile, the abundance of *Bacteroides* was increased in the Austrian elderly (Zwielehner et al., 2009), whereas in the Italian elderly an inverse trend was noted (Mueller et al., 2006).

To identify the sequential changes occurring in the gut microbiota during aging, a large number of studies have recently employed machine learning methods. Machine learning has unique advantages, including in cluster analysis of microbiota (Zhang et al., 2017), the identification and classification of microbiota (Wang et al., 2007; Zielinski et al., 2017; Oudah and Henschel, 2018), and the prediction of host phenotype (Johnson et al., 2016; Thompson et al., 2019).Using permutational multivariate analysis of variance (PERMANOVA), a method used in the supervised machine learning method, Odamaki et al. (2016) investigated fecal samples obtained from 367 healthy Japanese individuals aged less than 104 years, and revealed that the gut microbiota of those aged less than 20 years matured with age, while that of subjects over 70 years of age changed into the elderly type. The authors found that the aging process was accompanied by an increased abundance of *Bacteroides*, *Eubacterium*, and *Clostridiaceae*. Sequential changes occurred in the relative abundance of *Bacteroides*, Lachnospiraceae, and Bifidobacteria in the gut microbiota during childhood and adolescence (<20 years old), while *Megamonas* and *Peptoniphilus* were relatively enriched in the elderly (>70 years old). Using the same data samples, Xu et al. (2019) applied an unsupervised algorithm called Sample Progression Discovery (SPD) on genera abundance profile, and identified 35 genera associated with a continuous progression in the composition of the human gut microbiota with aging. Among these 35 genera, the abundance of some beneficial genera taxa, such as *Lactobacillus*, *Oscillospira*, *Oxalobacter*, *Prevotellaceae*, *Parascardovia*, and *Butyrivibrio*, increased with age, but decreased in the extremely elderly. In contrast, some taxa that are frequently linked with inflammation and diseases, including *Parvimonas*, *Anaerotruncus*, *Corynebacterium*, *Lachnospiraceae*, *Desulfovibrio*, *Bilophila wadsworthia*, *Odoribacter,* and *Butyricimonas*, showed monotonically increasing patterns with respect to aging. Yatsunenko et al. (2012) investigated 531 fecal samples of individuals from three different countries using Spearman's rank correlation and Random Forest methods and reported a decline in the abundance of *Bifidobacterium longum* species but an increase in the overall bacterial diversity with aging (0–70 years). However, another study obtained different results from the gut microbiota of more than 1,000 healthy Chinese individuals aged from 3 to over 100 years using PERMANOVA. These authors reported that the gut microbiota differed little among individuals from the ages of 30 to >100 and that the major between-group differences in gut microbiota profiles were found before 20 years of age (Bian et al., 2017). The similarity between the microbial diversity of the elderly and that of people decades younger may have been due to the participants being mostly very healthy or small sample size.

Collectively, despite the considerable inter-individual variability and the impact of external factors such as the dietary habits and geography/culture of the host (Claesson et al., 2012; Yatsunenko et al., 2012), it is increasingly clear that an intrinsic aging progression in the composition of human gut microbiota exists (Xu et al., 2019; Ragonnaud and Biragyn, 2021; **Figure 1**). In contrast, less is known of the mechanisms underlying these changes owing to the complicated physiological conditions and huge differences between individuals.

THE ROLE OF THE MICROBIOTA DURING AGING

The crucial influence of microbial composition on the rate and quality of aging is well-documented (Candela et al., 2014; Saraswati and Sitaraman, 2014). The changes in gut microbiome composition occur gradually (O'Toole and Jeffery, 2015). The fecal microbiota differs significantly between community-dwelling individuals and those living in long-term care nursing facilities (van Tongeren et al., 2005; Claesson et al., 2012; Kinross and Nicholson, 2012; Collino et al., 2013), consistent with its role in healthy aging.

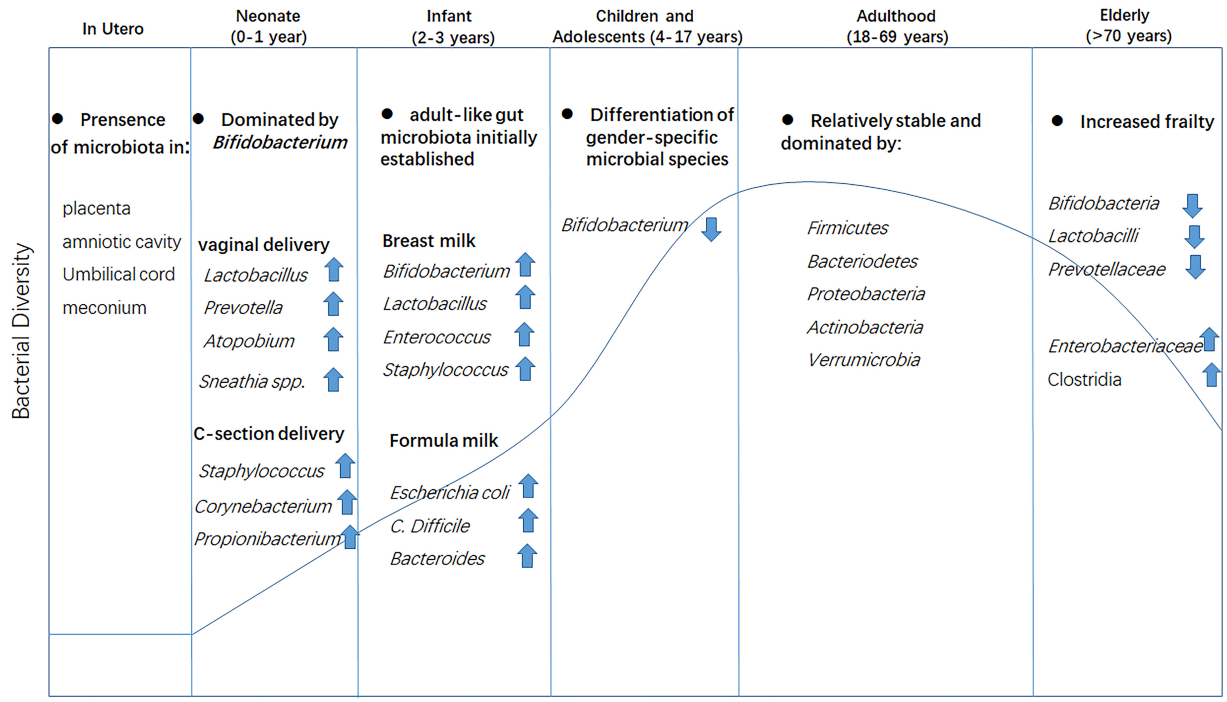

FIGURE 1 | Changes in gut microbiota composition at different life stages. Microbiota colonization may begin in the uterus as evidenced by the presence of bacteria in the placenta, amniotic cavity, umbilical cord, and meconium. The mode of delivery at birth has a direct effect on the bacterial community. The gut microbiota is initially established at approximately 3 years of age and matures at the onset of adulthood with the establishment of dietary habits, leading to a more diverse and stable gut microbial community. In old age, the gut microbiota is drastically altered and diversity is greatly reduced.

Immunosenescence, which refers to the gradual deterioration of the immune system with age, represents an important feature of the aging process. The chronic and low-grade activation of the innate and adaptive immune systems, also known as "inflammaging," is linked to immunosenescence, as evidenced by the persistence of NF-κB-mediated inflammation and the reduction of naïve $CD4^+$ T cell numbers (Franceschi, 2007; Claesson et al., 2012). Although the cause of inflammaging remains poorly understood, emerging evidence has connected it with reduced autoimmune tolerance of the gut microbiota and changes in its composition with aging (Guigoz et al., 2008). The gut microbiota is pivotal for maintaining the immune homeostasis of the human intestine (Honda and Littman, 2016). This can be directly evidenced in the germ-free (GF) mice, where the absence of gut microbes leads to the impaired the development and maturation of the immune system, while their presence induces the production of interleukin 10 (IL-10) and transforming growth factor beta (TGF-β)-producing regulatory T cells (Tregs), T helper 17 (Th17) cells, type-2 lymphoid innate cells (ILC2), and immunoglobulin A (IgA)-secreting B cells (Hansson et al., 2011; Atarashi et al., 2013, 2015; Furusawa et al., 2013; Satoh-Takayama et al., 2020). Besides, some beneficial bacterial species and their metabolites, particularly SCFAs and their precursors, have been reported to decline during aging in humans, and may be a primary cause of frailty in the elderly (Ragonnaud and Biragyn, 2021). Certain constituents of the gut microbiota were demonstrated to be predictors of exceptional human longevity (Vaiserman et al., 2017). The longevity of both Chinese and Italian centenarians has been positively associated with an abundance of beneficial commensals, such as *Clostridium* cluster XIVa, *Akkermansia muciniphila*, *Christensenellaceae,* and *Bifidobacteria* (Biagi et al., 2016; Kong et al., 2016). *Clostridium* cluster XIVa includes many genera involved in SCFAs production (van den Abbeele et al., 2013), while *A. muciniphila* helps maintain intestinal epithelial integrity by inducing the mucin production, supports beneficial SCFA-producing bacteria, and aids in reducing inflammation and metabolic impairments, such as insulin resistance (Schneeberger et al., 2015; Bodogai et al., 2018). The abundance of *Christensenellaceae* in the human gut shows an inverse correlation with the body mass index (BMI) and can help mitigate the effects of some inflammatory diseases in humans (Waters and Ley, 2019). *Bifidobacterium* can modulate the PH of the body and facilitate the digestion and absorption of essential nutrients and synthesis of vitamins through producing lactic acid, pyruvic acid, and butyrate; besides, it can inhibit the growth of pathogenic bacteria and enhance the immune response of the host (Yusof et al., 2000). These beneficial bacteria play important roles in gut homeostasis, and may help sustain a healthy state during aging.

Overall, the reduction of beneficial bacteria and enrichment of pro-inflammatory species, along with reduced gut microbial diversity, can exert detrimental effects on healthy aging and, consequently, longevity.

THE BRAIN-GUT-MICROBIOTA AXIS

The gut and brain are closely connected through a complex bidirectional communication system, known as the brain-gut-microbiota (BGM) axis, which primarily includes neural, immune, metabolic, and endocrine pathways (Martin et al., 2018).

The brain can directly or indirectly affect the composition and function of the gut microbiota by releasing signal molecules through lamina propria cells (intestinal chromaffin cells, neurons and immune cells) or modulating gastrointestinal motility, secretions, and intestinal permeability (Cryan et al., 2019). Inversely, the gut microbiota can influence the function, behavior, and health of the brain, and is suggested to be a key regulator of brain development, aging, and neurodegeneration (Dinan and Cryan, 2017). A normal gut microbiota–brain interaction is essential for the maintenance of a healthy physiological condition and normal cognitive functions.

Gut microbes can communicate with the brain *via* neuronal signaling that involves the central nervous system (CNS), autonomic nervous system, and enteric nervous system (ENS), often involving the stimulation of the vagus nerve. The vagus nerve is a bundle of parasympathetic motor and sensory fibers that provide a direct means of neurocommunication between the ENS and the CNS (Forsythe et al., 2014; Fung et al., 2017). Studies have found the genes encoding γ-aminobutyric acid (GABA) receptors are highly expressed in vagal afferent neurons and the nodose ganglion in the gastrointestinal tract of mice. The vagus nerve can sense GABA signals in the intestine and transmit them to the brain, as evidenced by the increased numbers of $GABA_A$ receptors and glutamic acid decarboxylase (GAD)-positive cells in the cerebral cortex following stimulation of the peripheral end of the vagus nerve (Marrosu et al., 2003; Neese et al., 2007; Egerod et al., 2018). Moreover, supplementation of the probiotic *Lactobacillus rhamnosus* JB-1 to mice can increase the mRNA level of the $GABA_B$ receptor B1 subunit in the cerebral cortex and that of the $GABA_A$ receptor α2 subunit in the prefrontal cortex and apricot kernel; suppress the stress-induced increase in corticosterone levels and alleviate anxiety and depression in mice; however, these beneficial effects were abolished when the mice were vagotomized (Bravo et al., 2011).

The effects of microbiota on CNS functions can also be mediated through the circulatory system *via* several microbial-derived molecules, including neurotransmitters, hormones, their precursors, and SCFAs. Some metabolites can pass through the intestinal barrier and enter the systemic circulation, and some can even cross the blood–brain barrier (BBB), thereby regulating neurological functions (O'Mahony et al., 2015; Koh et al., 2016; Sharon et al., 2016).The BBB forms during gestation and controls the passage and exchange of molecules and nutrients between the circulatory system and the brain parenchyma, which ensures CNS homeostasis (Braniste et al., 2014). The gut microbiota is important for the maintenance of BBB integrity, as evidenced by the increased BBB permeability and lower expression of the tight junction proteins occludin and claudin-5 in different brain regions of GF mice, while exposure of GF adult mice to a pathogen-free gut microbiota decreased BBB permeability and up-regulated the expression of tight junction proteins (Braniste et al., 2014). SCFAs are biologically active molecules that are mainly produced by beneficial intestinal microbes through the digestion of dietary fiber. These molecules can transverse the BBB and serve as key signaling metabolites in BBB development and maintenance via entering cells and acting as histone deacetylase inhibitors for epigenetic modulation or by binding to G protein-coupled receptor (GPR) 41 and/or GPR43 receptors on the cell membrane (Braniste et al., 2014; Michel and Prat, 2016). Although some gut neurotransmitters, such as serotonin (5-hydroxytryptamine, 5-HT), GABA, and dopamine, cannot cross the BBB, they can act on the vagus nerve or affect signaling in the peripheral nervous system, thereby eventually influencing brain functions (Wu et al., 2020). Serotonin, a well-characterized neurotransmitter known for its roles in modulating neural activity and a wide range of neuropsychological processes, is also an important regulator of gastrointestinal motility and cardiovascular function, among other functions (Berger et al., 2009). However, more than 90% of the body's 5-HT is synthesized by the enterochromaffin cells (ECCs) of the gastrointestinal tract. Metabolites such as SCFAs and 2Bas that are produced by indigenous spore-forming microbes have been demonstrated to promote the biosynthesis and release of 5-HT by ECCs (Yano et al., 2015). Tryptophan, the 5-HT precursor, is an essential amino acid that must be supplied in the diet. The gut microbiota contributes to the peripheral availability of tryptophan. Once absorbed from the gut, it enters the circulatory system, crosses the BBB *via* large amino acid transporters, and is converted to 5-HT in the CNS.

Neuroimmune signaling is also an important pathway for the communication between the gut microbiota and the CNS. In general, microbial-derived metabolites or other components mediate immune system activities. SCFAs can promote microglial maturation and are needed for the maintenance of mature microglia (Erny et al., 2015). Microglia are the resident macrophages and major immune defense cells of the CNS. GF mice have fewer microglia, and their morphology and function are abnormal compared with those of specific-pathogen-free (SPF) mice; however, these effects can be reversed by the administration of SCFAs to GF mice, and this may be primarily dependent on the activation of GPR43 by SCFAs (Erny et al., 2015). In addition, microbiota-derived microbial-associated molecular patterns (MAMPs) derived from microbiota, such as lipopolysaccharide (LPS), bacterial lipoprotein (BLP), flagellin, and cytosine-phosphate-guanosine (CpG) DNA, can activate the immune cells of the peripheral innate immune system and subsequently induce the release of numerous pro-inflammatory cytokines, such as interleukin-1-β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Sampson and Mazmanian, 2015). These pro-inflammatory cytokines can act on receptors in afferent nerves or cross the BBB and enter the brain parenchyma, eventually leading to changes in neurological functions in the CNS (Dantzer et al., 2000; Fung et al., 2017).

Recently, a number of studies have highlighted the potential role of bacteriophages as regulators of aging and neurodegeneration. Bacteriophages are the most abundant members of the microbial community, and have the potential

to shape gut bacterial communities and modulate microbiota stability (Gogokhia et al., 2019). Bacteriophages can influence the development of human diseases either directly through interactions with eukaryotic cells and proteins, or indirectly by inducing changes in the abundance of certain bacteria and increasing the levels of pathogen-associated molecular patterns (PAMPs). Many bacteriophages have the ability to maintain intestinal barrier integrity by embedding themselves within the mucus layer and controlling invasive bacterial populations (Barr et al., 2013), and can also alter mucosal immunity, thereby influencing mammalian health (Gogokhia et al., 2019). In a rodent model, bacteriophage administration induced shifts in the gut microbiota, leading to increased intestinal permeability and triggering chronic inflammation (Tetz and Tetz, 2016). Recent studies have demonstrated that bacteriophages are present in the cerebrospinal fluid (CSF) of subjects with neurodegenerative pathologies (Tetz and Tetz, 2018), and that PD patients display a greater abundance of lytic *Lactococcus* phages and a 10-fold reduction in neurotransmitter-producing *Lactococcus* bacteria compared with that in healthy individuals, suggestive of an association with and the possible role of phages in neurodegeneration (Tetz et al., 2018). Bacteriophages may affect the CNS through pathways involved in increasing intestinal permeability, altering the abundance of bacteria that are important for the maintenance of a healthy CNS, and inducing a chronic systemic inflammatory response (Tetz and Tetz, 2018). However, substantial research is still needed to elucidate these underlying mechanisms.

GUT MICROBIOTA AND COGNITIVE IMPAIRMENT

Unimpaired cognitive skills are crucial for the daily functioning of older people. However, some of these cognitive skills including memory, learning and problem-solving activities, decline during aging. Multiple risk factors have been proposed to be related to cognitive impairment (Klimova et al., 2017). Studies have increasingly emphasized the influence of microorganisms on host behavior and cognitive function, such as those involving GF animal models in which behavioral disorders and reduced cognitive function are prominent (Lee et al., 2020).

Gut microbiota can play an intrinsic role in aging-related impairments in a range of cognitive processes. The dysbiosis of gut microbiota is involved in aging-related diseases in multiple pathways (**Figure 2**). Aging is associated with low-grade inflammation, and prolonged exposure of the brain to inflammatory cytokines can impair cognition. Studies on aged mice have shown that acute inflammation can induce memory defects (Chen et al., 2008; Barrientos et al., 2009). Microbial dysbiosis or increased gut permeability with aging has similarly been linked with inflammation through inducing the production and release of bacterial components; such as LPS, lipoproteins, and double-stranded RNA into the bloodstream. This leads to the activation of the immune system and the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Sampson and Mazmanian, 2015; Komanduri et al., 2019). Age-associated cognitive decline may also be attributed to a decrease in synaptic connections in the brain (Vanguilder et al., 2010). Meanwhile, gut microbes are capable of producing neurotransmitters (such as GABA, acetylcholine, and dopamine) and neurotrophic factors (such as brain-derived neurotrophic factor [BDNF] and nerve growth factor [NGF]), which are essential for the transmission of nerve signals (Diaz Heijtz et al., 2011; Sampson and Mazmanian, 2015). *Lactobacilli* and *Bifidobacteria* can alleviate anxiety and depression-like symptoms by converting glutamate in the gut into GABA (Yunes et al., 2016). The numbers of *Alcaliganeceae* and *Porphyromonadaceae* were shown to be positively correlated with cognitive decline (Caracciolo et al., 2014). Besides, gut microbiota can also affect cognition through promoting oxidative stress, as evidenced by the sharp rise in the abundance of cyanobacteria in Alzheimer's disease (AD) patients. The cyanobacterial-derived metabolite (β-N-methylamino-L-alanine) can activate glutamate receptors, leading to oxidative stress in neurons and, ultimately, neuronal apoptosis (Banack et al., 2010).

Many preclinical studies have highlighted the crucial role of alterations in brain-gut-microbiome communication not only in the pathogenesis and pathophysiology of classic brain-gut disorders such as irritable bowel syndrome (IBS), and obesity, but also in psychiatric and neurological disorders, such as depression, anxiety neurosis, AD, and Parkinson's disease (PD) (Collins et al., 2012; Martin et al., 2018; **Table 1**).

Alzheimer's Disease

Alzheimer's disease is a progressive neurodegenerative syndrome and the most common cause of dementia. The pathogenesis of AD includes the excessive aggregation of amyloid-β (Aβ) peptides, the presence of neurofibrillary tangles (NFTs) induced by the hyperphosphorylation of tau protein, neuroinflammation, and metabolic disturbances; however, the mechanisms underlying the pathogenesis of AD is not fully understood (Jagust, 2018).

Accumulating evidence supports a close connection between gut microbiota dysbiosis and AD (Jiang et al., 2017). For example, the structure of the mucosa of the small intestine of AD transgenic mice differs from that of normal mice, while the number of Gram-negative bacteria in the colon is also significantly reduced in the AD mice (Karri et al., 2010). The McCarthy survey showed that the gut microbiota was altered in 85% of dementia patients in the United Kingdom (Xiao et al., 2014). Recent studies revealed that fecal samples of AD patients display higher relative abundances of taxa known to promote a pro-inflammatory state, such as *Escherichia Shigella*, *Odoribacter splanchnicus,* and *Klebsiella pneumonia*, and reductions in key butyrate-producing anti-inflammatory species, such as members of the genera *Butyrivibrio* and *Eubacterium*, compared with those of non-AD individuals (Cattaneo et al., 2017; Haran et al., 2019). Additionally, the diversity of the gut microbiota community was reported to be significantly reduced in AD patients compared with non-AD patients (Vogt et al., 2017). An increased prevalence of *Bacteroides* was proved to be independently associated with the presence of mild cognitive impairment (MCI) in patients without dementia based on graphical modeling and multivariable

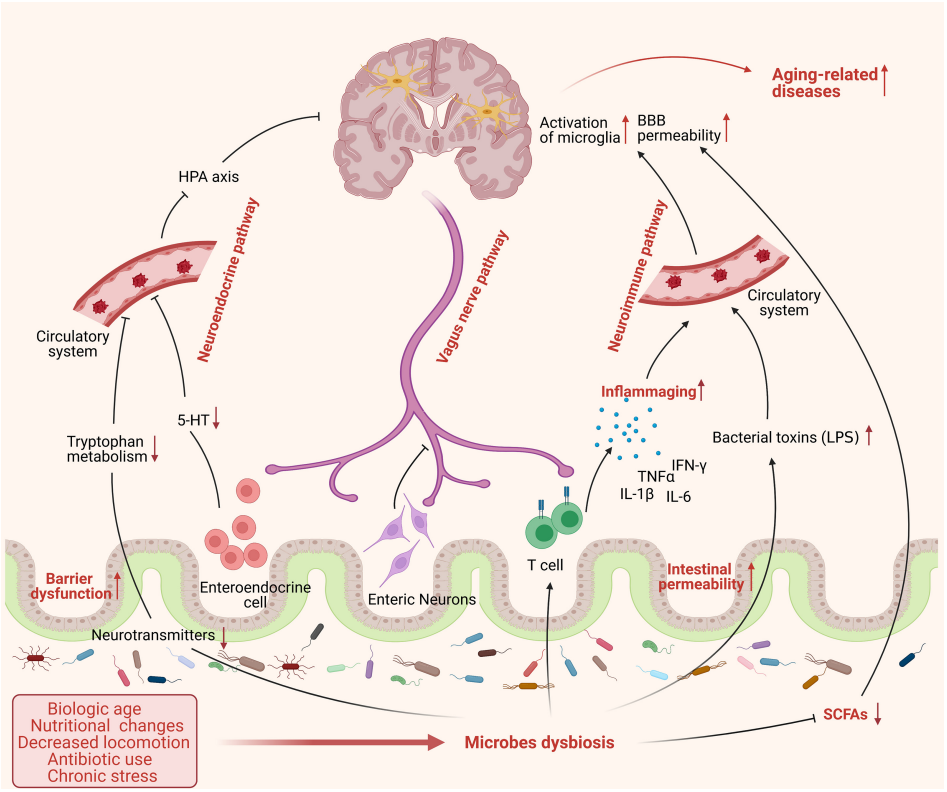

FIGURE 2 | The dysbiosis of gut microbes in aging process. Aging is accompanied by significant changes in lifestyle, such as biological age, nutritional changes, decreased locomotion, antibiotic use, and chronic stress, which may cause an alteration of microbes' composition and exacerbate the dysbiosis of gut microbiota in frail elderly people. Microbes dysbiosis in aging shows a severe decrease of beneficial bacteria, such as SCFA-producing bacteria, and increased pathogenic bacteria, such as Enterobacteriaceae, affecting gut physiology and leading to reduced intestinal motility, gut-barrier dysfunction, and increased intestinal permeability. Furthermore, the dysbiosis of microbiota could influence the brain function through various routes including: (1) promote the release of pro-inflammatory cytokines and bacterial toxins to the circulatory system, such as LPS, causing systemic inflammation via neuroimmune pathway; (2) inhibit the transmission of neural signal from enteric nervous system (ENS) to central nervous system (CNS) mainly via vagus nerve; (3) suppress the production and release of microbial neurotransmitters and hormones to circulatory system and subsequently to brain through neuroendocrine pathway. All these factors cause a chain of detrimental events to the brain that enhancing the risk of developing aging-related pathologies. (This figure was created with BioRender.com).

logistic regression analysis in a cross-sectional study (Saji et al., 2019). Besides, the latest research has shown a sex-dependent association between gut microbiota and cognitive impairment in a mice model of AD, indicating that sex itself may exert a specific impact on the composition of the microbiota in AD pathology (Cuervo-Zanatta et al., 2021).

Gut microbiota may participate in the development and progression of AD in multiple ways. A key player in the complex pathogenesis of AD in neuroinflammation, which is characterized by the excessive activation of microglia, leading to significant changes in their structure and function and resulting in the production of large amounts of pro-inflammatory factors in the CNS. Emerging evidence from both animal and human studies supports an association between dysbiosis and microglia activation during AD development (Vogt et al., 2017; Zhuang et al., 2018). Induced by certain gut bacteria, Aβ protein deposited in the brain can bind to the CD14 receptor, methyl peptide receptors, and Toll-like receptors 4 (TLR4) on the surface of microglia. This activates these cells, which release large amounts of pro-inflammatory factors such as IL-6 and TNF-α, thereby activating astrocytes, and further aggravating inflammatory responses in the brain (Lehnardt, 2010). Gut dysbiosis can also alter the production of tight junction proteins in the intestinal mucosa, which can destroy the intestinal barrier, leading to LPS leakage into the blood circulation and causing neuroinflammation (Cani et al., 2008). Recent evidence has implicated infiltrating peripheral immune cells, such as $CD4^+$ and $CD8^+$T cells in AD-associated neuroinflammation (Merlini et al., 2018). Wang X. et al. (2019), suggested that the combination of specific bacteria (e.g., Th1/M1-associated bacteria), amino acids (e.g., phenylalanine and isoleucine), and the composition of brain-infiltrating immune cell (e.g., Th1- dominant) may serve as an early diagnostic biomarker for MCI in AD patients.

Amyloid-β accumulates in the cerebral cortex and hippocampus of AD patients to form toxic senile plaques, which play an important role in the pathogenesis and progression of this disease. A variety of bacterial species resident in the human gut can produce amyloid, including *Pseudomonas*, *Streptomyces*, *Bacillus,* and *Escherichia coli*, which may contribute to both the systemic and CNS-associated amyloid burden in aging humans

(Zhao and Lukiw, 2015). The amyloid secreted by *E.coli* has a similar structure and immunogenicity to that of Aβ42, and can promote the release of pro-inflammatory factors by binding to the Toll-like receptors 2 (TLR2) on the surface of microglia and subsequently exacerbate the inflammatory response in the AD brain (Hill and Lukiw, 2015). Tau protein hyperphosphorylation is another pathological feature of AD. Gut microbiota may directly or indirectly regulate tau hyperphosphorylation through multiple mechanisms: (1) The interaction between deposited Aβ and tau protein could induce tau hyperphosphorylation, and microbes can influence this process by regulating Aβ deposition. (2) Through alleviating oxidative stress-induced damage by producing antioxidants or enhancing the activity of superoxide dismutase (SOD) and glutathione (GSH), gut microbiota can to some extent inhibit tau hyperphosphorylation (Wall et al., 2014; Yan et al., 2016). (3) Gut microbiota can interfere with the insulin signaling pathway by inducing inflammation and promoting adipogenesis, leading to the activation of glycogen synthase kinase 3β (GSK-3β), which promotes tau hyperphosphorylation. (4) The systemic inflammation induced by LPS secreted by specific bacteria can accelerate the hyperphosphorylation of tau protein (Savignac et al., 2016).

The gut microbiota is important for the synthesis of brain neurotransmitters (such as GABA and 5-HT), BDNF, and SCFAs, which are all associated with AD (Hu et al., 2016). GABA is a major inhibitory neurotransmitter in the human CNS. Studies have shown that the GABA level in the gut is affected when the gut microbiota is disturbed, especially when the numbers of *Bifidobacteria* and *Lactobacilli* decrease, which in turn leads to a decline in GABA levels in the CNS (Bhattacharjee and Lukiw, 2013). Glutamate is a crucial excitatory neurotransmitter in the human CNS, and is known to bind the N-methyl-D-aspartate (NMDA) receptor, an important regulator of neuronal activation, dendritic and axon structure, and synaptic plasticity. Neufeld et al. (2011) reported that the mRNA expression of NMDA receptor subunit 2B (NR2B) was significantly downregulated in the hippocampus of sterile mice, indicating that a correlation exists between the gut microbiota and the expression of the NMDA receptor. SCFAs are important metabolic products of gut microbiota, regulating the metabolism of free fatty acids, glucose, and cholesterol in the body through various cell-signaling cascades involving G protein-coupled receptors (Den Besten et al., 2013). SCFAs also serve as important modulators in immunity via NF-κB signaling, regulating the responsiveness of microglial cells to LPS (Jiang et al., 2017). Besides, SCFAs exert neuro-modulatory and epigenetic effects through histone acetylation and were shown to improve cognitive function in animal models of neurodevelopmental and neurodegenerative diseases (Stilling et al., 2014). Given these important physiological functions of SCFAs, reduced SCFA production by the gut microbiota of AD patients may represent another mechanism underlying the development of AD (Zhuang et al., 2018).

Parkinson's Disease

Parkinson's disease is a long-term degenerative disorder of the CNS, and is characterized by the abnormal aggregation of α-synuclein and the degeneration and necrosis of substantia nigra dopaminergic neurons. The clinical manifestations of PD include both motor symptoms and autonomic non-motor symptoms such as cognitive impairment and sleep disturbance. Several lines of evidence indicate that the gut may be involved in the origin of PD. For instance, gut symptoms appear in the early stages of PD, while α-synuclein misfolding and aggregation also initially occur in the ENS and dorsal motor nucleus of the vagus nerve (Braak and Del Tredici, 2017). Furthermore, LPS released by gut bacteria can also modulate α-synuclein aggregation, which is a well-characterized interaction associated with this alternative pathway of PD progression (Bhattacharyya et al., 2019). Holmqvist et al. (2014) for the first time demonstrated that various forms of α-synuclein can spread into the brain along the vagus nerve after being injected into the gut of mice, regardless of whether the injected material was a human PD brain lysate containing abnormally folded α-synuclein or recombinant forms (monomers, oligomers, or fibrils) of α-synuclein protein.

The gut microbiota is disordered in PD patients (Scheperjans et al., 2015). The abundance of *Prevotellaceae* in the gut of PD patients was reported to be 77.6% lower than that of healthy controls, and this decline may result in reduced mucin synthesis and a subsequent increase in gut permeability (Caputi and Giron, 2018; Gerhardt and Mohajeri, 2018). One study revealed that the abundance of "anti-inflammatory" butyrate-producing bacteria from the genera *Blautia*, *Coprococcus*, and *Roseburia* was significantly lower in the colonic mucosa and fecal samples of PD patients than in those of control subjects (Keshavarzian et al., 2015). *Verrucomicrobia*, *Mucispirillum*, *Porphyromonas*, *Lactobacillus*, and *Parabacteroides* were reported to be the most abundant taxa in PD patients, while *Prevotella* is more abundant in the gut of healthy individuals. The abundance of *Bacteroides* is significantly higher in PD patients without tremor symptoms than in those presenting with tremors, and increases with motor symptom severity (Lin et al., 2019). This study indicated that a correlation existed between changes in the gut microbiota of PD patients and the severity of their clinical phenotype. Epidemiological studies involving Danish and Swedish patients have also shown that truncal vagotomy is protective against PD (Svensson et al., 2015; Liu et al., 2017), providing clinical evidence for a link between gut microbiota and this disease. Additionally, a preclinical study using a mouse model of PD showed the beneficial effect of a nutritional supplement containing prebiotic fibers (fructo-oligosaccharide [FOS] and galacto-oligosaccharide [GOS]), on motor, cognitive, and gut symptoms (Perez-Pardo et al., 2017). Gut bacteria may be implicated in PD through several pathways. Gut dysbiosis can induce intestinal inflammation, leading to the accumulation of α-synuclein in the ENS, which may subsequently spread to the CNS through the vagus nerve (Houser and Tansey, 2017). Additionally, SCFAs were shown to be significantly reduced in the gut of PD patients, which may result in mucosal barrier impairment and increased gut permeability, thereby affecting the immune system, ENS, and CNS, and exerting a profound effect on the condition of PD patients (Vizcarra et al., 2015). Additionally, a recent study identified significant changes in the phage/lactic acid bacteria ratio in PD patients. The bacteria are

TABLE 1 | Alterations of the gut microbiota in different geriatric diseases.

Disease	Subjects	Gut microbiota alterations	References
Alzheimer's disease	AD human patients	*Bacteroides*↓; *Ruminococcus*↑; *Actinobacteria*↑; *Lachnospiraceae*↓	Zhuang et al., 2018
	AD human patients	*Escherichia/Shigella* (pro-inflammatory taxon) ↑; *E. rectale* (anti-inflammatory taxon) ↓	Cattaneo et al., 2017
	AD human patients	*Butyrivibrio/Eubacterium/Clostridium* (key butyrate-producing species)↓; *O. splanchnicus*↑; *K. pneumonia*↑	Haran et al., 2019
	AD human patients	microbial richness and diversity↓; *Firmicutes*↓; *Bacteroidetes*↑; *Bifidobacterium*↓	Vogt et al., 2017
	APP/PS1 mice	Microbiota diversity↓; *Helicobacteraceae*↑; *Desulfovibrionaceae*↑; *Odoribacter*↑; *Helicobacter*↑; *Prevotella*↓	Shen et al., 2017
	5 × FAD mice	*Firmicutes:Bacteroidetes* ratio↑; *Clostridium leptum*↑	Brandscheid et al., 2017
Parkinson's disease	PD human patients	Prevotellaceae↓; Enterobacteriaceae↑	Scheperjans et al., 2015
	PD human patients	Intestinal permeability↑; *E. coli* bacteria↑;	Forsyth et al., 2011
	PD human patients	*Blautia*↓; *Coprococcus*↓; *Roseburia*↓; *Faecalibacterium*↓; *Ralstonia*↑	Keshavarzian et al., 2015
	PD human patients	*Lactobacillus*↑; *Faecalibacterium*↓; *Coprococcus*↓; *Blautia*↓; *Bifidobacteriu*↑;*Verrucomicrobiaceae*↑	Gerhardt and Mohajeri, 2018
	PD human patients	*Bacteroides*↑; *Prevotella*↓; *Parabacteroides*↑; *Verrucomicrobia*↑; *Akkermansia*↑; *Butyricimonas*↑; *Veillonella*↑; *Odoribacter*↑; *Mucispirillum*↑; *Bilophila*↑; *Enterococcus*↑; *Lactobacillus*↑	Lin et al., 2019
Hypertension	Hypertension patients	Microbial richness and diversity↓; *Prevotella*↑; *Klebsiella*↑; *Porphyromonas*↑; *Actinomyces*↑; *Faecalibacterium*↓; *Oscillibacter*↓; *Roseburia*↓; *Bifidobacterium*↓; *Coprococcus*↓; *Butyrivibrio*↓	Li et al., 2017
	Hypertension patients and SHRs rat	Microbial richness and diversity↓; *Firmicutes*/*Bacteroidetes* ratio↑; Acetate- and butyrate-producing bacteria (*Coprococcus* and *Pseudobutyrivibrio*)↓; lactate-producing bacteria (*Streptococcus* and *Turicibacter*)↑	Yang et al., 2015
Atherosclerosis	Atherosclerosis patients	*Collinsella*↑; *Eubacterium*↓; *Roseburia*↓; *Bacteroides*↓	Karlsson et al., 2012
	Atherosclerosis patients	*Escherichia coli*↑; *Klebsiella* spp.↑; *Enterobacter aerogenes*↑; *Streptococcus* spp.↑;*Lactobacillus salivarius*↑; *Solobacterium moorei*↑; *Atopobium parvulum*↑; *Bacteroides* spp.↓; *Prevotella copri*↓; *Alistipes shahii*↓	Jie et al., 2017
Type 2 diabetes mellitus	T2DM patients	*Firmicutes*/*Bacteroidetes* ratio↓; *Clostridia*↓; *Proteobacteria*↑; *Bacilli*↑	Larsen et al., 2010
	T2DM patients	Verrucomicrobiae↓; butyrate producing bacteria (*Akkermansia muciniphila* and *Faecalibacterium prausnitzii*)↓	Zhang et al., 2013
	T2DM patients	*Lactobacillus*↑; *Bifidobacteria*↓	Sedighi et al., 2017
		Faecalibacterium prausnitzii↓; *Akkermansia muciniphila*↓	Fassatoui et al., 2019

known to produce dopamine and regulate intestinal permeability and are major factors implicated in PD pathogenesis (Tetz et al., 2018). However, more research is needed to understand the mechanism underlying how the gut microbiota affects the development of PD.

Hypertension

Hypertension (HTN) is a major risk factor for cardiovascular, cerebrovascular, and kidney diseases in the elderly. Longitudinal studies have indicated that HTN during midlife may be a risk factor for subsequent cognitive decline and dementia (Wysocki et al., 2012; Walker et al., 2019). HTN may be induced by a complex interplay between genetic and environmental factors; however, the precise cause of this morbidity has not been elucidated to date (Li et al., 2017). Genetic, environmental, and dietary factors profoundly influence both gut microbiota and blood pressure, suggesting a link between gut dysbiosis and HTN (Yang et al., 2015). Li et al. (2017) found dramatically decreased microbial richness and diversity, a *Prevotella*-dominated gut enterotype, distinct metagenomic composition with reduced numbers of beneficial bacteria, and disease-linked microbial function in both pre-hypertensive and hypertensive human populations compared with that of healthy controls. Additionally, blood pressure is elevated in GF mice after the transplantation of fecal microbiota from hypertensive humans, demonstrating that gut microbiota can directly influence the blood pressure of their host. Diet is an important factor in the onset of HTN. A recent study reported that the detrimental effects resulting from low-fiber Westernized diets may underlie hypertension, which may be due to a deficiency in SCFA production by gut microbiota (Kaye et al., 2020). Evidence has suggested that LPS overproduction by gut bacteria may be directly linked to

HTN development, whereas amino acid biosynthesis, fatty acid utilization, and purine metabolism by bacteria might have a role in HTN prevention (Li et al., 2017). Overall, despite the mechanism being undefined, gut dysbiosis has been closely linked with hypertension, which is likely to be associated with bacterial metabolites, such as SCFAs.

Atherosclerosis

Atherosclerosis is the most common vascular brain pathology in the elderly, and is characterized by the formation of atherosclerotic plaques mainly consisting of accumulated modified lipids, hyperplastic smooth muscle cells and collagen fibers (Gui et al., 2012). Studies have shown that cerebral atherosclerosis can increase the risk of dementia, particularly that associated with AD (Arvanitakis et al., 2016). Accumulating evidence suggested that a link exists between gut microbiota and atherosclerosis development, and changes in the function and composition of bacterial population may increase the risk for atherosclerosis through complex mechanisms (Drosos et al., 2015). Compared with that of healthy controls, the microbiota of atherosclerosis patients exhibits an increased abundance of *Enterobacteriaceae* and *Streptococcus spp*., resulting in a less fermentative and more inflammatory gut environment (Jie et al., 2017). Gut microbiota might be involved in the progression of atherosclerosis primarily through modulating inflammation and the production of microbial metabolites such as trimethylamine-N-oxide (TMAO), SCFAs, and bile acids (Brown and Hazen, 2018). TMAO, a gut microbe-dependent metabolite, was shown to be important for the development of atherosclerosis through the regulation of the host's immune system (Jandhyala et al., 2015) and cholesterol metabolism (Geng et al., 2018), contributing to oxidative stress (Mohammadi et al., 2018) and inflammation (Ma et al., 2017), and increasing the risk of thrombosis (Zhu et al., 2016). Studies have found that the level of TMAO in plasma can be reduced by remodeling the gut microbiota through the intake of probiotics and prebiotics (Qiu et al., 2018; Tenore et al., 2019).

Type 2 Diabetes Mellitus

Diabetes mellitus (DM) is one of the most important public health challenges of the 21st century and poses a serious threat to the health of the elderly. Epidemiological studies have shown that diabetic patients are more susceptible to dementia than healthy individuals (Sun et al., 2020). Type 2 diabetes mellitus (T2DM) is associated with an increased risk of cognitive impairment through multiple-mechanisms, among which vascular disease may be a key factor (Biessels et al., 2014). Altered gut microbial composition has been implicated in diabetes. Diabetic patients were reported to have a low abundance of butyrate-producing bacteria and reduced proportions of *Verrucomicrobiae* compared with healthy controls (Tai et al., 2015). Additionally, the abundance of *Lactobacillus* was reported to be significantly increased in T2DM patients, whereas that of *Bifidobacteria* was decreased (Sedighi et al., 2017), Another study found a markedly lower abundance of *Lactobacillus acidophilus* in fecal samples of diabetic patients, suggestive of a correlation between Lactobacillus and T2DM (Halawa et al., 2019). In addition, the amounts of *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* were lower in the guts of T2DM patients than in those of non-diabetic patients (Fassatoui et al., 2019). The effect of microbiota on T2DM has been proposed to be mediated through mechanisms involving modifications in butyrate and incretin secretion (Baothman et al., 2016). Although there is evidence for a link between gut dysbiosis with T2DM, differences in research populations, sequencing technologies, analysis methods, diets, and drugs utilized have led to varied results. Consequently, further studies are needed to uncover the exact relationship between T2DM and the gut microbiomes.

POTENTIAL INTERVENTION STRATEGIES TARGETING GUT MICROBIOTA IN BRAIN AGING AND COGNITIVE IMPAIRMENT

Dietary Fiber

Lifestyle greatly influences diet structure in humans, while the gut microbiome can rapidly adapt to dietary alterations (David et al., 2014). Specific diets (low-fermentable, oligo-, di-, mono-saccharides, and polyols [FODMAPs] and gluten-free diet [GFD]) and eating habits can have a positive effect on balanced microbiota composition and thus contribute to the enhancement of cognitive functions, important for any learning process (Novotny et al., 2019). Numerous evidences have shown that certain types of dietary fiber can regulate the number of microorganisms and their metabolites, i.e., the intake of fructan and GOS can increase the abundance of *Bifidobacteria* and *Lactobacilli* (So et al., 2018). A high-fat diet not only leads to weight gain, but also increases gut permeability and systemic inflammation levels, and reduces defense functions (Lam et al., 2012). In contrast, dietary fiber can correct the composition of the gut microbiota and promote the production of SCFAs (Bishehsari et al., 2018). SCFAs, including butyric acid, acetic acid, and propionic acid, are beneficial for metabolism and physiological health, helping to maintain the acidic environment of the intestinal cavity, increasing the abundance of beneficial bacteria, promoting mucus secretion, and sustaining the barrier function of the intestinal mucosal. Butyric acid can cross the BBB and function as an anti-depressive ability (Han et al., 2014), and can also help prevent colon cancer by inhibiting histone deacetylase (Hamer et al., 2008). Propionic acid contributes to cholesterol synthesis, plays an important role in liver gluconeogenesis, and exerts a preventive effect against metastatic liver cancer (Comalada et al., 2006). Acetic acid, used in liver fat production and cholesterol synthesis, can regulate the blood supply of the colon and protect against liver cancer (Chambers et al., 2002). SCFAs may also modulate the hypothalamic-pituitary-adrenal axis by directly affecting the mucosal immune system, and further affect information transmission in the CNS through these mechanisms (Perry et al., 2016). De Filippo et al. (2010) compared the fecal microbiota of African children who consumed a high fiber diet and European children who consumed a Westernized diet, and found that

the African children had greater gut microbial diversity and abundance of SCFA-producing bacteria and reduced quantities of potentially pathogenic strains compared with their European counterparts. This suggested that diet plays a dominant role in shaping the gut microbiota and highlighted the beneficial effect of dietary fiber on maintaining a healthy intestinal tract.

Probiotics and Prebiotics

Using probiotics and prebiotics to regulate the gut microbiota represents a viable strategy to delay aging. In addition to providing health benefits for individuals with underlying pathologies, probiotic supplementation can also improve the gut microbiota composition in healthy adults (Khalesi et al., 2019). The probiotics currently used in humans mainly include *Lactobacillus*, *Bifidobacterium*, *S. thermophilus*, *Enterococcus,* and *Bacillus*. Probiotics can regulate the release of neurotransmitters, increase the levels of tryptophan-derived neurotrophic factors, and contribute to the prevention and early treatment of cognitive dysfunction-related diseases (Bhattacharjee and Lukiw, 2013). Certain *Lactobacillus* and *Bifidobacterium strains* secrete important neurotransmitters such as GABA, acetylcholine, dopamine, or 5-HT, which play important roles in controlling the neural excitatory-inhibitory balance, mood, cognitive functions, and learning and memory processes (Bravo et al., 2011; Barrett et al., 2012; O'Mahony et al., 2015). In animal models, *Lactobacillus helveticus* NS8 can increase the expression of 5-HT and BDNF in the hippocampus, and induce significant improvements in cognitive function (Liang et al., 2015). Prebiotics, mainly including FOS, GOS, manno-oligosaccharides (MOS), and xylo-oligosaccharide (XOS), are non-digestible food ingredients that can regulate the gut microbiota by stimulating the growth of some beneficial bacteria (Duncan and Flint, 2013). GOS administration by gavage significantly increased the expression of BDNF in the brains of rats (Savignac et al., 2013). A recent study showed that prebiotic supplementation (FOS-inulin) can inhibit Con A-induced systemic inflammation in middle-aged mice, particularly by reducing TNF-α, indicating that prebiotics might have specific systemic effects on the immune priming of T and NK cells (Boehme et al., 2020).

Probiotics can improve intestinal defense mechanisms against pathogenic microorganisms and enhance the immune system (Rooks and Garrett, 2016). Studies have demonstrated that probiotic intake can reduce systemic inflammation by decreasing the levels of pro-inflammatory cytokines, such as IL-1β (Ait-Belgnaoui et al., 2012; Luo et al., 2014), IL-6, and TNFα (Ait-Belgnaoui et al., 2012), as well as those of microglial activation markers (Ait-Belgnaoui et al., 2014). In the CD1 mouse, prebiotic treatment can significantly suppress the LPS-induced inflammatory response, decrease the expression of IL-1β in the cortex, and increase the expression levels of 5-HT and those of components of the neuronal protective glutamatergic system (Savignac et al., 2016). In a mouse model of cognitive impairment, gastric infusion of *Lactobacillus pentosus* var. *plantarum* C29 significantly improved D-galactose-induced memory dysfunction, increased the expression of BDNF in the brain, and decreased the expression of the senescence marker p16 and that of the inflammation markers TNF-α, p-p65, p-foxo3a, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS), indicating that C29 may ameliorate aging-related memory impairment and inflammation (Woo et al., 2014). A recent study has revealed that the SLAB51 formulation, a mixture of bifidobacteria and lactobacilli, can counteract the detrimental effect induced by 6-hydroxydopamine (6-OHDA) *in vitro* and *in vivo* models of PD through modulating the BNDF pathway, increasing the PPARγ, activating the Nrf2/HO-1 pathway and inhibiting NFκB, which suggested that SLAB51 can be a promising candidate for PD prevention or treatment or as coadjuvant therapy (Castelli et al., 2020). In addition, probiotics may delay senescence through their antioxidant and free radical scavenging abilities (Li et al., 2012). *Lactobacillus fermentum* can increase the total serum antioxidant capacity in the pig by reducing malondialdehyde levels and increasing those of SOD and glutathione peroxidase, and has the capacity for scavenging free radicals (Wang et al., 2009).

Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) is defined as the transplantation of the functional bacteria present in the feces of healthy people into the gastrointestinal tract of patients to rebuild the gut microbiota and treat both intestinal and extra-intestinal diseases. FMT has been used in the treatment of and exploratory research into various microbiota-related diseases, such as *Clostridium diffcile* infection (CDI), and is regarded as a medical breakthrough. A recent study reported that transferring the gut microbiota of aged mice to young GF mice promoted inflammation in the small intestine of the latter and enhanced the leakage of inflammatory bacterial components into the systemic circulation (Fransen et al., 2017). Ridaura et al. (2013) transplanted the fecal microbiota of adult twin pairs of mice, one obese and one not, into GF mice fed a low-fat chow, and found that mice transplanted with the fecal microorganisms of obese mice became obese, while those transplanted with the fecal microorganisms of lean mice became lean, revealing that microbiota transplantation can change the composition of the host microbiota. Another study demonstrated that gut microbiota may play a key role in modulating the life span of vertebrates. Recolonizing the gut of middle-aged African turquoise killifish with bacteria from young killifish increased life span and delayed behavioral decline, while also helping to maintain an overall healthier physiological status, and a highly diverse and young-like gut microbial community (Smith et al., 2017). In summary, the transplantation of gut microbiota has been shown to have the potential to prolong life; however, the unraveling of the underlying mechanisms requires extensive further investigation.

CONCLUSION AND FUTURE PERSPECTIVES

The association between gut microbiome and conditions of the host are complicated. The composition of gut microbiota and

cellular metabolism varies with aging. Whether the alteration of gut microbiota is inducer of or consequence of cognitive disorder and its mechanisms need to explored. Emerging evidences have emphasized the importance of the preservation of a healthy microbiome to maintain brain functions during aging. They have supported a causal or contributory role of gut microbiota in the progress of cognitive impairment. The contribution of gut microbiota in the origin of PD provided clear confirmation of causality, evidenced by early appearance of α-synuclein inclusions in the ENS, glossopharyngeal and vagal nerves, and the reduced risk for PD in vagotomized individuals (Braak et al., 2003; Shannon et al., 2012; Svensson et al., 2015). Moreover, it was demonstrated that a 12-week consumption for probiotic (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum*) can positively affect cognitive function and some metabolic statuses in the AD patients through a randomized, double-blind, and controlled clinical trial (Akbari et al., 2016). GF mice and antibiotic-induced gut dysbiosis are two methods to investigate the causality in gut microbiota-brain relationships (Frohlich et al., 2016). GF mice showed changes in anxiety-like, social and cognitive behavior compared with SPF mice (Diaz Heijtz et al., 2011; Desbonnet et al., 2014; Stilling et al., 2014). The GF APP transgenic mice showed a drastic reduction of cerebral Aβ amyloid pathology compared with control mice, while the Aβ pathology increased after colonization with microbiota from conventionally raised APP transgenic mice (Harach et al., 2017). One study has showed the pseudo GF mice that received fecal bacteria transplants from senescence-accelerated mouse resistant 1 (SAMR1) mice but not from senescence accelerated mouse prone 8 (SAMP8) mice (a mouse model of AD) showed improvements in behavior and in α-diversity and β-diversity indices of microbiota (Zhan et al., 2018). Furthermore, the microbiota dysbiosis induced by antibiotics in mouse model was verified to be associated with the dysregulation of cerebral signaling molecules, including reduced BDNF, increased neuropeptide Y and serotonin transporter, which subsequently led to cognitive impairment (Frohlich et al., 2016).

However, changes of gut bacterial composition may also simply be a consequence of the disease state. Several systems are at work to ensure the efficient functioning of the gut. The CNS, ENS, the sympathetic and parasympathetic branches of the autonomic nervous system, and neuroendocrine and neuroimmune pathways are all involved in communication with the gut microbes (Cryan and Dinan, 2012). In the BGM axis, the brain can directly or indirectly affect the composition and function of the gut microbiota by releasing signal molecules through lamina propria cells or modulating motility, secretions, and permeability of the gastrointestinal tract (Cryan et al., 2019).Therefore, it is also reasonable to assume that the neuronal dysfunction, the major phenotype of neurodegenerative diseases, may contribute to the microbiota dysbiosis. A latest study supported that gut microbiota-dependent metabolite TMAO or its predecessors including choline, carnitine, and betaine do not play causal roles in the development of AD through a bidirectional mendelian randomization approach (Zhuang et al., 2021). From another point of view, both these disease models that mentioned above have limitations. As a highly artificial model, GF mice also display alterations in the blood-brain barrier and brain ultrastructure (Diaz Heijtz et al., 2011; Braniste et al., 2014) and their physiologic deficits caused by the life-long absence of microbiota are likely to be dampened by compensatory processes (Frohlich et al., 2016). As in antibiotic-induced gut dysbiosis, the antibiotics are likely to evoke systemic effects, or even act directly on the brain rather than from gut to brain. All these factors may shield the real cause behind the outcomes in probing the microbiota-dependent effects.

Although the mouse models possess plenty of advantages in the research of neurodegenerative disorders and many therapeutic approaches show considerable efficacy, there is still no effective treatment of these diseases. This may, at least in part, be related to the fact that these models are highly artificial and only mimic selected aspects representing the human disease. Pet species may be a rational model to study cognitive disorders because (i) disease phenotypes develop spontaneously as they are usually maintained by their owners, (ii) they are not required to be genetically manipulated, and (iii) the long life span and late onset of the conditions is a great parallel to human patients who are also not genetically modified. In addition, the non-human primate is another promising model in investigating human aging and cognitive impairment due to the high similarity to the human brain and nervous system regardless of their high cost and longer research period (Teng et al., 2000).

On all accounts, insights into the mechanisms involved in how gut microbes affect aging and aging-related cognitive impairment may provide new directions for the diagnosis, treatment, and prevention of aging-related diseases. Gut microbiota can be a promising intervention target for aging and cognitive disorders. The future research based on microbiota-directed interventions require more well-designed, large, randomized, double-blind, and placebo-controlled clinical trials. Moreover, research needs to illustrate the physiological and pathological function of the microbiota that are present and investigate precise mechanisms by which changed microbe composition contributes to the pathophysiology of disease and by which potential probiotic bacteria exert beneficial effects on host health.

AUTHOR CONTRIBUTIONS

JN and HQ contributed to organizing this article and revising its content. HL was responsible for writing the manuscript and figure design. All authors read and approved the final manuscript.

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Administration of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI Improves Cognitive and Memory Function in the Mouse Model of Alzheimer's Disease

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Recent evidence indicates that gut microbiota could interact with the central nervous system and affect brain function, including cognition and memory. In this study, we investigated whether *Bifidobacterium bifidum* BGN4 (*B. bifidum* BGN4) *and Bifidobacterium longum* BORI (*B. longum* BORI) alleviated the pathological features in a mouse model of Alzheimer's disease (AD). Administration of *B. bifidum* BGN4 and *B. longum* BORI effectively suppressed amyloidosis and apoptotic processes and improved synaptic plasticity by ameliorating the neuroinflammatory response and BDNF expression. Moreover, behavioral tests indicated that *B. bifidum* BGN4 and *B. longum* BORI attenuated the cognitive and memory disability of AD mice. Taken together, the present study highlights the therapeutic potential of *B. bifidum* BGN4 and *B. longum* BORI for suppressing the pathological features of AD.

Keywords: Alzheimer's disease, probiotics, gut microbiota, *Bifidobacterium*, cognitive and memory impairment

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that causes cognitive decline and memory loss. Its major pathological hallmarks are an accumulation of amyloid peptides, which are products of amyloid precursor protein (APP), and intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Jucker and Walker, 2011). Mutations in APP, presenilin, and tau genes cause the development of relevant molecular pathologies inside neurons, leading to neuroinflammation and altered synaptic plasticity, and eventually resulting in neuronal death (Goate et al., 1991; Wolfe et al., 1999). Previous studies have shown that alternative therapies, including acupuncture, meditation, and medical foods, can treat AD (Thaipisuttikul and Galvin, 2012; Jia et al., 2017; Innes et al., 2018). Additionally, dietary supplements, such as unsaturated fatty acids, and vegetables, legumes, fruits, and omega prevent AD by reducing LDL cholesterol levels, have antioxidant and anti-inflammatory effects, and attenuate cognitive decline (Hu et al., 2013; El-Sayyad, 2015; Mendiola-Precoma et al., 2016).

Recently, studies have implicated gut microbiota in several neurological disorders, such as AD (Vogt et al., 2017), autism spectrum disorder (Sgritta et al., 2019), and Parkinson's disease (Lee et al., 2019). Based on the concept of the microbiota-gut-axis, the relationship between the CNS and gut microbiota contributes a key role in disease risk, and the activity and progression of neurological disorders. For example, the gut microbiota, including Ruminococcaceae, Rikenellaceae, Clostridiaceae, and Enterobacteriaceae, acts as key regulators of neuroinflammation in aged mice (Conley et al., 2016; Matt et al., 2018). Moreover, an increase in the abundance of the pro-inflammatory gut microbiota taxon, Escherichia/Shigella, accelerates brain amyloidosis in cognitively impaired elderly individuals (Cattaneo et al., 2017). Moreover, many studies showed that the altered gut microbiota secretes immunogenic compounds such as amyloid, lipopolysaccharides (LPS), and other microbial exudates which mediate the effects of microbiota in several neurological disorders. For example, LPS can be translocated from the gut to the brain, exacerbating amyloid deposition in the AD model (Zhan et al., 2016). Sgritta et al. (2019) also reported that alteration of *L. reuteri* in gut microbiota modulates the expression of oxytocin *via* the vagus nerve in mouse models of autism spectrum disorder (Sgritta et al., 2019). Additionally, truncal vagotormy in mice prevented the spread of α-synucleinopathy from the gut to the brain (Kim et al., 2019), indicating that these immunogenic compounds mediate afferent and efferent pathways such as the vagus nerve or the hypothalamic-pituitary adrenal pathway (Grenham et al., 2011; Sgritta et al., 2019). Taken together, these results suggest that alterations in gut microbiota composition by probiotics may provide a novel therapeutic approach or ameliorating strategy for AD. Consistent with these results, recent studies have shown that the treatment of *Bifidobacterium breve* strain A1 alters gut microbiota and ameliorates cognitive dysfunction in an Aβ-induced AD mouse model (Kobayashi et al., 2017). In addition, the administration of xylooligosaccharides attenuates surgery-induced cognitive dysfunction in APP/PS1 AD mice by altering intestinal microbiota (Han et al., 2020). Moreover, in one of our previous our study, probiotic supplementation containing *B. bifidum* BGN4 and *B. longum* BORI was shown to improve mental flexibility and alleviate stress in healthy older adults (Kim et al., 2021). Therefore, from this perspective, we asked whether probiotics containing *B. bifidum* BGN4 and *B. longum* BORI can alleviate AD pathologies, such as cognitive dysfunction and memory loss.

In this study, in order to examine the therapeutic effects of *B. bifidum* BGN4 and *B. longum* BORI administration for AD, probiotic supplementation containing *B. bifidum* BGN4 and *B. longum* BORI was treated by oral administration in a mouse model of AD. We first determined the effects on the suppressed amyloidosis and apoptotic process in the hippocampus of AD mice. We subsequently examined behavioral changes using the Y-maze, fear conditioning, and Morris water maze tests in probiotics treated AD mice. Importantly, we found that treatment with *B. bifidum* BGN4 and *B. longum* BORI significantly improved the cognitive and memory ability of 5xFAD mice, indicating that the administration of probiotic *B. bifidum* BGN4 and *B. longum* BORI in gut microbiota can effectively ameliorate the pathological features of AD.

MATERIALS AND METHODS

Animals and Treatment Protocol

Five familial AD mutations (APP K670N/M671L, V717I, I716V, and PS1 harboring two FAD mutations, M146L and L286V)-expressing 5xFAD transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched C57BI/6 and 5xFAD mice were used for all experiments and randomly assigned to each group. The mice were assigned into four groups: Control-BGN4/BORI group (n = 10), Control+BGN4/BORI group (n = 10), 5xFAD-BGN4/BORI group (n = 10), and 5xFAD+BGN4/BORI group (n = 10). For probiotic treatment, freeze dried *B. bifidum* BGN4 and *B. longum* BORI powder (BIFIDO, Gangwon, Korea) were orally administrated to 3-month-old mice daily *via* oral gavage (1×10^9 CFU in 0.2 ml sterile water) for 30 days. All experiments were performed in accordance with the institutional guidelines for animal use and received ethical approval from the Institutional Animal Care and Use Committee at Dongguk University.

Genomic DNA Extraction

To collect murine fecal samples, each group was placed in a separate sterile cage. The mice were assigned into four groups: Control-BGN4/BORI group, Control+BGN4/BORI group, 5xFAD-BGN4/BORI group, and 5xFAD+BGN4/BORI group. Fecal pellets (for n = 3 per group) were collected directly from the anal orifices once a week for 5 weeks and stored at −80°C for analyses. Total bacterial DNA was isolated from the stool by using ZymoBIOMICS^{TM} DNA Miniprep Kit (D4304, Zymo Research Corporation, Irvine, USA). The total DNA was isolated and purified following the manufacturer's protocol. After extraction, the DNA purity and concentration were measured with a spectrophotometer, Nano-Drop ND-2000 (Thermo Scientific, Waltham, MA) and Qubit 3.0 fluorometer (Thermo Scientific). Samples were stored at −20°C until further analysis.

Amplification of 16S rRNA Gene and Sequencing

16S rRNA gene amplification and index PCR were conducted following the Illumina 16S Metagenomic Sequencing Library preparation guide (Illumina, San Diego, CA, USA). The 16S sequence was amplified using forward primer and reverse primer. PCR was initially performed using the primer set, MiSeq 341F (5′-TCGTCGGCAGCGTCAG ATGTGTATAA GAGACAGCCTACGGGNGGCWGCAG-3′) and MiSeq 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC-3′) using 2× Kapa HiFi Hot start Ready Mix DNA polymerase (Kapa Biosystems, Wilmington, MA, USA). PCR products were purified using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA). Amplification was performed at 95°C (3 min) with 25 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s), and a final extension of 72°C (5 min). Quantification and size estimation of the library

was carried out on the QIAxcel Advanced using QIAxcel DNA High Resolution Kit (QIAGEN, Hilden, Germany). Sequencing was performed using the Illumina MiSeq System (2 × 250 bp paired-end reads; Illumina, USA).

Bioinformatic Analysis of Sequencing Data

Microbial sequences were processed using QIIME2 (version 2020.6). Raw sequence reads were demultiplexed using the q2-demux plugin, followed by denoising to remove the sequences with low-quality score using DADA2. All amplicon sequence variants (ASVs) were aligned using the MAFFT and were used to create a rooted phylogenetic tree for phylogenetic diversity analysis with FastTree 2 (Price et al., 2010). ASVs were assigned based on pre-built branches from the 99% SILVA 132 database with a naïve Bayes taxonomy classifier developed for the sklearn classifier (Bokulich et al., 2018). All samples were rarefied to a maximum depth (19,431 reads) that could retain all samples. Faith's phylogenetic diversity and observed features were calculated to measure microbial diversity. Unweighted and weighted UniFrac distance metrics were used to compare the microbial community structure (Lozupone and Knight, 2005; Lozupone et al., 2007). The non-parametric Kruskal-Wallis test was used to determine significant differences in microbial diversity. To evaluate the difference in community structure, PERMANOVA (with 999 random permutations) was used. The linear discriminant analysis effect size (LEfSe) method and LDA effect size (cut-off ≥3) was used to detect significant differences in comparison to the bacteria abundance (Segata et al., 2011). Correlations between gut microbiota and parameters were calculated by using the Spearman's rank correlation coefficient in R package.

Accession Number

The accession number for the gut microbiome data reported in this article is PRJNA731317.

Behavioral Tests

The Y-maze test was performed 30 days after probiotic administration to assess short-term memory. Spontaneous alternation and the number of alternations were tested using white polyvinyl plastic with three open arms (300 mm deep, 150 mm high, 50 mm wide,) at angles of 120° from each other. Mice were placed at the end of one arm and recorded during the 10-min test period. The maze was washed with 70% ethanol after each trial. Data were acquired using the Y-maze software. The sequence of arm entry and the total number of entries was counted by the software to calculate the alternation ratio.

A contextual fear conditioning test was performed using standard chamber with shock floors (20 × 20 cm) and conducted over 3 days. On the 1st day, each mouse was placed in a fear conditioning chamber for 5 min and exposed to white background noise. On the 2nd day of the conditioning session, mice received a 1-s 1.7 mA electric foot shock at 3 min. The training was repeated twice. Twenty-four hours later, mice were exposed to the chamber for 5 min without an electric foot shock. The freezing behavior of mice was videotaped, and the freezing and exploring in the fear conditioning chamber was calculated using specialized behavioral tracking software (EthoVision).

The Morris water maze was performed to test the acquisition of spatial memory and long-term spatial memory. This test was performed in a circular pool (diameter: 90 cm, height: 45 cm) filled with water. An escape platform was placed below the water surface. The water maze test was conducted over 5 days with three trials per day. Each trial was recorded over a 60 s test period. Twenty-four hours after the last training trial, mice were exposed to a 60 s probe test, in which the platform had been removed from the pool. Performance in the probe trial of the water maze was videotaped. The time spent in each quadrant was analyzed using specialized behavioral tracking software (EthoVision).

Western Blot Analysis

Mouse brains were lysed in RIPA buffer containing 1% NP-40, 0.5% DOC, 0.1% SDS, and 150 mmol/L NaCl in 50 mmol/L Tris (pH 8.0) supplemented with 1× proteinase inhibitor mixture (GenDepot). After adding 5× SDS loading buffer and boiling for 5 min, the samples were centrifuged for 10 min at 12,000 *g*. The supernatants were electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to 0.2 μm nitrocellulose membranes. The membrane was then probed with the following antibodies: Homer1 (1:1,000, Millipore, ABN37), PSD95 (1:1,000, Cell Signaling, 2507S), BDNF (1:1,000, Invitrogen, OSB00017W), APP c-terminal (1:500, Sigma, A8717), and β-actin (1:1,000, Abfrontier, LF-PA0207).

Immunofluorescence Staining Analysis

Brain samples of control and 5xFAD mice were sliced to 40 μm with a microtome. Brain sections were washed with 1× phosphate-buffered saline after being fixed in 4% paraformaldehyde in phosphate-buffered saline. Brain sections were immunostained according to standard protocols using the following primary antibodies: ChAT (Millipore, AB144P), NeuN (Millipore, MAB377), MAP2 (Invitrogen, 13-1,500), BDNF (Invitrogen, OSB00017W), β-amyloid 1–42 (Biolegend, 805501), Cleaved-caspase3 (Cell Signaling, 9661S) and appropriate fluorescent secondary antibodies (Invitrogen). Next, brain sections were mounted in Fluoromount-G mounting medium. Representative images were then captured using a confocal laser-scanning microscope (*ZEISS*, LSM800). The number of ChAT-expressing neurons and NeuN-expressing cells was assessed in the hippocampal CA1, CA2, and CA3 region using the automatic cell counter plugin in ImageJ. An investigator blinded to the experimental conditions obtained bilateral counts of ChAT- and NeuN-ir from anatomically matched images to produce an average score.

Quantitative RT-PCR Analysis

Total RNA for all conditions was extracted from the hippocampus and purified using an RNeasy Kit (QIAGEN) according to the manufacturer's protocols. The AccuPower RT-PCR PreMix (Bioneer) was used to synthesize cDNA from isolated RNA. Quantitative RT-PCR analysis was performed using Platinum SYBR green qPCR SuperMix (Invitrogen) in a Rotor-Gene Q real-time PCR cycler (QIAGEN) with following conditions: 95°C for 15 min followed by 40 cycles of 95°C for 10 s, 55°C for 15 s, 72°C for 20 s; melting curve from 72°C

to 95°C every 0.2°C for 1 s per step. Target gene expression was normalized against the expression of a housekeeping gene, GAPDH, in control and 5xFAD mice administrated with sterile water or *B. bifidum* BGN4 and *B. longum* BORI. The following PCR primers were used: *IL-17* Forward: 5′-CCAGGGAGAG CTTCATCTGT-3′ Reverse: 5′-AGGAAGTCCTTGGCCTCA GT-3′, *IL-1*β Forward: 5′-GGATGAGGACATGAGCAA CCT-3′ Reverse: 5′-AGCTCATATGGGTCCGACAG-3′, *IL-6* Forward: 5′-CCGGAGAG GAGACTTCACAG-3′ Reverse: 5′-CAGA ATTGCCATTGCACAAC-3′, *IL-10* Forward: 5′-TGCTGCCT GCTCTTACTGAC-3′ Reverse: 5′-TGGCAACCCAAGTAAC C CTT-3′, *NF-kB* Forward: 5′-AGGCTCCTGTGCGTGTCT CC-3′ Reverse: 5′-AGGTCCACTGCG AGGTGAAGG-3′, *COX2* Forward: 5′-CTACAAGACGCCACATCCCC-3′ Reverse: 5′-AT GCGTAGAGAGGGGAGAGC-3′.

TNF-α and IL-12 Quantification

ELISA was performed using a cytokine ELISA combo kit (KOMA Biotech, K033KIT-02) to detect TNF-α and IL-12 concentrations. Whole blood samples were collected from sterile water or probiotic-treated mouse hearts. Serum samples were separated by centrifugation at 1,000 *g* for 10 min in a refrigerated centrifuge. The collected samples were assessed *via* TNF-α and IL-12 ELISA kits, according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as the mean ± SEM of three independent experiments. One-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test was performed with GraphPad Prism (La Kolla, California, USA). *N* values represent the number of independent experiments, number of individual experiments, or mice. All statistical details of experiments can be found in the figure legends.

RESULTS

B. bifidum BGN4 and *B. longum* BORI Reduced Hippocampal Neuronal Death in 5xFAD Mice

To assess the effect of *B. bifidum* BGN4 and *B. longum* BORI in AD, we first examined the number of hippocampal neuronal cells in the CA3 region of 3 months old probiotic-treated 5xFAD mice. Thirty days after BGN4/BORI treatment (once a day, 1×10^9 CFU in 0.2 ml sterile water), we observed significantly increased NeuN positive cells in the CA3 region of probiotic-treated AD mice hippocampus (**Figures 1A,B**). Since choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine, is expressed during hippocampus-based learning and memory processes (Hawley et al., 2015), we next examined the number of ChAT+ neurons in the AD hippocampus. Consistent with previous results, we observed a significant increase in ChAT+/NeuN+ cells in the CA3 region of probiotic-treated 5xFAD mice hippocampus (**Figures 1A,C**). Moreover, we confirmed a significant increase in the number of ChAT+/NeuN+ cells in the CA1 region of probiotic-treated 5xFAD mice (**Figures 1D–F**). However, we observed that the number of NeuN+ and ChAT+/NeuN+ cells was not changed in the CA2 region between groups (**Figures 1G–I**). These data suggest that *B. bifidum* BGN4 and *B. longum* BORI attenuated hippocampal neuronal death in CA3 and CA1 regions of AD mice.

B. bifidum BGN4 and *B. longum* BORI Restored BDNF and Synaptic Scaffolding Proteins in the Hippocampus of 5xFAD Mice

To evaluate the effect of *B. bifidum* BGN4 and *B. longum* BORI on functional synaptic plasticity, we next examined the expression level of BDNF, which regulates an important role in hippocampal synaptic plasticity and cognition (Vaynman et al., 2004). We found a significant increase in the number of Map2+/BDNF+ neurons in the hippocampus of 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI when compared with control-treated 5xFAD mice (**Figures 2A,B**). Semiquantitative Western blotting confirmed the increased protein expression of BDNF in the hippocampus of probiotic-treated 5xFAD mice (**Figures 2C,D**). Further, the expression of synaptic scaffolding proteins, including PSD95 and Homer1, was significantly restored in probiotic-treated 5xFAD mice (**Figures 2C,E**), demonstrating that *B. bifidum* BGN4 and *B. longum* BORI improved the molecular composition of postsynaptic machinery in the mouse model of AD.

B. bifidum BGN4 and *B. longum* BORI Ameliorated the AD Phenotypes of 5xFAD Mice

Next, we examined whether treatment of *B. bifidum* BGN4 and *B. longum* BORI can ameliorate the AD-associated phenotypes in the hippocampus of 5xFAD mice. Initially, we found reduced amyloid-β42 positive cells in the hippocampus of AD mice at 4 weeks after the oral administration of probiotics (**Figures 2F,G**). Additionally, we observed a decrease in cleaved caspase-3 positive cells, which are expressed in the apoptotic process, in probiotic-treated 5xFAD mice (**Figures 2F,H**). The production of APP-CTFs, C99 and C83, which are cleaved by β- and α-secretase contributes to the accumulation of amyloid aggregates (Checler, 1995; Lauritzen et al., 2012). Detection of C99 and C83 using the APP c-terminal antibody revealed a decrease in C99 protein expression in the hippocampus of probiotic-treated 5xFAD mice (**Figures 2I–K**). These results indicated that AD pathology was attenuated by *B. bifidum* BGN4 and *B. longum* BORI administration.

Next, we investigated the effect of *B. bifidum* BGN4 and *B. longum* BORI on brain inflammatory responses in AD mice. Previous studies have shown that the neuroinflammatory cascade aggravates AD pathogenesis, reduces neuroprotective factors, enhances synaptic dysfunction, and causes neuronal damage, leading to neurodegeneration (Kauwe et al., 2014; Chen et al., 2015). Thus, we determined the expression of inflammatory related genes and cytokines in the probiotic-treated 5xFAD mice. We first observed a statistically marginal increase in the expression of these genes from 5xFAD mice compared to control mice ($n = 5$). Interestingly, *B. bifidum* BGN4 and *B. longum* BORI

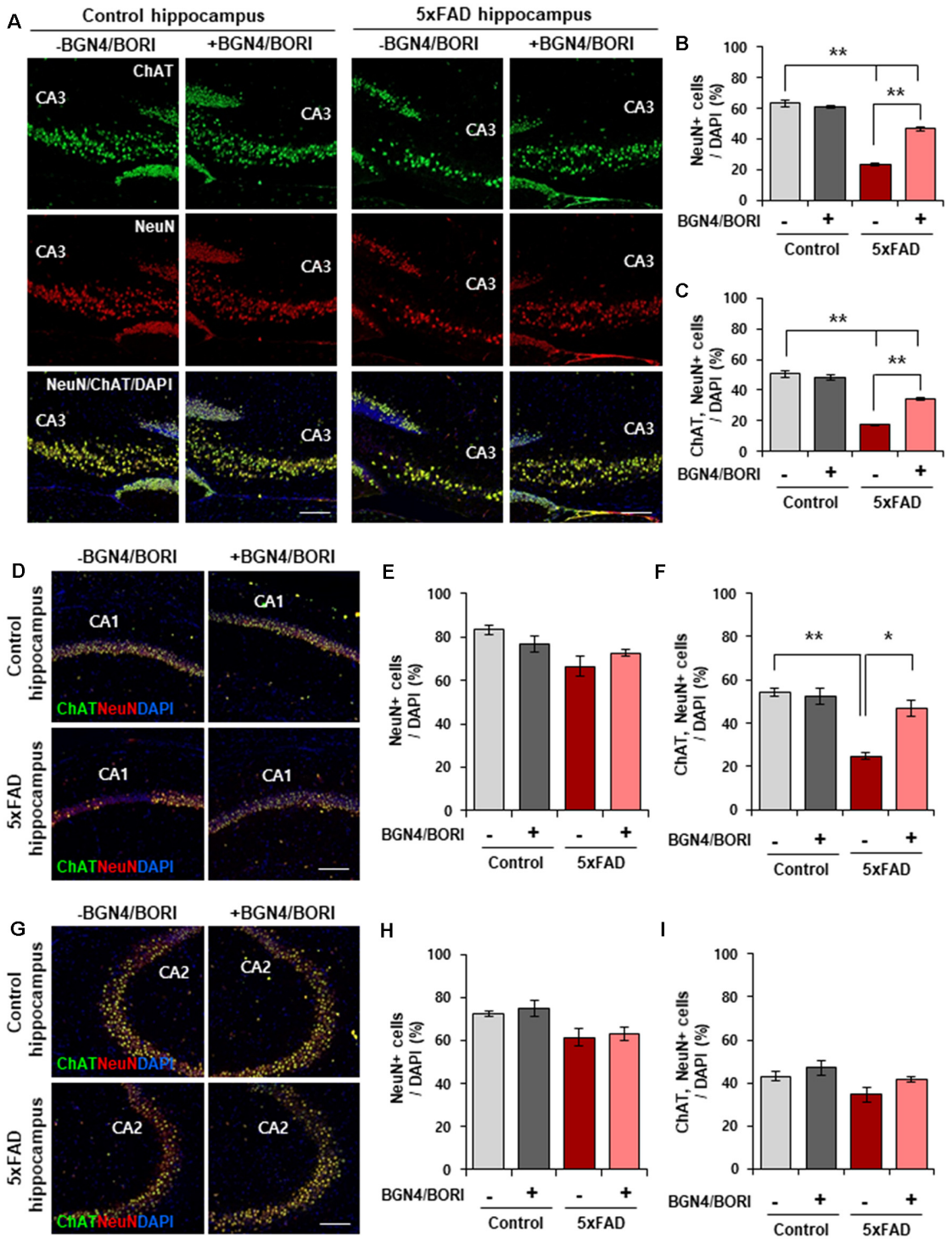

FIGURE 1 | *B. bifidum* BGN4 and *B. longum* BORI reduced hippocampal neuronal death in 5xFAD Mice. **(A)** Immunofluorescence for ChAT and NeuN positive cells in the CA3 hippocampal subfields of control (left) or 5xFAD mice (right) treated with *B. bifidum* BGN4 and *B. longum* BORI (BGN4/BORI). Scale bar = 50 μm. **(B,C)** Quantifications of the NeuN+ **(B)** and ChAT+/NeuN+ **(C)** neurons in the CA3 region at 30 days. Data represent the mean ± SEM. *ANOVA*, $^{**}p < 0.01$ ($n = 5$). **(D)** Representative images of ChAT and NeuN positive cells in the CA1 hippocampal subfields of control or 5xFAD mice treated *B. bifidum* BGN4 and *B. longum* BORI. Scale bar = 50 μm. **(E,F)** Quantifications of the NeuN+ **(E)** and ChAT+/NeuN+ **(F)** neurons in the CA1 hippocampal region at 30 days. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 5$). **(G)** Representative images of ChAT and NeuN positive cells in the CA2 hippocampal subfields of control or 5xFAD mice treated *B. bifidum* BGN4 and *B. longum* BORI. Scale bar = 50 μm. **(H,I)** Quantifications of the NeuN+ **(H)** and ChAT+/NeuN+ **(I)** neurons in the CA2 hippocampal region at 30 days. Data represent the mean ± SEM ($n = 5$).

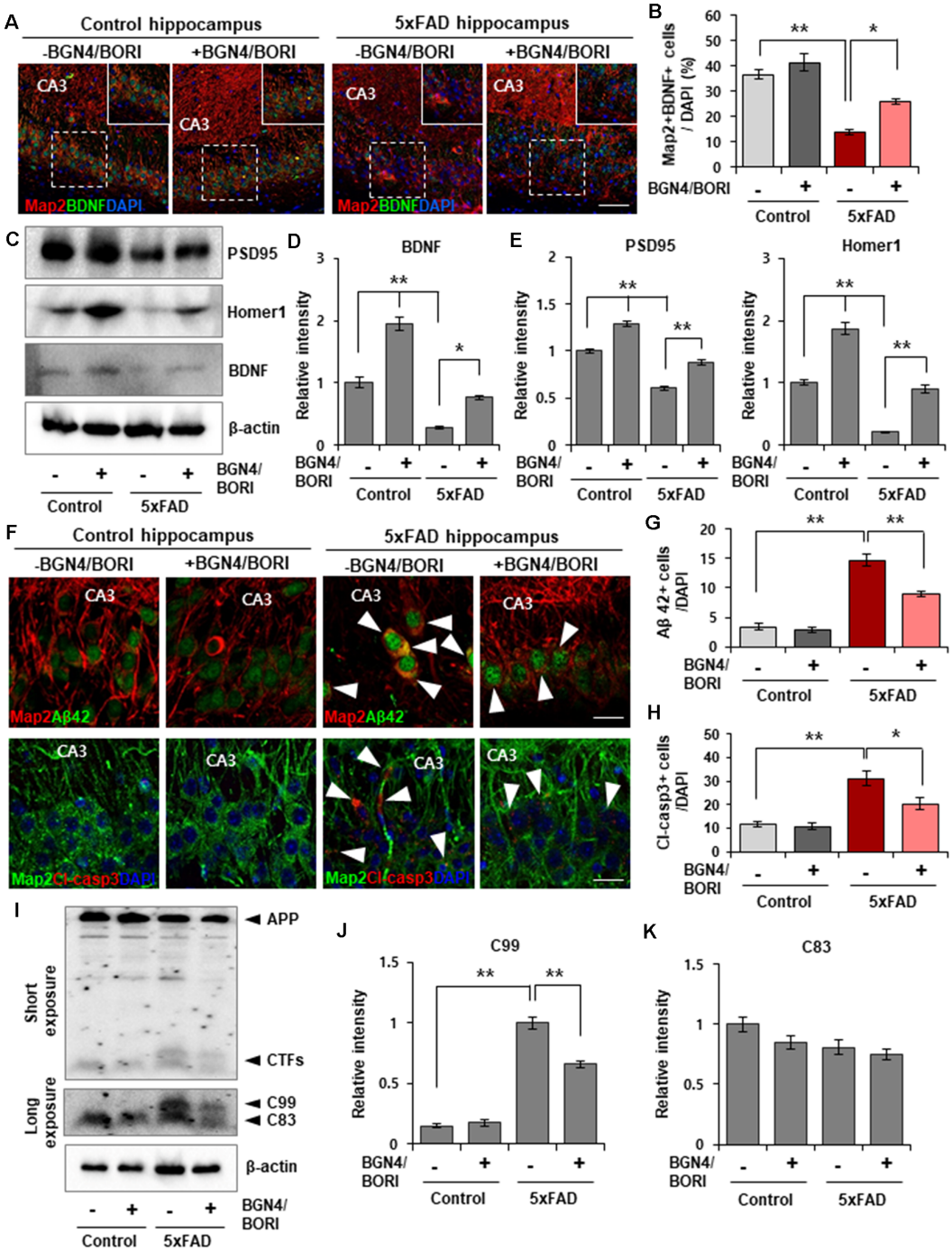

FIGURE 2 | *B. bifidum* BGN4 and *B. longum* BORI reduced Alzheimer's disease (AD) pathogenesis in the 5xFAD Mouse Brain. **(A)** Representative images of Map2 and BDNF positive cells in the CA3 hippocampal subfields of control or 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI (BGN4/BORI). Scale bar = 50 μm. **(B)** Quantification of the Map2+/BDNF+ neurons in the CA3 hippocampal region at 30 days. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 5$). **(C)** Western blot analysis of the scaffolding proteins, PSD95 and Homer1, and blood brain-derived neurotrophic factor, BDNF, in the hippocampus of control or 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI. **(D)** Hippocampal quantification of BDNF expression were normalized to β-actin. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 5$). **(E)** Hippocampal quantifications of PSD95 (left) and Homer1 (right) expression were normalized to β-actin. Data represent the mean ± SEM. *ANOVA*, $^{**}p < 0.01$ ($n = 5$). **(F)** Representative images showing the production of amyloid-β42 (top) and cleaved-caspase3 (bottom) in the CA3 hippocampal subfields of control or 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI. Scale bar = 20 μm. **(G,H)** Quantifications of the amyloid-β42+ **(G)** and cleaved-caspase3+ **(H)** cells in the CA3 hippocampal region at 30 days. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 6$). **(I)** Immunodetection of amyloid precursor protein (APP), C99, C83 in Western blots with protein lysates derived from the hippocampus. **(J,K)** Hippocampal quantifications of C99 **(J)** and C83 **(K)** expression were normalized to β-actin. Data represent the mean ± SEM. *ANOVA*, $^{**}p < 0.01$ ($n = 5$).

significantly decreased the expression of *L-17* and *IL-6* in 5xFAD mice, whereas no differences in the expression of *IL-1β* and *IL-10* were observed (**Figures 3A–D**). To determine how the AD phenotype was ameliorated by *B. bifidum* BGN4 and *B. longum* BORI, we next assessed the expression of the inflammatory pathway factor, *NF-kB*, and its downstream marker, *COX2*. Consistently, *NF-kB* expression was attenuated in *B. bifidum* BGN4 and *B. longum* BORI-treated 5xFAD mice, similar to control levels (**Figure 3E**, right panel). In addition, we confirmed that the expression of *COX2* was significantly decreased in probiotic-treated 5xFAD mice (**Figure 3E**, left panel). In the serum, the concentration of the proinflammatory proteins, TNF-α and IL-12, were significantly restored in 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI (**Figure 3F**). Finally, we observed that the expression of microglial marker, Iba1, and astrocytic marker, Gfap, were significantly decreased in the hippocampus of probiotic-treated 5xFAD mice compared with control-treated 5xFAD mice (**Figures 3G,H**). Taken together, these results suggest that *B. bifidum* BGN4 and *B. longum* BORI ameliorates the neuroinflammatory response, which may prevent the progression of the AD phenotype.

B. bifidum BGN4 and *B. longum* BORI Attenuated Cognitive Impairments in 5xFAD Mice

Next, to investigate whether cognitive deficits can be attenuated by *B. bifidum* BGN4 and *B. longum* BORI treatment, we assessed spatial recognition memory using spontaneous alternation behavior in the Y-maze test. Remarkably, probiotic-treated 5xFAD mice showed an improved alternation performance behavior when compared with non-treated 5xFAD mice (**Figure 4A**). In the contextual fear conditioning test, we found a significant increase in the freezing ratio in 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI (**Figures 4B,C**). Next, we sought to determine whether AD-associated memory loss could be rescued by *B. bifidum* BGN4 and *B. longum* BORI in the Morris water maze test. Importantly, on probe test without a platform, we found that *B. bifidum* BGN4 and *B. longum* BORI treatment dramatically increased time spent in the target quadrant when compared with non-treated 5xFAD mice (**Figures 4D,E**). These results indicated that *B. bifidum* BGN4 and *B. longum* BORI treatment improved AD-associated memory deficits in 5xFAD mice.

Altered Gut Microbiome by *B. bifidum* BGN4 and *B. longum* BORI in 5xFAD Mice

To assess the effect of probiotic supplementation on intestinal bacterial communities, mouse fecal microbiota profiles were analyzed during supplementation. To collect murine fecal samples, fecal pellets were collected directly from the anal orifices once a week for 5 weeks in the probiotic treated mice. Interestingly, we found that the 5xFAD mice group fed with *B. bifidum* BGN4 and *B. longum* BORI showed depletions in the bacterial genus *Parvibacter*, *Incertae_Sedis*, and *Oscillibacter*, and enrichments in *Akkermansia*, *Faecalibacterium*, *Erysipelatoclostridium*, and *Candidatus_Stoquefichus* when compared with the control group (LDA >3.0; **Figures 5A,B**). Moreover, control mice fed with *B. bifidum* BGN4 and *B. longum* BORI showed lower relative abundances of the genus *NK4A214_group*, *Alistipes*, *Lachnoclostridium*, *Desulfovibrio*, and the family Peptococcaceae when compared with non-treated control mice (LDA >3.0; **Figures 5A,C**). The most relevant change was the enrichment of the genus *Akkermansia*. *Akkermansia* was less abundant in the probiotic-treated 5xFAD mice (0.25%) compared with the untreated 5xFAD mice (1.21%) before the administration of probiotics; this showed a tendency to increase at weeks 1–3 in the 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI (1.83–5.86%) and decrease in the untreated 5xFAD group (0.15–0.51%; **Figure 5D**). The relative abundance of *Akkermansia* was strongly correlated with probiotic supplementation periods ($\rho = -0.81$). In the 5xFAD mice group treated with *B. bifidum* BGN4 and *B. longum* BORI, the marked decline was mitigated, showing a weak negative correlation ($\rho = -0.26$; **Figure 5E**). Thus, these results indicated that *B. bifidum* BGN4 and *B. longum* BORI treatment altered Gut Microbiome in 5xFAD mice.

DISCUSSION

AD is the most common dementia. It is caused by an accumulation of Aβ peptides in the adult brain. Amyloid pathogenesis starts with a nucleation phase that leads to the formation of small aggregates in 2-month-old 5xFAD mice (Oakley et al., 2006). Next, the accumulation of intraneuronal Aβ is rapidly increased during the amyloid growth stage (Takahashi et al., 2002; Eisenberg and Jucker, 2012). Recent studies have identified intestinal microbiome dysbiosis, including decreased Firmicutes, increased Bacteroidetes, and decreased *Bifidobacterium*, in patients with AD (Vogt et al., 2017; Li et al., 2018). These findings suggested that microbiome modulation *via* supplementation of a beneficial and safe probiotic can ameliorate physiological functions, such as the epithelial barrier, gut homeostasis, and the inflammatory response, and improve psychiatric and neurological disease symptoms through the regulation of the gut-brain axis (Ait-Belgnaoui et al., 2014; Akbari et al., 2016). These findings lead us to examine the effects of *B. bifidum* BGN4 and *B. longum* BORI administration on AD pathological features, such as amyloid aggregation, neuroinflammatory response, and AD-associated memory.

In our study, we should note that probiotic supplementation was orally administrated to 3-month-old 5xFAD mice which display early amyloid growth stages; therefore, our data clearly indicate that *B. bifidum* BGN4 and *B. longum* BORI treatment can efficiently reduce amyloid aggregation and cellular apoptosis in the early stages of AD. However, since AD shows a progressive neurodegenerative phenotype, it is important to determine the subsequent effects of this treatment in the middle and late stages of AD. A previous study has demonstrated that treatment with a probiotic mixture containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* significantly improves the Mini-mental state examination (MMSE) score of patients with severe AD (Akbari et al., 2016). Further studies are required to

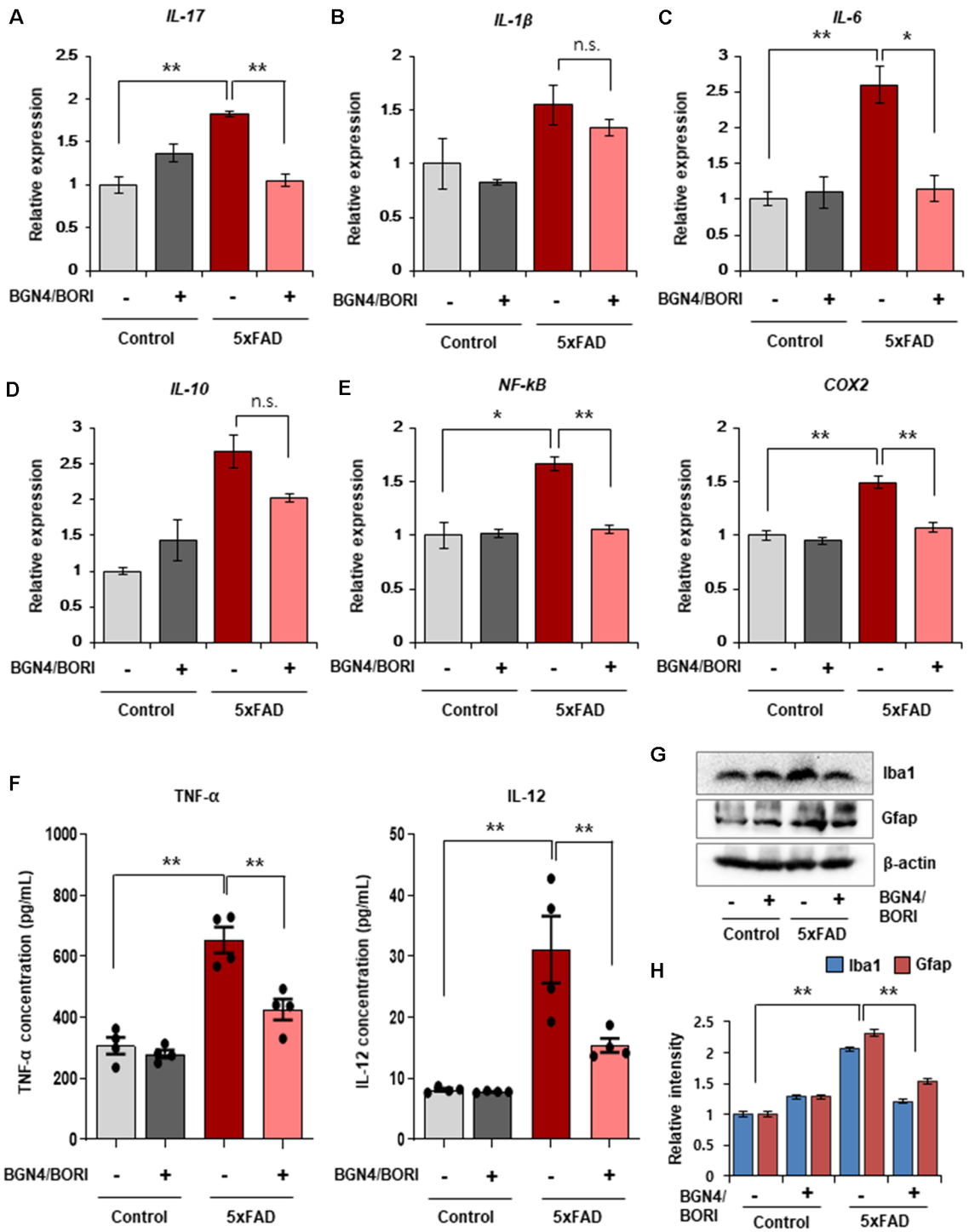

FIGURE 3 | Effects of *B. bifidum* BGN4 and *B. longum* BORI on the neuroinflammatory responses in 5xFAD Mice. **(A–C)** Quantitative RT-PCR analysis of the pro-inflammatory cytokines, *IL-17* **(A)**, *IL-1*β **(B)**, and *IL-6* **(C)** in the hippocampus of control and 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI (BGN4/BORI). Data represent the mean ± SEM. *ANOVA*, data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$; n.s., not significant ($n = 5$). **(D)** Quantitative RT-PCR analysis of the anti-inflammatory cytokine, *IL-10*, in the hippocampus of control or 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI. Data represent the mean ± SEM, n.s., not significant ($n = 5$). **(E)** Quantitative RT-PCR analysis of inflammation pathway genes, *NF-kB* and *COX2*, in the hippocampus of control or 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 5$). **(F)** The concentration of TNF-α and IL-12 in the serum of control or 5xFAD mice treated with *B. bifidum* BGN4 and *B. longum* BORI. Data represent the mean ± SEM. *ANOVA*, $^{**}p < 0.01$ ($n = 4$). **(G)** Immunodetection of Iba1 and Gfap in Western blots with protein lysates derived from the hippocampus. **(H)** Hippocampal quantification of Iba1 and Gfap expression were normalized to β-actin. Data represent the mean ± SEM. *ANOVA*, $^{**}p < 0.01$ ($n = 5$).

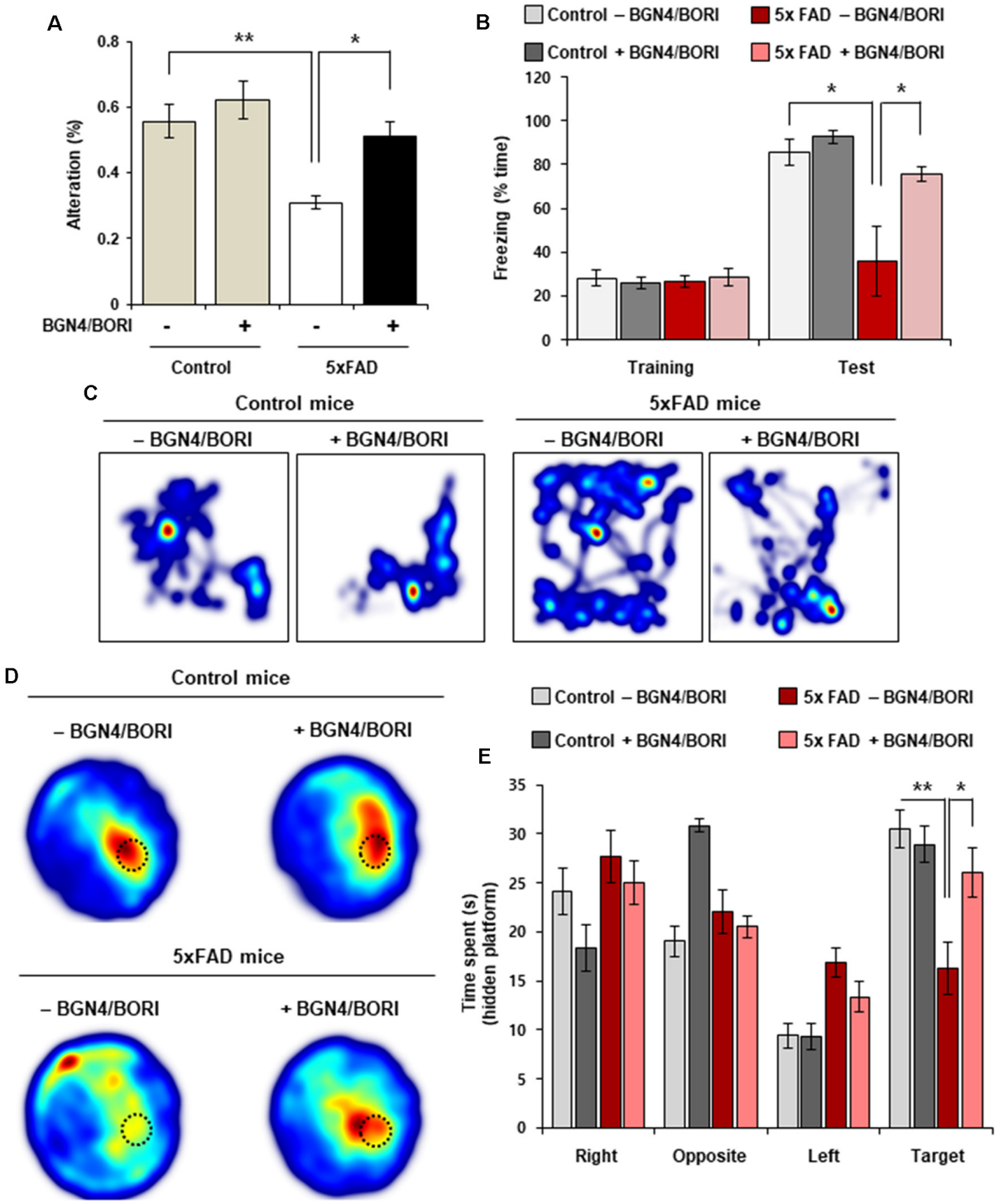

FIGURE 4 | Cognitive impairments was attenuated by *B. bifidum* BGN4 and *B. longum* BORI in 5xFAD Mice. **(A)** Effect of *B. bifidum* BGN4 and *B. longum* BORI (BGN4/BORI) treatment on the performance of control and 5xFAD mice in the Y-maze test. The percent spontaneous alternation was recorded over 5-min in each trial. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 6$ per group). **(B)** Effect of *B. bifidum* BGN4 and *B. longum* BORI treatment on contextual fear conditioning test. Freezing levels of control ($n = 5$), BGN4/BORI-treated control ($n = 5$), 5xFAD ($n = 5$), and BGN4/BORI-treated 5xFAD ($n = 6$) on day 2. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$. **(C)** Representative heatmaps during fear conditioning test. **(D)** Effect of *B. bifidum* BGN4 and *B. longum* BORI on the performance of control and 5xFAD mice in the Morris water maze. Representative heatmaps showing that the time spent in the circular pool to assess long-term spatial learning memory. The dotted circle indicates the target region. **(E)** Quantification of duration in the probe quadrant in the prove trial. Data represent the mean ± SEM. *ANOVA*, $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 8$ per each group).

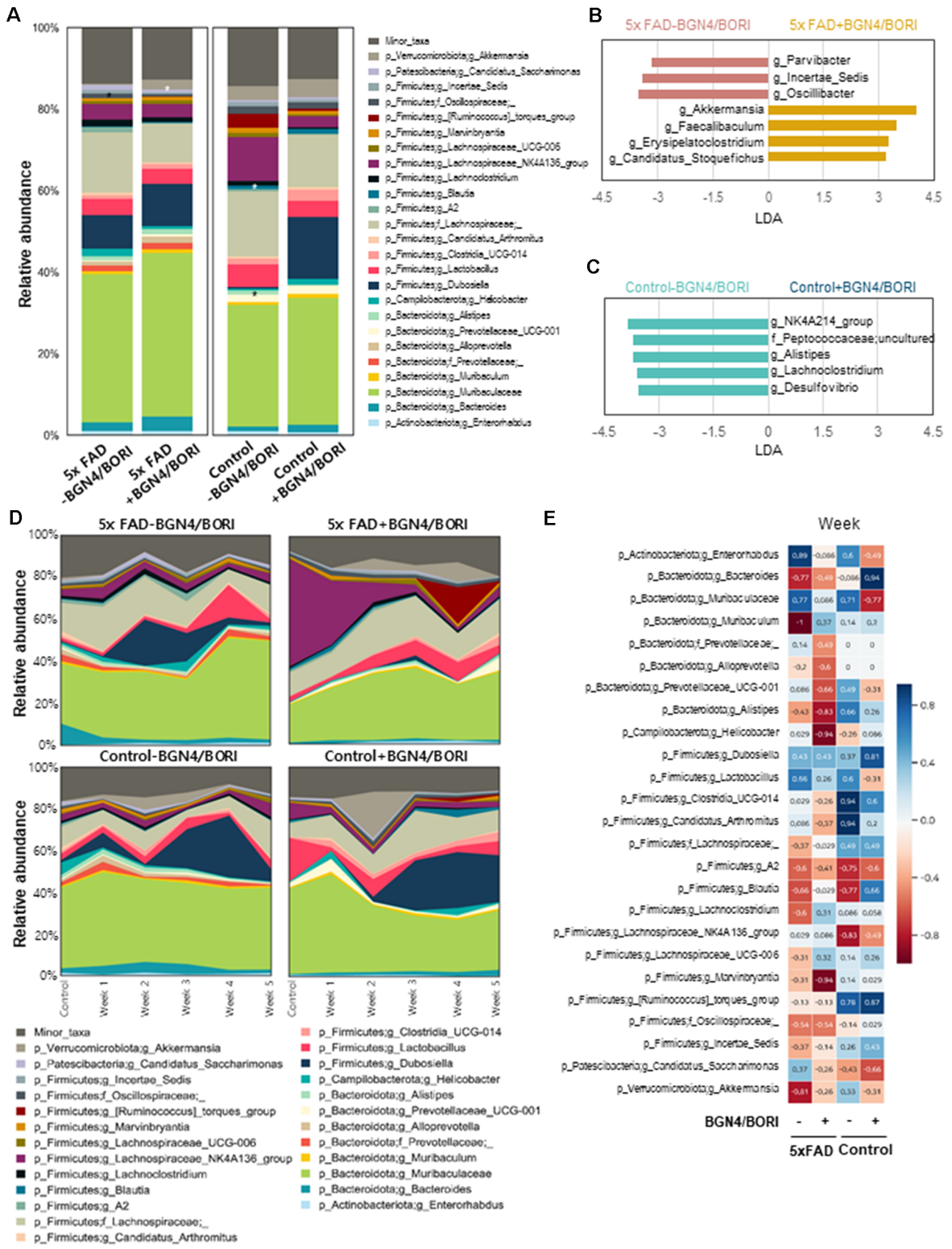

FIGURE 5 | Differences in the gut microbiome following *B. bifidum* BGN4 and *B. longum* BORI Treatment in 5xFAD Mice. **(A)** Comparison of differences in microbial composition between the probiotics intake and the non-intake group (Week 1–5 samples) of 5xFAD or control mice. *Indicates linear discriminant analysis (LDA) score >3.0. Microbial taxa differing **(B)** between 5xFAD and BGN4/BORI-treated 5xFAD mice and **(C)** between control and BGN4/BORI-treated control mice determined by LEfSe analysis (LDA scores >3.0 are shown). **(D)** Changes in microbial composition at genus level in each group over time. **(E)** Spearman's rank correlation showing the relationship between bacterial abundance at the genus level and time.

assess whether probiotic supplementation attenuates the progression of the AD phenotype during the mid and late amyloid-seeding stages.

Moreover, we found that 5xFAD mice group treated with *B. bifidum* BGN4 and *B. longum* BORI, depleted bacterial from the genus, *Parvibacter*, *Incertae_Sedis*, and *Oscillibacter*, and enriched *Bifidobacterium, Akkermansia*, *Faecalibacterium*, *Erysipelatoclostridium*, and *Candidatus_Stoquefichus*, when compared with untreated 5xFAD mice. Among them, the most relevant change was the enrichment of the genus *Akkermansia* and *Faecalibacterium*. Both bacteria possess anti-inflammatory properties (Thursby and Juge, 2017). Interestingly, supplementation of *Akkermansia muciniphila* reduces Aβ 40–42 in the cerebral cortex of APP/PS1 mice, shortens study time, and improves completion rate in Y-maze tests (Ou et al., 2020). Further, this treatment improves intestinal barrier function. Another interesting genus that was enriched was *Faecalibacterium*. This is a representative butyrate-producing bacteria; this substance acts as an anti-inflammatory agent by suppressing the nuclear factor kappa-light-chain-enhancer of the signaling pathways of activated B cells (Schwab et al., 2007). Butyrate drives microglial maturation and is required for the maintenance of mature microglia (Cresci and Bawden, 2015). However, fecal microbiota may not be fully representative of those in the contents or mucosa of the gastrointestinal (GI) tract (Lyra et al., 2012; Lavelle et al., 2015). Thus, the expression of microbial population in fecal samples might be quite different to the intestinal population, which has the capacity to induce changes in the brain. Nevertheless, because of the convenience and non-invasiveness of fecal sampling, many studies have used fecal samples as a proxy to study the gut microbiota. Therefore, a comprehensive understanding of microbial populations between fecal and GI microbiota would help improve longitudinal analyses of microbiota and the application of fecal samples (Lo Presti et al., 2019).

We also explored the neuroinflammatory response that contributes to the progression of AD pathology. Our results showed that probiotic treatment mitigated neuronal inflammation and elevated BDNF expression in AD mice. Previous studies have demonstrated that activated microglia cells were accompanied by increased pro-inflammatory cytokines, such as interleukins and TNF-α, in the AD brain (Vukic et al., 2009; Wang et al., 2015). However, these activated microglia switch to the anti-inflammatory M1-like phenotype which secrete anti-inflammatory cytokines, such as interleukin-4 and interleukin-10, and BDNF that are responsible for inhibiting the innate and adaptive immune reaction and restore synaptic function (Sánchez-Sarasúa et al., 2020). Consistent with this, elevating BDNF expression in the hippocampus of 5xFAD mice was associated with improved cognition (Choi et al., 2018). Thus, our results support that treatment of the *B. bifidum* BGN4 and *B. longum* BORI effectively improved the cognitive functions through an increased BDNF and a decreased neuroinflammatory response in the AD mice.

Moreover, we showed the increase in the synaptic plasticity by oral administration of the *B. bifidum* BGN4 and *B. longum* BORI in control mice (**Figures 2C–E**), suggesting that altered gut microbiota can modulate the synaptic function through the gut-brain axis even in the control healthy mice. Since increased synaptic plasticity by oral administration of the *B. bifidum* BGN4 and *B. longum* BORI was not previously reported in control mice, our findings provide important implications for the neurotherapeutic effect of the *B. bifidum* BGN4 and *B. longum* BORI.

Taken together, our results demonstrated that treatment with *B. bifidum* BGN4 and *B. longum* BORI ameliorated cognitive dysfunction and memory loss in an AD mouse model *via* elevated BDNF expression. These results indicate the therapeutic potential of *B. bifidum* BGN4 and *B. longum* BORI in preventing the pathological features of AD.

CONCLUSION

Our data provide evidence for a therapeutic potential of *B. bifidum* BGN4 and *B. longum* BORI in the mouse model of AD. Administration of *B. bifidum* BGN4 and *B. longum* BORI effectively improves cognition and memory through an increased BDNF and a decreased neuroinflammatory response in the mouse AD hippocampus. Thus, these results indicated that oral treatment with *B. bifidum* BGN4 and *B. longum* BORI could be a novel therapeutic for AD. In the future, it is necessary to verify the efficacy of *B. bifidum* BGN4 and *B. longum* BORI in humans for clinical applications.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: NCBI BioProject, https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA731317.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Dongguk University (IACUC-2021-006-1).

AUTHOR CONTRIBUTIONS

HK and SK performed the experiments. HK, S-jP, GP, HS, and MP performed the data analysis. HK, MP, and JK designed the study and contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

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Lipopolysaccharide, Identified Using an Antibody and by PAS Staining, Is Associated With *Corpora amylacea* and White Matter Injury in Alzheimer's Disease and Aging Brain

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Corpora amylacea (CA) increase in number and size with aging. Their origins and functions remain unknown. Previously, we found that Alzheimer's disease (AD) brains have more *CA* in the periventricular white matter (PVWM) compared to aging controls. In addition, CA is associated with neurodegeneration as indicated by colocalization of degraded myelin basic protein (dMBP) with periodic acid-Schiff (PAS), a CA marker. We also found that bacterial lipopolysaccharide is present in aging brains, with more LPS in AD compared with controls. Periodic acid-Schiff staining is used to identify CA by virtue of their high polysaccharide content. Despite the growing knowledge of CA as a contributor to AD pathology, the molecules that contribute to the polysaccharides in CA are not known. Notably, lipopolysaccharides (LPS) are important cell-surface polysaccharides found in all Gram-negative bacteria. However, it is unknown whether PAS could detect LPS, whether the LPS found in aging brains contribute to the polysaccharide found in CA, and whether LPS associate with myelin injury. In this study, we found that aging brains had a myelin deficit zone (MDZ) adjacent to the ventricles in PVWM. The MDZ contained vesicles, most of which were CA. LPS and dMBP levels were higher in AD than in control brains. LPS was colocalized with dMBP in the vesicles/CA, linking white matter injury with a bacterial pro-inflammatory molecule. The vesicles also contained oxidized fibers, C-reactive protein, NG2, and GALC, markers of oligodendrocyte precursor cells (OPCs) and oligodendrocyte cells (OLs), respectively. The vesicles/CA were surrounded by dense astrocyte processes in control and AD brains. LPS was co-localized with CA by double staining of PAS with LPS in aging brains. The relationship of LPS with PAS staining was confirmed by PAS staining of purified LPS on nitrocellulose membranes. These findings reveal that LPS is one of the polysaccharides found in CA which can be stained with PAS. In addition, vesicles/CA are associated with oxidized and damaged myelin. The LPS in these vesicles/CA may have contributed to this oxidative myelin damage and may have contributed to oxidative stress to OPCs and OLs which could impair the ability to repair damaged myelin in AD and control brains.

Keywords: lipopolysaccharide vesicles, *Corpora amylacea*, white matter injury, Alzheimer's disease, neuroinflammation, innate immune response, astrocytes, human brain

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INTRODUCTION

Corpora amylacea are glycoprotein-containing inclusions found in the aging brain and other organs. *Corpora amylacea* (CA) were not considered to be of any pathological significance for more than a century. However, recently, CA are reported to be associated with various neurodegenerative diseases including Alzheimer's disease (AD) (Averback, 1981; Tate-Ostroff et al., 1989; Cisse et al., 1993; Singhrao et al., 1993; Renkawek and Bosman, 1995; Zhan et al., 2014), multiple sclerosis (Gati and Leel-Ossy, 2001; Selmaj et al., 2008), amyotrophic lateral sclerosis (Atsumi, 1981; Gati and Leel-Ossy, 2001), Parkinson's disease (Buervenich et al., 2001; Pisa et al., 2016), and Huntington's disease (Averback, 1981). CA usually increase in number with advanced age in normal human brains.

Corpora amylacea are positive for periodic acid-Schiff (PAS) staining due to their high polysaccharide content. Besides containing glucose polymers, many other components derived from the breakdown products of neurons, oligodendrocytes, and astrocytes are reported (Ramsey, 1965; Anzil et al., 1974; Palmucci et al., 1982; Singhrao et al., 1993, 1994; Leel-Ossy, 2001; Auge et al., 2019). In addition, components from blood plasma, blood cells, and other cells of the human body are identified in the CA of human brains. These components include ubiquitin (Sahlas et al., 2002), heme oxygenase-1 (Sahlas et al., 2002), thrombospondin (Meng et al., 2009), complement (Singhrao et al., 1995), S100 proteins (Hoyaux et al., 2000), and calprotectin, a soluble protein contained in neutrophil granules. Calprotectin is a mammalian antimicrobial protein that has antibacterial and antifungal properties. Recently, pathological structures associated with fungal infections have been demonstrated in CA in human brains (Pisa et al., 2016, 2018). In addition, a recent proteomic analysis identified several peptides associated with Gram-negative and lipopolysaccharide (Peters et al., 2005) containing proteobacteria in CA (Pisa et al., 2018). These findings suggest that CA are not just inert structures that occur in the brain but may be part of an orchestrated immune system response that involves vascular components, damage to white matter and adjacent brain tissues, and microbial components including Gram negative bacteria.

We have previously reported that neuronal and myelin breakdown products, including dMBP, neurofilament, and myelin lipids are detected in CA of aging brains. We have also reported that AD brains have more CA compared to control aging brains (Zhan et al., 2014). AD brains have more dMBP$^+$ vesicles in the periventricular white matter (PVWM) compared to controls (Zhan et al., 2014). The ependymal cells that separate the ventricles from PVWM are denuded in aging brains, with the ependymal damage being more extensive in the AD brain compared to controls (Zhan et al., 2014). We also found that bacterial components including lipopolysaccharides (LPS) are present in AD and aging brains with much more LPS in AD compared to controls (Zhan et al., 2016). LPS was localized to neurons, oligodendrocytes, oligodendrocyte progenitor cells (OPCs), microglia, and ependymal cells (Zhan et al., 2016). LPS also localized to virtually all amyloid plaques in AD brains. These findings show that several features of AD pathology associate with the presence of bacterial LPS, and raise the question of whether LPS contribute to AD pathology, or whether AD pathology leads to the accumulation of LPS.

Lipopolysaccharides are pro-inflammatory molecules that can elicit a potent innate immune response and lead to the production of cytokines and inflammation that could contribute to the formation of PAS positive vesicles/CA that associate with damaged myelin. Thus, we determined that PAS positive vesicles/CA that contained dMBP also contained LPS, and that dMBP and LPS levels were higher in AD compared to control brains. We also showed significantly more LPS$^+$ CA/vesicles in AD compared to control brains. Since an antibody was used to detect LPS immunocytochemically, we used an independent enzymatic Limulus Amoebocyte Lysate (LAL) assay to also show higher LPS activity levels in AD compared with control brains. Since PAS stains polysaccharides and LPS are cell-surface polysaccharides found in all Gram-negative bacteria, we performed double staining of LPS and PAS in AD and aging brains. The association of LPS and PAS was further confirmed by PAS staining of purified LPS on nitrocellulose membranes. The relationship between LPS$^+$ and PAS positive vesicles/CA to oligodendrocytes, OPCs, oxidative stress markers, C-reactive protein (CRP), microglia, and astrocytes are also shown.

MATERIALS AND METHODS

Brain Samples

Postmortem brain samples were provided by the Alzheimer's Disease Center at the University of California Davis (UCD ADC). The study was approved by the UCD Institutional Review Board. A written informed consent to share research tissues after death was obtained from all participants or their proxy prior to their death. The clinical diagnosis of AD was made by the board-certified neurologists and pathological diagnosis was confirmed by the board-certified neuropathologists. AD pathology was rated using Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria and staging of Braak. Controls were normal individuals, those with a low likelihood of clinical AD and individuals who did not meet the criteria for AD neuropathology. Controls were matched to AD based on age and sex. A total of 50 brains, including 30 AD and 20 control brains were studied. Blocks of tissue including frontal PVWM at the level of the head of the caudate nucleus from each brain were removed. For immunostaining, brains were fixed in formalin and brain tissue was embedded in paraffin. Sections were cut in the coronal plane. For Western blot analyses and the LAL assays, the brain tissue was frozen at −70°C.

Immunohistochemistry

Detailed methods are described in our previous studies (Zhan et al., 2008, 2014, 2015). Briefly, after removing paraffin with xylene (2 min × 3 times) and rehydrating through graded alcohols (2 min in 100% × 2 times; 2 min in 95% and 2 min in 75%), brain sections were incubated in endotoxin-free 0.1M PBS antigen retrieval buffer containing 1 mM EDTA and 0.05% Tween 20 at 95°C for 20 min. Endogenous peroxidase activity was quenched with 3% H_2O_2 in endotoxin-free PBS for 20 min.

The endotoxin-free 0.1M PBS blocking buffer contained 2% goat serum, 1% BSA, and 0.3% Triton 100.

Primary antibodies used in immunohistochemistry included mouse monoclonal antibodies against Gram-negative bacterial LPS (MD-05-0148, RayBiotech, Norcross, GA, USA), MBP (MAB382, Millipore, Burlington, MA, USA), rabbit polyclonal antibodies against 8-Iso-PGF2α (ADI-905-015-100, Enzo Biochem, Farmingdale, NY, USA), and 13-14-DH-15keto-PGF2α (ADI-905-051-100, Enzo Biochem). The secondary antibody was a biotinylated goat anti-mouse IgG (1:200 dilution, Vector Labs, Burlingame, CA, USA). The antibody complex was detected using ABC reagent, alkaline phosphatase (AP), Vector® Blue Substrate Kit, or a Horseradish peroxidase (HRP), and Vector® VIP Substrate Kit or DAB Substrate Kit according to the instructions of the manufacturer (Vector Labs). The primary antibody was omitted to assess non-specific staining.

Immunofluorescence

Immunofluorescence methods are described in our previous studies (Zhan et al., 2008, 2014, 2015). Briefly, after removing paraffin, rehydrating, and antigen retrieval, as mentioned above, sections were treated with Autofluorescence Eliminator Reagent (2160, Millipore). The primary antibodies included mouse monoclonals against myelin basic protein (MBP) (MBP382, Millipore), neuron-glial antigen 2 (NG2) (MAB5384, Millipore), galactocerebroside (GALC) (MAB342, Millipore), Gram-negative bacterial LPS (MD-05-0148, Ray Biotech), and rabbit polyclonal antibodies against dMBP (AB5864, Millipore), 8-Iso-PGF2α (ADI-905-015-100, Enzo Biochem), 13-14-DH-15keto- PGF2α (ADI-905-051-100, Enzo Biochem), CRP (PA1-29087, Thermo Fisher, Waltham, MA, USA), Iba1 (019-19741, Wako, Richmond, VA, USA), and glial fibrillary acidic protein (GFAP) (250661, Invitrogen, Waltham, MA, USA). Goat anti-mouse or goat anti-rabbit Alexa Fluor® 488 or 594 conjugated antibodies (Invitrogen) were used for secondary antibodies depending on the species of the primary antibody. Slides were cover slipped with a mounting medium containing DAPI and examined under a Nikon Eclipse E600 fluorescent microscope at excitation/emission wavelengths of 493/520 nm (for green fluorochrome), 590/619 nm (for red fluorochrome), or 358/463 nm (for blue fluorochrome). For controls, the primary antibody was deleted or immunodepleted with the target antigen of the antibody.

Autofluorescence occurs in aging brains, which can interfere with the detection of specific fluorescent signals and can be problematic if not removed. We treated all sections with Autofluorescence Eliminator Reagent prior to immunostaining, which eliminated most autofluorescence in both control and AD brains (**Supplementary Figure 1**). These results suggest that the immunofluorescent signals detected in this study were not due to autofluorescence.

The specificity of mouse monoclonal antibody against bacterial LPS was verified, previously (Zhan et al., 2016, 2018). Immunostaining controls for the other antibodies were carried out by omitting the primary antibodies in control and AD brain sections. The results showed that none of the vesicles and other brain structures were stained positively (**Supplementary Figure 2**). These results suggest that the staining was not due to the non-specific binding of the secondary antibodies used.

Western Blot Analysis

Detailed methods are described in our previous studies (Zhan et al., 2008, 2014, 2015). Briefly, frozen tissues were homogenized in ice-cold RIPA buffer containing a complete protease inhibitor (Sigma). Homogenates were centrifuged at 14,000 × *g* for 30 min at 4°C. Protein (12.5 μg each) from the supernatant was loaded on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to the nitrocellulose membrane. The primary antibody included rabbit polyclonal against dMBP (AB5864, 1:1,000 dilutions; Millipore). NIH Image J software was used to quantify band intensities. A mouse monoclonal against β-actin (sc-69879, Santa Cruz, Dallas, TX, USA) was used as a loading control for Western blots and optical densities of each target protein normalized to β-actin. Horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG (Bio-rad) was used to detect the primary antibody. The ECL chemiluminescent detection system (PIERCE Inc., Thermofisher Scientific, Waltham, MA, USA) was used to detect the signals. Blots were imaged on the Fluorchem 8900 system (Alpha Innotech, San Leandro, CA, USA). The ratio of the intensity of dMBP/β-actin bands was quantified with NIH Image J software. The relative band intensity in AD samples was averaged and compared to the averaged band intensity of control samples.

Limulus Amoebocyte Lysate Assay

The LAL enzymatic assay for LPS was performed using an Endpoint Chromogenic LAL Assay kit (50-647U, Lonza) according to the instruction of the manufacturer. A standard curve was generated from known amounts of LPS, and this curve was used to derive the values from brain samples. The LAL enzymatic assay for LPS was performed to confirm the results obtained using the monoclonal antibody to LPS.

Quantitative Analysis of LPS$^+$ Vesicles

Sections including frontal PVWM at the level of the head of the caudate nucleus from AD and control brains were used for counting LPS$^+$ vesicles. Three sections per brain were counted. The average of number of three brain sections in each case was used for the statistical analysis. Only clearly stained round vesicles were counted. PVWM was defined as the white matter within 1 mm of the ependymal layer. The numbers of vesicles were counted in random areas about 0.8 mm^2 (20X fields) by an investigator blinded to diagnosis using NIH Image J software.

PAS Staining of CA and Purified LPS

Brain sections with PVWM were incubated in 0.5% periodic acid (Sigma) for 5 min at room temperature followed by washing in tap water for 1 min. Sections were then incubated in Schiff reagent (Sigma) for 10 min followed by washing in tap water for 10 min. Sections were mounted with an aqueous mounting medium and cover slipped.

To further confirm that PAS stains LPS, purified LPS from *E. coli,* serotype O111:B4 (L2630, Sigma-Aldrich, St. Louis, MO,

TABLE 1 | Demographic data and neuropathological assessment of Alzheimer's disease and control patients.

	Controls ($n = 20$)	AD ($n = 30$)	p-values
Age (years ± SE)	83.6 ± 1.4	80.3 ± 1.6	0.078
Sex male: n (%)	9 (45.0)	12 (40.0)	0.73
Braak stage: median	2 (IQR 1, 2)	6 (IQR 5, 6)	<0.001
CERAD: median	0 (IQR 0, 1)	3 (IQR 3, 3)	<0.001

Differences between groups were analyzed suing a Student t-test (continuous), Kruskal-Wallis test (ordinal) or Fisher Exact test (categorical). IQR, interquartile range.

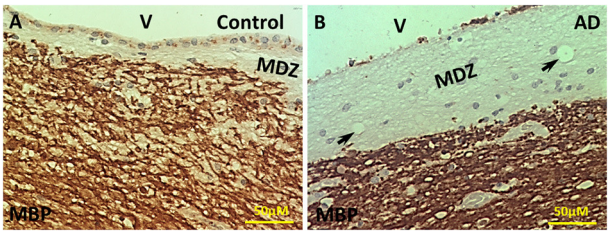

FIGURE 1 | Immunohistochemistry of MBP in the PVWM of AD and control brains. **(A)** Intact MBP immunostaining in the PVWM of control brain. A thin myelin-deficit zone (MDZ) was observed under the ependymal cells. Some ependymal cells were stained for Intact MBP. **(B)** Intact MBP staining in the PVWM of AD brain. The MDZ was much wider in AD brain. There was a loss of many ependymal cells. A few areas of no tissue staining were observed (arrows). Note: Dark Brown, positive staining; MBP, myelin basic protein; V, ventricle; MDZ, myelin-deficient zone; PVWM, periventricular white matter; AD, Alzheimer's disease; Bar = 50 μm.

USA) and its mutant form of *E. coli,* serotype J5 (ALX-581-014-L002, Enzo Biochem) were used for PAS staining. LPS from *E. coli* O111:B4 was purified by phenol extraction and dissolved in endotoxin free water. LPS from *E. coli* J5 is purified by a modification of the PCP extraction and dissolved in sterile pyrogen-free double distilled water. Bovine serum albumin (BSA, 23209, Pierce™, Thermofisher Scientific, Waltham, MA, USA) was used as a control. Three microliters of samples from each concentration of LPS and BSA were spotted onto the nitrocellulose membrane and air dried for 30 min. The membrane was washed with PBS followed by 0.5% periodic acid (Sigma) for 5 min at room temperature before washing in PBS. The membrane was then incubated in Schiff reagent (Sigma-Aldrich) for 10 min followed by washing in PBS. The intensity of PAS staining was measured using NIH Image J software.

Statistical Analyses

Differences between AD and control groups were analyzed using Student's *t*-test (continuous), Kruskal-Wallis test (ordinal), and Fisher Exact test (categorical). Differences among *E. coli* serotype J5 LPS, *E. coli* O111:B4 LPS, and BSA groups were analyzed using One Way ANOVA (SigmaStat). Values were expressed as mean ± SE. A $p \leq 0.05$ was considered significant.

RESULTS

Patient Characteristics

Characteristics of the 30 patients with AD and 20 control patients are shown in **Table 1**. There were no differences for controls compared to AD in age or sex. The differences in median Braak and Braak stage and CERAD between AD and control brains were significant, as expected.

Periventricular Myelin Is Deficient in Aging Control and AD Brain

Our previous studies showed the presence of dMBP in both control and AD brains, though there was more dMBP in AD brains (Zhan et al., 2014). Since it was unclear where the dMBP was coming from, we stained the control and AD brains with an antibody specific for intact MBP.

In control aging brains, there was intense MBP immunostaining in the PVWM except for a thin myelin-deficit zone (MDZ, **Figure 1A**). The width of the myelin-deficit zone in controls varied between 25-150 μm with an intact ependymal lining separating the ventricle (V) from the MDZ. Some ependymal cells stained positive for MBP (**Figure 1A**). In AD brains, the myelin-deficient zone was wider and varied between 75 and 450 μm (**Figure 1B**). Occasional clear vesicles were observed in the myelin-deficit zone in the AD brain (**Figure 1B**, arrows). Thus, we deduced that one source of dMBP in both control and AD brains was in the zone between the ependyma and intact myelin adjacent to the ventricle in the area we termed the "myelin-deficient zone" (MDZ).

Oxidized Vesicle Walls

Since there is evidence for increased oxidative stress in normal aging and AD brain, and since oxidative stress might play a role in the loss of myelin in the MDZ, we stained the control and AD brains for two different but related oxidative stress markers.

Staining for oxidative stress markers was performed in the MDZ in PVWM of control (**Figures 2A,B**) and AD brains (**Figures 2C,D**). The staining revealed vesicles that stained for 8-Iso-PDG2α in the control (**Figure 2A**) and AD brains (**Figure 2C**), and stained for PGFM, also known as 13,14,-Dihydro-15-keto-PGF2α, in control (**Figure 2B**) and AD brains (**Figure 2D**). Staining for the majority of vesicles was in the walls with a hollow center, though some of the vesicles were full of oxidized filaments (**Figure 2**). Vesicles varied in size from ∼4 to ∼20 μm in diameter (**Figure 2**). Thus, there is evidence of oxidative stress in the MDZ as manifested by vesicles that we have previously noted to be positive for dMBP.

Oxidized NG2 and GALC in the Vesicles

Since the oxidatively stressed vesicles occurred in the region of myelin loss adjacent to the ventricles, we reasoned that the vesicles might be made up partially from damaged oligodendrocytes and oligodendrocyte progenitor cells (OPCs). Thus, we stained sections for GALC and NG2, oligodendrocyte, and OPC markers, respectively.

The oligodendrocyte marker, Galactocerebroside (GALC), was localized to vesicles in the MDZ of control brains

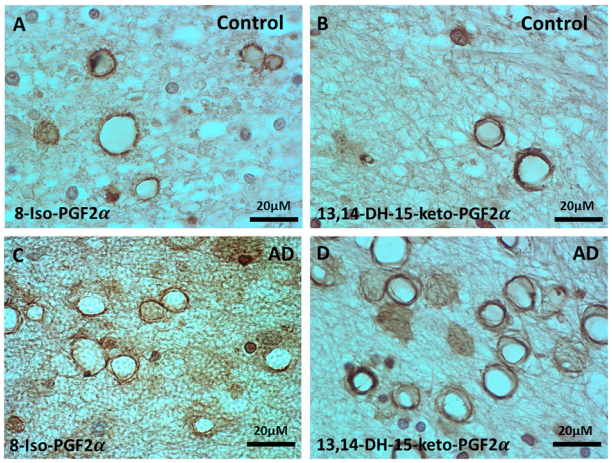

FIGURE 2 | Immunohistochemistry of oxidative markers, 8-Iso-PGF2α and PGFM, in the vesicles of control and AD brains. **(A)** 8-Iso-PGF2α stained vesicles of PVWM in control brain. **(B)** PGFM, also known as 13,14,-Dihydro-15-keto-PGF2α, stained vesicles in the PVWM of the control brain. **(C)** 8-Iso-PGF2α stained vesicles in AD PVWM. **(D)** PGFM stained vesicles in AD PVWM. Note: PGFM, Prostaglandin F2α metabolite; 8-Iso-PGF2α, 8-Iso-Prostaglandin F2α; PVWM, periventricular white matter; AD, Alzheimer's disease; Bar = 20 μm.

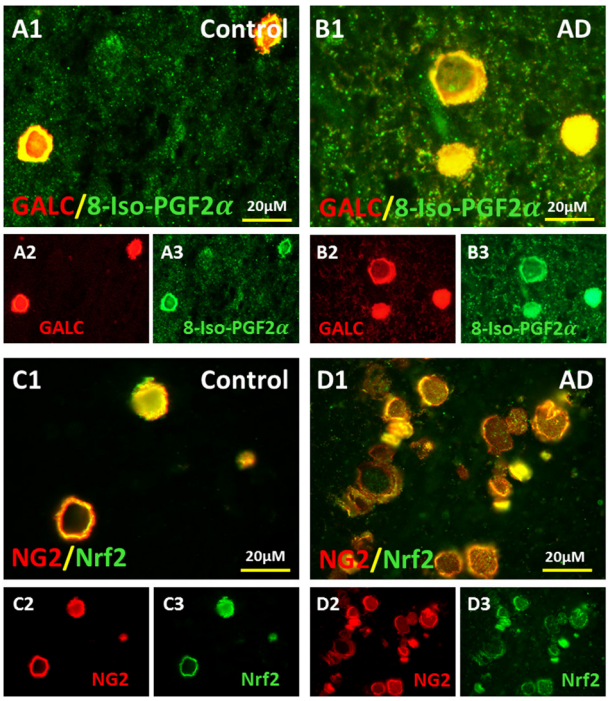

FIGURE 3 | Colocalization of OLs and OPCs markers with oxidative markers in the vesicles of AD and aging brains. Oligodendrocyte marker, GALC, in control **(A2)** and AD brains **(B2)** and 8-Iso-PGF2α in control **(A3)** and AD brains **(B3)** colocalized in control **(A1)** and AD brains **(B1)**. Oligodendrocyte precursor cell (OPCs) marker, NG2, in control **(C2)** and AD brains **(D2)** and oxidative stress marker Nrf2 in control **(C3)** and AD brains **(D)** colocalized in control **(C1)** and AD brains **(D1)**. Note: OL, oligodendrocyte; OPC, oligodendrocyte precursor cell; GALC, Galactocerebroside; NG2, neuron-glial antigen 2; Nrf2, Nuclear factor erythroid 2-related factor 2; AD, Alzheimer's disease; Bar = 20 μm.

(**Figures 3A2,A1**) and AD brains (**Figures 3B2,B1**), and co-localized with the oxidative stress marker 8-Iso-PGF2α in control (**Figures 3A1,A3**) and AD brain (**Figures 3B1,B3**). Similarly, the oligodendrocyte precursor cells (OPCs) marker, neuron-glial antigen 2 (NG2), was localized to vesicles in the MDZ of control brains (**Figures 3C2,C1**) and AD brains (**Figures 3D2,D1**), and co-localized with oxidative stress marker Nrf2 in control brain (**Figures 3C1,C3**) and AD brain (**Figures 3D1,D3**). Thus, oligodendrocyte and OPC markers are in the vesicle walls in the MDZ, and both markers are associated with oxidative stress.

Detection of Bacterial LPS in the Vesicles

We have previously shown that LPS, the polysaccharide found in the outer wall of all Gram-negative bacteria, is found in both control and AD brain, though there are much more LPS in AD brain. We wondered if one cause for the increased oxidative stress in the MDZ might be related to LPS.

Staining for LPS in the myelin-deficient zone (MDZ) of the control brain (**Figures 4A1,A2**) showed some LPS stained vesicles. In contrast, there were many more LPS stained vesicles in the MDZ of the AD brain (**Figures 4B1,B2**). A quantification confirmed this with more LPS^{+} stained vesicles in the myelin-deficit zone of AD brains (44.8 ± 6.5) compared to controls (20.1 ± 6.7, $p = 0.0085$) (**Figure 4C**). Thus, LPS were also found in the walls of vesicles in the MDZ of control and AD brain and could contribute to the oxidative stress associated with the vesicles.

Association of LPS With Myelin Degradation Within the Vesicles

We have previously shown that dMBP was associated with vesicles in the MDZ of the PVWM (Zhan et al., 2014). We reasoned that dMBP might co-localize with LPS in the vesicles in the MDZ.

We, therefore, stained for LPS and dMBP in control brains (**Figures 5A1–A3**) and AD brains (**Figures 5B1–B3**). LPS colocalized with dMBP in the vesicles of control brains (**Figure 5A3**) and AD brains (**Figure 5B3**).

To quantify LPS levels in the PVWM, we performed the LPS LAL enzymatic assay for LPS from PVWM of AD and control brains. The data showed greater LPS activity in AD PVWM (77.3 ± 3.1 EU/g) compared to controls (65.0 ± 2.1 EU/g, $p = 0.0022$, **Figure 5C**). To quantify dMBP levels in the PVWM, we performed Western blots from PVWM of AD and control brains. The blots showed significantly more dMBP in AD PVWM compared to control (**Figure 5E**). Quantitative analysis confirmed the density of the dMBP bands in AD PVWM (4.8 ± 0.8) was much higher than in controls (1.0 ± 0.3) (**Figure 5D**, $p = 0.007$).

These data show that LPS co-localizes with dMBP in PVWM vesicles, and that both LPS and dMBP are increased in AD PVWM compared to controls. Whether LPS causes myelin degradation in the vesicles requires further study.

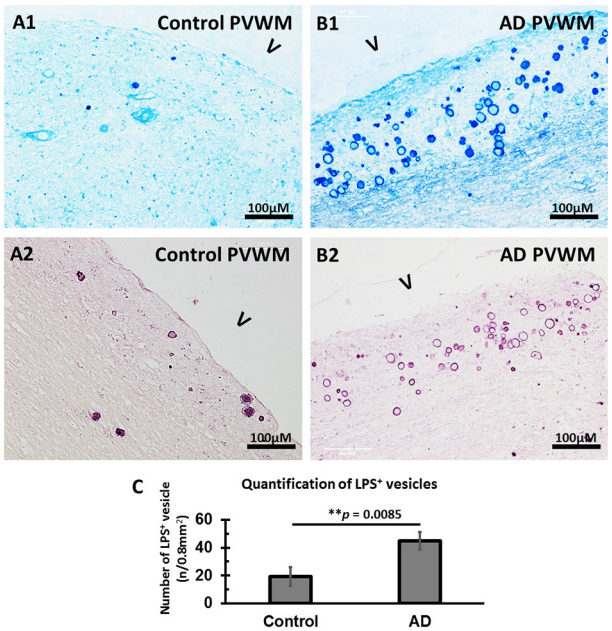

FIGURE 4 | Immunostained LPS vesicles in the PVWM of AD and aging control brains. **(A)** LPS was identified in the vesicles in control PVWM using an antibody against LPS and staining with alkaline phosphatase and Vector® Blue Substrate Kit **(A1)** or horseradish peroxidase and Vector VIP Substrate Kit **(A2)**. **(B)** LPS was identified in the vesicles in AD PVWM using an antibody against LPS and staining with alkaline phosphatase and Vector Blue Substrate Kit **(B1)** or horseradish peroxidase and Vector VIP Substrate Kit **(B2)**. **(C)** Quantitative analysis showed more LPS$^+$ vesicles in AD (44.8 ± 6.5, $n = 12$) PVWM compared to control PVWM (20.1 ± 6.7, $n = 9$, $p = 0.0085$). Note: LPS, lipopolysaccharide; V, ventricle; PVWM, periventricular white matter; AD, Alzheimer's disease; Bar = 100 μm.

Inflammation Related to LPS$^+$ Vesicles

LPS are potent pro-inflammatory agents that provoke a strong immune response and induce many molecules including C-reactive protein (CRP). Thus, we stained PVWM for CRP and LPS. Indeed, CRP was found in the walls of the vesicles in MDZ of PVWM in both the control brain (**Figure 6A2**) and AD brain (**Figure 6B2**). CRP co-localized with LPS in vesicles in the MDZ in control (**Figures 6A3,A1**) and AD brains (**Figures 6B3,B1**). These data are consistent with the suggestion that LPS promotes inflammation manifested in part by the induction of CRP.

Since microglia and astrocytes are the resident immune cells that defend the host against infection or injury, and since bacterial LPS was detected in the PVWM of AD brains, we determined whether microglia and astrocytes were associated with LPS in the PVWM of AD brains compared to controls using the Iba1 microglial marker and the GFAP astrocyte marker. The results showed that Iba1 stained microglia were sparse in the myelin-deficit zone (MDZ) of the aging control brain that contained little LPS (**Figures 7A1–A3**). Unexpectedly, Iba1 stained microglia were also sparse in the MDZ zone of AD brains even around LPS$^+$ vesicles (**Figures 7B1–B3**). Iba1 did not colocalize with LPS in either control (**Figure 7A1**) or AD (**Figure 7B1**) MDZ.

In contrast, there was intense GFAP staining in the myelin-deficit zone of control brains (**Figures 8A1–A3**) and AD brains (**Figures 8B1–B3**). There were scattered astrocytes in the white matter adjacent to the MDZ in both control and AD brains. Some of the astrocytes in deep white matter in control brains (**Figures 8C1,C3**) and AD brains (**Figures 8D1,D3**) co-localized with LPS (**Figures 8C2,C3,D2,D3**). In the periventricular white matter, reactive astrocytes were observed both in control (**Figures 8E1,E3**) and AD (**Figures 8F1,F3**) brains and LPS was colocalized with GFAP or was immediately adjacent in control (**Figures 8E2,E3**) and AD (**Figures 8F2,F3**) brain. Negative control for GFAP immunostaining (deleting antibody to GFAP) showed no signal in PVWM of AD and control brains (**Supplementary Figure 3**). The data suggest an intense astrocyte response to the loss of myelin in the MDZ of both control and AD brains, but little ongoing microglial response.

LPS$^+$ Vesicles Stain for PAS and Are CA

The LPS$^+$ or dMBP stained vesicles were morphologically similar to *corpora amylacea* which are usually identified by Periodic acid-Schiff (PAS) staining. Since PAS stains polysaccharides and LPS are important polysaccharides in the outer membrane of Gram-negative bacteria, we hypothesized that PAS might stain LPS. We, therefore, performed double staining for PAS and LPS in the myelin-deficient zone in the PVWM. The results showed LPS-stained vesicles in control (**Figure 9A1**) and AD brains (**Figure 9B1**), and PAS-stained CA in control (**Figure 9A2**) and AD brains (**Figure 9B2**). Overlay of the images showed that LPS was co-localized with PAS in the control (**Figure 9A3**) and AD brains (**Figure 9B3**), though some LPS vesicles did not contain PAS and some PAS-stained *corpora amylacea* were negative for LPS in AD brains (**Figure 9B3**).

These findings indicate that PAS probably does stain polysaccharides in bacterial LPS, and LPS staining vesicles constitute most of the *corpora amylacea* found in aging control and AD brains.

Purified LPS Stains for PAS

To further confirm that PAS stains LPS, we performed PAS staining for purified LPS from *E. coli,* serotype O111:B4 and its mutant form of *E. coli,* serotype J5. The results showed that PAS did stain both *E. coli* J5 LPS and *E. coli* O111:B4 LPS at higher concentrations (1,000 ng/μl and 500 ng/μl) with much greater staining intensity for *E. coli* J5 LPS than *E. coli* O111:B4 LPS at the same concentrations (**Figures 9C,D**; $p = 0.001$ at 1,000 ng/μl and $p = 0.029$ at 500 ng/μl, respectively). When the LPS concentrations were 250 ng/μl, PAS only stained *E. coli* J5 LPS but not the *E. coli* O111:B4 LPS. However, the difference was not significant. When the LPS concentrations were 125 ng/μl, PAS did not stain either *E. coli* J5 LPS or *E. coli* O111:B4 LPS.

These findings suggest that PAS stains LPS in a dose-dependent and strain-dependent manner.

DISCUSSION

A myelin deficient zone was observed in PVWM in AD and control aging brains. This zone contained vesicles, many of

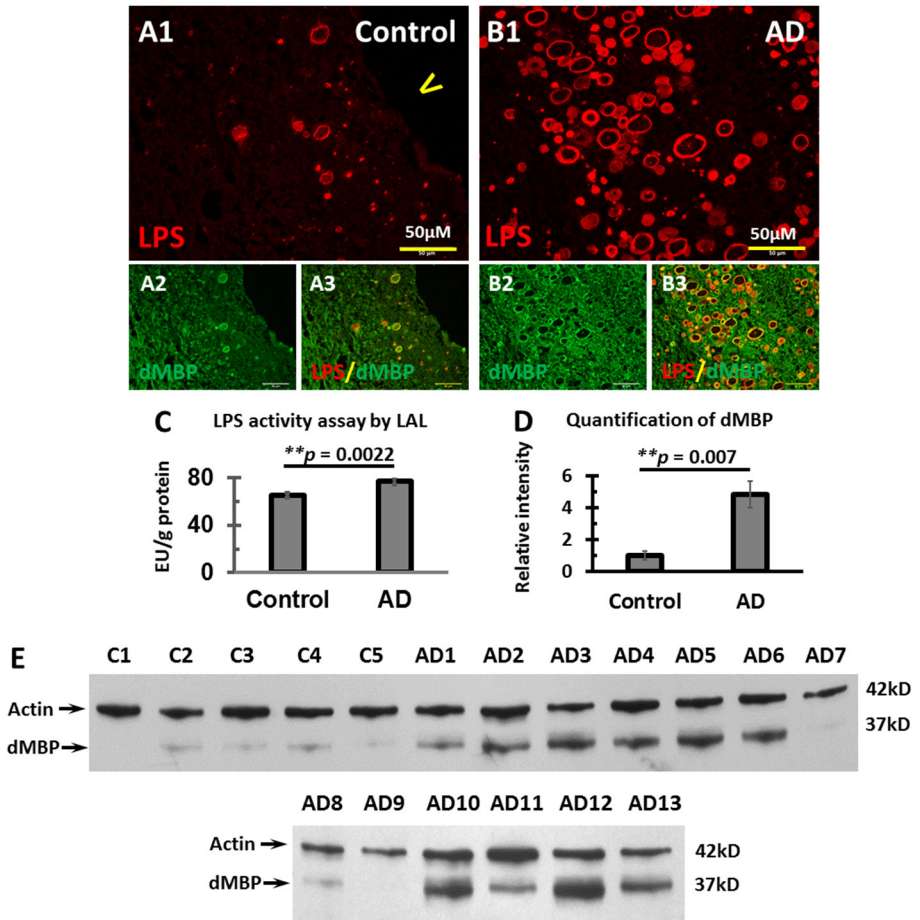

FIGURE 5 | Colocalization of LPS with dMBP, LPS LAL assay, and Western blot analysis of dMBP in the PVWM of AD and control brains. **(A)** LPS **(A1)** and dMBP **(A2)** in control PVWM were colocalized **(A3)**. **(B)** LPS **(B1)** and dMBP **(B2)** in AD PVWM were colocalized **(B3)**. **(C)** The LAL assay for LPS of PVWM showed significantly greater LPS activity in AD (77.3 ± 3.1 EU/g, $n = 12$) compared to controls (65 ± 2.1 EU/g, $n = 10$, $p = 0.0022$). **(D)** The dMBP band intensity in PVWM was greater in AD (4.8 ± 0.8, $n = 13$) compared to controls (1 ± 0.3, $n = 5$, $p = 0.007$). **(E)** Western blots of PVWM for dMBP and β-actin for 5 controls (C1-C5) and 13 AD samples (AD1-AD13). β-actin was used as a loading control. Note: LAL, Limulus Amoebocyte Lysate assay for LPS; dMBP, degraded myelin basic protein; LPS, lipopolysaccharide; AD, Alzheimer's disease; Bar = 50 μm.

which were corpora amylacea stained with PAS and co-localized with LPS. The CA/vesicles had evidence of oxidative stress to GALC and NG2, markers for oligodendrocytes (OLs), and oligodendrocyte precursor cells (OPCs), respectively. There were more LPS^+ vesicles in AD than control PVWM, and LPS activity was greater in AD than control PVWM. The LPS^+ vesicles were associated with the acute phase protein CRP, with degraded and oxidized myelin products, and were surrounded by astrocytes. Degraded MBP was found in the LPS^+ vesicles and dMBP levels were significantly greater in AD compared to control PVWM. PAS, the CA marker, was shown to directly stain LPS in nitrocellulose membranes. We postulate that CA/LPS^+ vesicles participate in a cerebral innate immune defense against LPS, damaged myelin, and other reactive molecules.

Inflammation of PVWM in AD and Aging Brains

We have previously demonstrated that AD brains have significant loss of intact MBP and an increase in dMBP in PVWM adjacent to a denuded ependymal layer (Zhan et al., 2014). In regions of myelin loss, vesicles that stained positive for dMBP, myelin lipid, and neurofilament but not for intact MBP were identified (Zhan et al., 2014). AD brains have significantly more vesicles in the PVWM compared to control brains (Zhan et al., 2014). In addition, we have also demonstrated that LPS in AD gray matter and AD white matter were greater than in control brains (Zhan et al., 2016, 2018). In this study, we show that the dMBP containing vesicles also contained Gram-negative bacterial LPS and that LPS co-localized with PAS in most of the vesicles, identifying the LPS-dMBP stained vesicles as *corpora amylacea* (CA).

Lipopolysaccharides are the major components of the outer membrane of Gram-negative bacteria and are potent inflammatory agents. LPS has been identified in human blood (Klimiec et al., 2016, 2018; Hakoupian et al., 2021) and brains (Zhan et al., 2016, 2018) with higher levels of LPS in blood (Zhang et al., 2009) and brains (Zhan et al., 2016, 2018; Zhao et al., 2017) of AD compared to controls. CRP is a plasma acute-phase protein

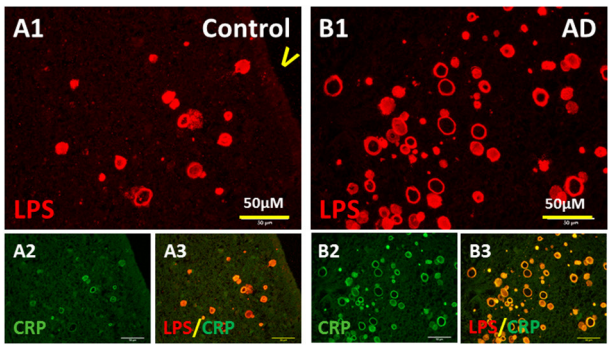

FIGURE 6 | Colocalization of LPS with CRP. **(A)** LPS **(A1)** and CRP **(A2)** in control PVWM were co-localized **(A3)**. **(B)** LPS **(B1)** and CRP **(B2)** in AD PVWM were co-localized **(B3)**. Note: LPS, lipopolysaccharide; CRP, C-reactive protein; V, ventricle; PVWM, periventricular white matter; AD, Alzheimer's disease; Bar = 50 μm.

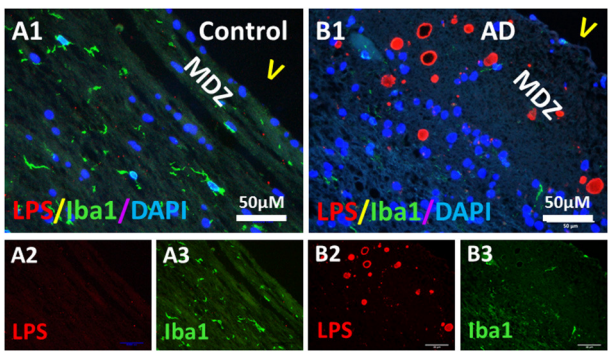

FIGURE 7 | Double immunostaining of LPS and microglial Iba1 in the PVWM of AD and aging control brains. **(A)** LPS **(A2)** and Iba1 **(A3)** in control PVWM did not co-localize **(A1)**. Iba1 stained microglia were sparse **(A1,A3)**. **(B)** LPS **(B2)** and Iba1 **(B3)** in AD PVWM did not co-localize **(B1)**. Iba1 stained microglia were sparse in AD PVWM **(B1,B3)**. Note: MDZ, myelin deficient zone; V, ventricle; LPS, lipopolysaccharide; Iba1, microglial marker; AD, Alzheimer's Disease; Bar = 50 μm.

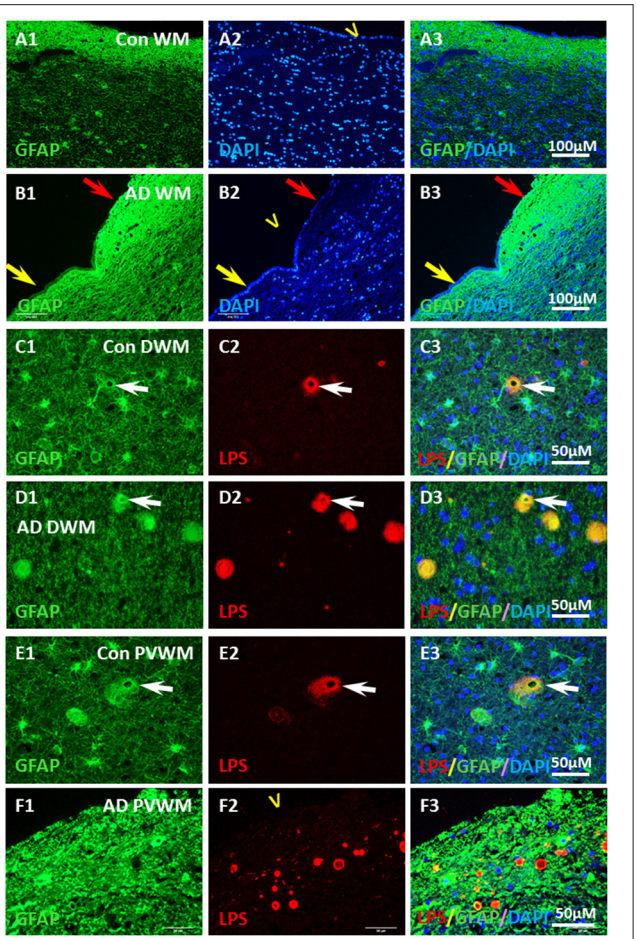

FIGURE 8 | Localization of GFAP and LPS in the vesicles in AD and aging WM. **(A)** GFAP was intensely stained in the control PVWM myelin deficient zone, which forms a GFAP reactive layer immediately beneath the ependymal lining **(A1,A3)**. DAPI stained all nuclei in the section, including the ependymal cells **(A2,A3)**. **(B)** GFAP was intensely stained in the AD PVWM myelin deficient zone, which forms a GFAP reactive layer immediately beneath the ependymal cells **(B1,B3)**. The GFAP stained layer was thicker where ependymal cells were partially lost (**B1–B3**, red arrow) compared to areas where ependymal cells were intact (**B1–B3**; yellow arrow). DAPI staining showed loss of ependymal cells in some areas (**B2,B3** red arrow). **(C)** In control deep white matter (Con DWM), scattered astrocytes were observed **(C1)** a few of which stained for LPS **(C2,C3)**. **(D)** In Alzheimer's Disease deep white matter (AD DWM) scattered astrocytes **(D1)** were also observed, many of which stained for LPS (**D1–D3**, arrow). **(E)** In control periventricular white matter (Con PVWM) scattered astrocytes were observed **(E1)** some of which were stained for LPS (**E2,E3**, arrow). **(F)** In Alzheimer's Disease periventricular white matter (AD PVWM) there was dense GFAP staining throughout the myelin deficient zone **(F1)**. LPS staining showed discrete vesicles in this zone **(F2)**, the margins of which appeared to co-localize with or were immediately adjacent to GFAP positive astrocytic processes **(F3)**. Note: LPS, lipopolysaccharide; V, ventricle; WM, white matter; PVWM, periventricular white matter; DAPI, nuclear stain; DWM, deep white matter; AD, Alzheimer's disease; Bar = 100 μm in **(A,B)**; Bar = 50 μm in **(C–F)**.

whose concentrations increase in response to inflammation. Our recent study has demonstrated that plasma LPS levels positively correlated with plasma CRP levels in humans (Hakoupian et al., 2021). Other studies demonstrated that CRP levels are elevated in blood years before the onset of AD (Schmidt et al., 2002; Engelhart et al., 2004), and plasma CRP levels are higher in patients with AD compared to controls (Song et al., 2015). Though CRP is not normally found in normal brains, it has been reported in the tangles (Duong et al., 1997), plaques (Iwamoto et al., 1994), and pyramidal neurons of AD brains (Yasojima et al., 2000). It is unclear whether CRP plays a role in AD pathogenesis, or is simply a response to LPS and other pro-inflammatory molecules in the AD brain.

How LPS entered brain PVWM is still unclear. However, we have previously found that the ependymal cells were injured in aging brains and that the ependymal cell loss was more severe in AD compared to controls brains (Zhan et al., 2014). Most of the LPS in deep regions of the brain was co-localized with the vesicles in the myelin deficient zone adjacent to the ependymal layer. This finding suggests that ependymal injury might be associated

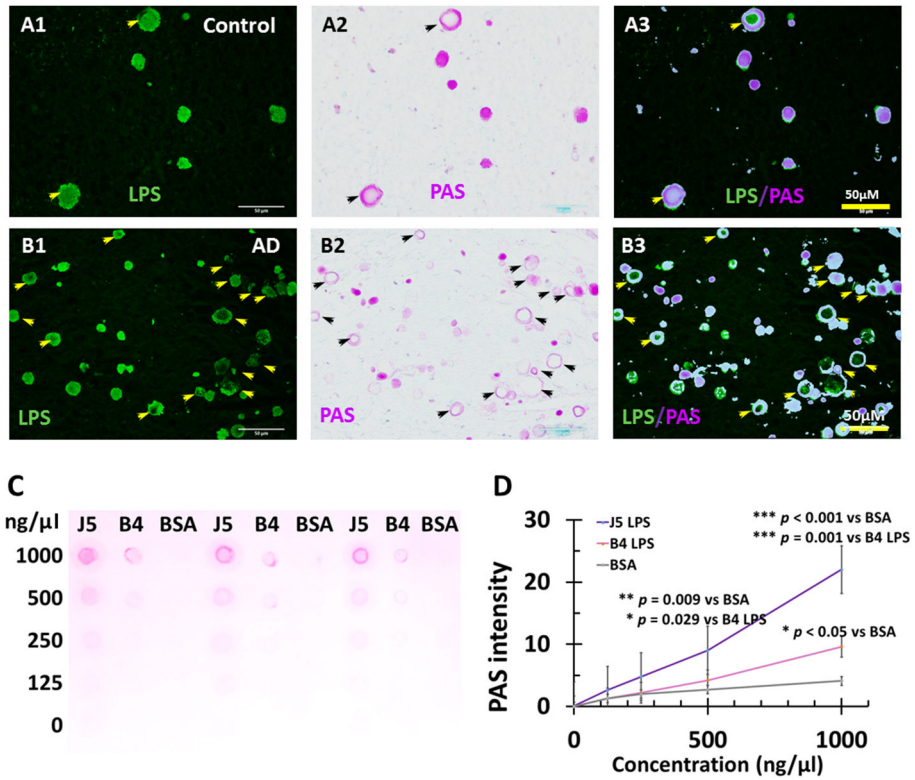

FIGURE 9 | PAS staining of LPS in *Corpora amylacea* of AD and aging brains, and PAS staining of purified LPS. In control, PVWM LPS **(A1)** and PAS **(A2)** were co-localized **(A3)**. IN AD PVWM there were more LPS stained vacuoles **(B1)** and PAS-stained *Corpora amylacea* **(B2)** which were mostly co-localized **(B3)**. Most but not all LPS stained vesicles were PAS positive and therefore were *Corpora amylacea* (CA). PAS stained both *E. coli* J5 LPS and *E. coli* O111:B4 LPS at the higher concentrations (1,000 ng/μl and 500 ng/μl) with much greater staining intensity for *E. coli* J5 LPS than *E. coli* O111:B4 LPS at the same concentrations **(C,D)**. When the LPS concentrations were 250 ng/μl, PAS only stained *E. coli* J5 LPS but not the *E. coli* O111:B4 LPS. However, the difference was not significant. When the LPS concentrations were 125 ng/μl, PAS did not stain either *E. coli* J5 LPS or *E. coli* O111:B4 LPS. Note: LPS, lipopolysaccharide; PAS, Periodic acid–Schiff, a marker for CA; J5, *E. coli* J5 LPS; B4, *E. coli* O111:B4 LPS; AD, Alzheimer's disease; Bar = 50 μm.

with LPS entry into the PVWM which could be carried into the PVWM by CSF from the lateral ventricles.

The myelin injury was associated with oxidative damage. Degraded MBP was co-localized with LPS in the vesicles located in the myelin-deficit zone of aging and AD brains. Higher levels of LPS and dMBP were found in AD than controls. LPS was also associated with oxidized myelin molecules in the vesicles. We speculate that the LPS contributed to the oxidative stress that caused the ependymal and myelin injury.

Glial fibrillary acidic protein positive astrocytes were intensely stained in the myelin-deficit zone that was adjacent to the lateral ventricles. Astrocytes are involved in forming the blood-brain barrier (BBB) which modulates the passage of molecules including nutrients, ions, glucose, water, and amino acids and restricts the passage of pathogens. Since the astrocytes appeared to be intimately associated with the walls of the vesicles, we postulate they might form a barrier to help prevent bacterial components such as LPS from spreading to other parts of the brain.

We have previously shown that Iba1$^+$ microglia associate with LPS in the gray matter of AD brains (Zhan et al., 2016). Activated microglia are involved in AD pathology in gray matter and amyloid plaques (Dickson et al., 1988; Serrano-Pozo et al., 2013; Zhan et al., 2016; Hansen et al., 2018; Ahmad et al., 2019; Dionisio-Santos et al., 2019). We expected increased numbers of activated microglia in areas of myelin injury in the PVWM of AD brains. Surprisingly, Iba1$^+$ microglia were quite sparse in the PVWM of aging and AD brains. Presumed microglial dysfunction has been suggested in frontotemporal lobar degeneration (Sakae et al., 2019) and AD (Piirainen et al., 2017; Yoshino et al., 2017; Andreone et al., 2020; Gabande-Rodriguez et al., 2020). Alternatively, it is possible that the phagocytic function of the microglia has already been completed with the removal of most of the injured myelin, compartmentation of damaged myelin, and LPS in the vesicles resulting in little ongoing microglial activity at this point.

Lipopolysaccharides were localized to microglia, neurons, OPCs, oligodendrocytes, and extracellular amyloid plaques in AD gray matter in our previous study (Zhan et al., 2016). We also found Gram-negative bacterial DNA in AD and aging brains. Recent studies demonstrated that bacterial DNA promotes aggregation of β-amyloid and tau, two hallmarks of

AD pathology (Tetz et al., 2020; Tetz and Tetz, 2021). The DNA was derived from *Porphyromonas gingivalis*, *Burkholderia burgdorferi*, and different strains of *E. coli,* all of which are gram-negative and have been associated with AD (Tetz et al., 2020). Other bacteria and viruses have also been linked to AD pathology (Hashioka et al., 2008; Hammond et al., 2010; Miklossy, 2011, 2015, 2016; Lim et al., 2014; Fulop et al., 2018). These microorganisms and their molecular components are derived from the gut, skin, gums, and other organs, and presumably cross the BBB and might play a role in the pathogenesis of AD. This study supports the existence of some role for LPS in AD pathogenesis since it is localized to dMBP-PAS positive vesicles which appear to be CA.

LPS Positive Vesicles Are CA and PAS Directly Stains LPS

The vesicles described in the myelin deficient zone adjacent to the ventricles appear mostly to be CA in both control and AD brains. Therefore, the vesicles and CA might have similar functions. Based upon the molecules found in the vesicles/CA, possible functions include being part of the innate immune response, inflammation, oxidative stress, myelin degradation, and compartmentalizing microbial molecules. The findings suggest that CA are waste depots where deleterious molecules like LPS are deposited, and damaged organelles and damaged myelin are restricted by actions of the innate immune system (Auge et al., 2017; Riba et al., 2019).

Lipopolysaccharides were co-localized with PAS in many vesicles of AD and control brains. Since PAS is a staining method used to detect polysaccharides in CA, and LPS are important bacterial cell-surface polysaccharide, it is possible that LPS stained positive for PAS in CA in aging control and AD brains. In AD brains, most LPS^+ vesicles stained positive for PAS, which suggests that the LPS in the vesicles can be detected by PAS staining. However, some PAS-stained CA did not contain LPS, which suggests that other polysaccharides other than LPS might be present. In addition, some LPS positively stained vesicles did not stain for PAS, which suggests that not all LPS can be detected by PAS.

In our previous studies, LPS was detected by immunostaining, which involves antibody signal detection and amplification that can detect very small amounts of LPS. We hypothesized that even if PAS does stain LPS, perhaps it does not stain low levels of LPS. This hypothesis was confirmed by the PAS staining of purified LPS on nitrocellulose membranes at different concentrations. PAS stained both *E. coli* J5 LPS and *E. coli* O111:B4 LPS at high concentrations of LPS (1000 ng/μl and 500 ng/μl) with much greater staining intensity for *E. coli* J5 LPS than *E. coli* O111:B4 LPS at the same LPS concentration. When LPS concentrations were 125 ng/μl, PAS did not stain either *E. coli* J5 LPS or *E. coli* O111:B4 LPS. These findings show that PAS stains LPS in a dose-dependent manner.

One notable finding was that PAS-stained *E. coli* J5 LPS more intensely than *E. coli* O111:B4 LPS at the same concentrations of LPS. *E. coli* J5 LPS is a mutant form of *E. coli* O111:B4 LPS lacking the O-antigen. Therefore, the molecular mass of *E. coli* J5 LPS is smaller than *E. coli* O111:B4 LPS. Thus, more LPS molecules are in *E. coli* J5 LPS solution than in *E. coli* O111:B4 LPS solution given a certain concentration and volume. This might contribute to the higher PAS staining intensity of *E. coli* J5 LPS than *E. coli* O111:B4 LPS. Even lacking the O-antigen (O-polysaccharide), *E. coli* J5 LPS was still stained by PAS, which suggests that O-antigen is not the key element that is recognized by PAS. These findings revealed a previously unknown feature of CA that PAS does stain LPS on nitrocellulose membranes, and therefore bacterial LPS is likely at least one of the polysaccharides stained by PAS in CA.

In summary, myelin injury occurs in the PVWM of aging brains and more so, in AD brains. Degraded MBP levels are greater in AD brains compared to controls. The myelin injury in aging control and AD brain was associated with oxidative damage and neuroinflammation that may be due, in part, to bacterial LPS. LPS activity was greater in AD compared to control PVWM, and LPS^+ vesicles were more abundant in AD compared to control PVWM. Bacterial LPS was one of the polysaccharide sources in CA and PAS stained LPS on nitrocellulose membranes in a dose-dependent and strain-dependent manner. CA found in AD and aging control brains are associated with bacterial LPS and the innate immune response.

LIMITATIONS

This is a preliminary postmortem study on a limited number of sections from a limited number of brains. This is primarily an observational study that presents some novel findings. Though we attempted to infer mechanisms that could have produced the pathology, there is no way to determine cause and effect based upon the findings reported here. Future mechanistic studies will be required to better understand the significance of these results to normal aging and AD.

A vesicle is often a subcellular structure, bounded by a membrane. We chose to use the term "vesicle" simply because it was convenient. It is possible that all of the vesicles described here are all CA, in which case *corpora amylacea* would be the preferred name because of the history and many studies of CA.

The monoclonal antibody to LPS used in this study does seem to bind LPS since immunoprecipitation of the antibody with LPS eliminated all immunostaining. As pointed out in our prior studies, however, this antibody produces a single band on Western blots that is greater than the expected molecular weight of LPS. Thus, we have suggested that the antibody likely detects LPS bound to another unknown molecule. Thus, we used a completely independent LAL enzymatic LPS assay to show LPS activity was higher in AD PVWM compared to controls.

Future studies will be needed to explore the birth and death of oligodendrocytes and OPCs in the myelin deficient zone and in areas adjacent to the MDZ. It is likely the MDZ region studied here is identical to what is referred to as periventricular White Matter Hyperintensities (WMH) on human MRI brain scans. This needs to be confirmed by comparing the brain pathology and MRI in individual cases in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of California at Davis Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XZ designed the studies and wrote the manuscript. MH performed experiments under the supervision of XZ. L-WJ provided samples. L-WJ and FS reviewed subjects for correct diagnosis. MH and XZ performed statistical analysis. All authors made changes to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.705594/full#supplementary-material

Supplementary Figure 1 | Autofluorescence removal test. At red fluorochrome wavelength, autofluorescence was detected in the PVWM of aging brains including control **(A1)** and AD **(B1)** brains. Similarly, at green fluorochrome wavelength, autofluorescence was detected in the PVWM of control **(C1)** and AD **(D1)** brains. After applying Autofluorescence Removal Reagent, fluorescent signals at red fluorochrome wavelength in control **(A2)** and AD **(B2)** were nearly eliminated. Similarly, after applying Autofluorescence Removal Reagent, fluorescent signals at green fluorochrome wavelength in control **(C2)** and AD **(D2)** were nearly eliminated. These data indicated that immunofluorescent signals detected in this study were not due to autofluorescence. Note: $Auto^{+}$, Autofluorescence before removal; $Auto^{-}$, Autofluorescence after removal; AD, Alzheimer's disease; PVWM, periventricular white matter; Bar = 100 μm.

Supplementary Figure 2 | Negative control test for immunofluorescence. A negative control test for immunofluorescence was carried out the same as the regular immunofluorescence used in the study except primary antibodies were omitted. There were no fluorescence signals either at the red fluorochrome wavelength in control **(A1)** or in AD **(B1)** PVWM or at the green fluorochrome wavelength in control **(A2)** or AD **(B2)** PVWM. These data indicate the immunofluorescence signals were not due to non-specific staining from the secondary antibodies. Note: AD, Alzheimer's disease; PVWM, periventricular white matter; Bar = 100 μm.

Supplementary Figure 3 | Immunofluorescence of GFAP and its negative control. Immunofluorescence of GFAP was detected in control PVWM **(A1)** and AD PVWM **(B1)**. In controls, GFAP positively stained astrocytes as well as the vesicle (**A1**, arrow). In AD PVWM **(B1)**, GFAP positively stained astrocytes and vesicles (**B1**, arrows) as well. Negative controls with deletion of primary GFAP antibody showed no signals in both control **(A2)** and AD **(B2)** brains. Note: AD, Alzheimer's disease; PVWM, periventricular white matter; Bar = 50 μm.

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REVIEW
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The Gut–Brain Axis and Its Relation to Parkinson's Disease: A Review

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Parkinson's disease is a chronic neurodegenerative disease characterized by the accumulation of misfolded alpha-synuclein protein (Lewy bodies) in dopaminergic neurons of the *substantia nigra* and other related circuitry, which contribute to the development of both motor (bradykinesia, tremors, stiffness, abnormal gait) and non-motor symptoms (gastrointestinal issues, urinogenital complications, olfaction dysfunction, cognitive impairment). Despite tremendous progress in the field, the exact pathways and mechanisms responsible for the initiation and progression of this disease remain unclear. However, recent research suggests a potential relationship between the commensal gut bacteria and the brain capable of influencing neurodevelopment, brain function and health. This bidirectional communication is often referred to as the microbiome–gut–brain axis. Accumulating evidence suggests that the onset of non-motor symptoms, such as gastrointestinal manifestations, often precede the onset of motor symptoms and disease diagnosis, lending support to the potential role that the microbiome–gut–brain axis might play in the underlying pathological mechanisms of Parkinson's disease. This review will provide an overview of and critically discuss the current knowledge of the relationship between the gut microbiota and Parkinson's disease. We will discuss the role of α-synuclein in non-motor disease pathology, proposed pathways constituting the connection between the gut microbiome and the brain, existing evidence related to pre- and probiotic interventions. Finally, we will highlight the potential opportunity for the development of novel preventative measures and therapeutic options that could target the microbiome–gut–brain axis in the context of Parkinson's disease.

Keywords: Parkinson's disease, gut–brain axis, microbiome, alpha-synuclein, dysbiosis

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder with an estimated prevalence of approximately 1% among individuals over the age of 65 (Nussbaum, 2003). The worldwide burden of PD has more than doubled over the past three decades, increasing from 2.5 million patients in 1990 to over 6.1 million in 2016 (GBD 2016 Parkinson's Disease Collaborators, 2016; Rocca, 2018). This trend is expected to continue in the coming generations

as the global population continues to increase in age. In addition, period effects also suggest the age-adjusted incidence of PD is increasing among males (Rocca, 2018). Hence, the prevalence of Parkinson's Disease (PD) is estimated to reach nearly 1,238,000 cases by the year 2030 in the United States alone (Marras et al., 2018). PD is a progressive neurodegenerative disease characterized by the pathological misfolding of alpha-synuclein (α-syn) protein which subsequently impacts the function of the central, peripheral, and enteric nervous systems (Kouli et al., 2018). PD is also a multifactorial disease with both genetic (e.g., 23 genes are linked to Mendelian forms of parkinsonism, and 187 genes in ∼90 loci have been associated with idiopathic PD) and environmental (e.g., head injury, cigarette smoking, caffeine consumption, exposure to certain pesticides/herbicides or heavy metals, etc.) risk factors (Kouli et al., 2018). Misfolded α-syn aggregates, otherwise known as Lewy body deposits, contribute to the degeneration of dopaminergic neurons within the *substantia nigra* and other related circuitry (Spillantini et al., 1997, 1998; Trojanowski and Lee, 1998), and ultimately the onset of several cardinal motor and non-motor features of PD including dementia, gastrointestinal (GI) dysfunction, tremor, postural instability, bradykinesia, and rigidity (Váradi, 2020). Although most commonly located in the brain, α-syn aggregates have also been found in peripheral locations, such as the enteric nervous system (ENS), lending support to the idea of a "gut–brain axis," a bidirectional communication pathway between the central and enteric nervous systems and the GI system (Chao et al., 2020; **Figure 1**).

ALPHA-SYNUCLEIN PATHOLOGY IN PARKINSON'S DISEASE

Alpha-synuclein is a neuronal protein found at synapses and is abundantly found in the brain (**Figure 1A**) and is associated with the neuropathology observed in PD and related neurodegenerative disorders (e.g., Lewy Body Disease and Multiple System Atrophy), predominantly through the formation of aberrant aggregates that may disrupt cellular homeostasis, synaptic function, and induce neuronal degeneration (Spillantini et al., 1997, 1998; Trojanowski and Lee, 1998; Stefanis, 2012). Notably, α-syn is released through an unique secretory pathway (Lee et al., 2005, 2016; Emmanouilidou et al., 2010; Gustafsson et al., 2018), and although the exact mechanisms of fibrillar α-syn cellular secretion and uptake are not well understood, extracellular forms of this protein have been found in both rodent and human brain and interstitial fluid, providing a potential avenue for intercellular propagation (Emmanouilidou et al., 2011). A recent study using primary neuronal cell cultures and *in vivo* microdialysis found that approximately 70% of extracellular α-syn originates from neuronal activity-dependent pathways (e.g., glutamatergic neurotransmission) (Yamada and Iwatsubo, 2018). Further, microglial activation and subsequent neuroinflammation is associated with misfolded α-syn in PD (Alvarez-Erviti et al., 2011; Xia et al., 2019). Whether neuroinflammation triggers protein misfolding or, alternatively, the presence of misfolded α-syn promotes a neuroinflammatory response capable of initiating PD pathology, has not yet been definitively determined and neither is mutually exclusive. While several potential factors associated with α-syn misfolding and aggregation have been identified [e.g., intra- and extra-cellular pH (Buell et al., 2014), ionic concentration (de Oliveira and Silva, 2019), presence of metals (Villar-Piqué et al., 2016)], the underlying mechanisms are still largely unknown. Clearly, chronic and constitutive α-syn gene expression is a major risk factor as gene dose is inversely correlated with age at onset (Book et al., 2018).

POTENTIAL CONTRIBUTIONS OF MICROBIOTA TO ALPHA-SYNUCLEIN PATHOLOGIES: BRAAK'S HYPOTHESIS

The presence of healthy intestinal microbiota promote the integrity of the blood–brain barrier (BBB) through regulation of tight junction protein expression (e.g., occludin and claudin-5) mediated by short chain fatty acids (SCFAs) (Hoyles et al., 2018; Tran and Mohajeri, 2021). SCFAs play an important role in maintaining intestinal barrier integrity by preventing microbial translocation, that is known to be associated with local intestinal inflammation, systemic inflammation and neuroinflammation (Houser and Tansey, 2017; Wang et al., 2020). However, dysbiosis of the microbiome, associated with an increased abundance of potentially detrimental bacteria, can compromise gut barrier integrity through bacterial production of endotoxins (e.g., lipopolysaccharide) capable of altering immune response, initiating proinflammatory pathways, and directly damaging intestinal epithelial cells (Ghosh et al., 2020). In the bloodstream, lipopolysaccharide (LPS) interacts with immune cells, upregulates systemic expression of proinflammatory cytokines (e.g., TNF and interleukins) and inflammation (Ghosh et al., 2020), and, in high concentrations, may directly disrupt the BBB to induce neuroinflammation (Banks et al., 2015). Further, LPS has been found to induce a structurally distinct strain of self-renewing α-syn fibrils in mice capable of initiating hallmark patterns of synucleinopathy similar to that induced by the wild-type form of α-syn commonly observed in PD (Kim et al., 2016). The modulation of α-syn amyloidogenesis caused by interaction with LPS has been characterized as a heteromolecular interaction resulting in the formation of intermediate nucleating species which then mature into divergent fibrillar forms of α-syn, affecting both cellular internalization and related cytotoxicity (Bhattacharyya et al., 2019).

Several studies have explored the potential connection between α-syn-related pathology and GI symptoms of PD. While α-syn is found copiously in the brain, it is also found in the ENS and is produced by enteric neurons to mediate neurotransmitter release and uptake (Grathwohl et al., 2013). In individuals with PD, pathological α-syn aggregates have been found in GI tissue biopsies (Sánchez-Ferro et al., 2015), lending support to the theory that PD pathology could be initiated in the ENS. Importantly, α-syn has also been found in the salivary glands, esophagus, and stomach (Fayyad et al., 2019), potentially corresponding to common non-motor symptoms

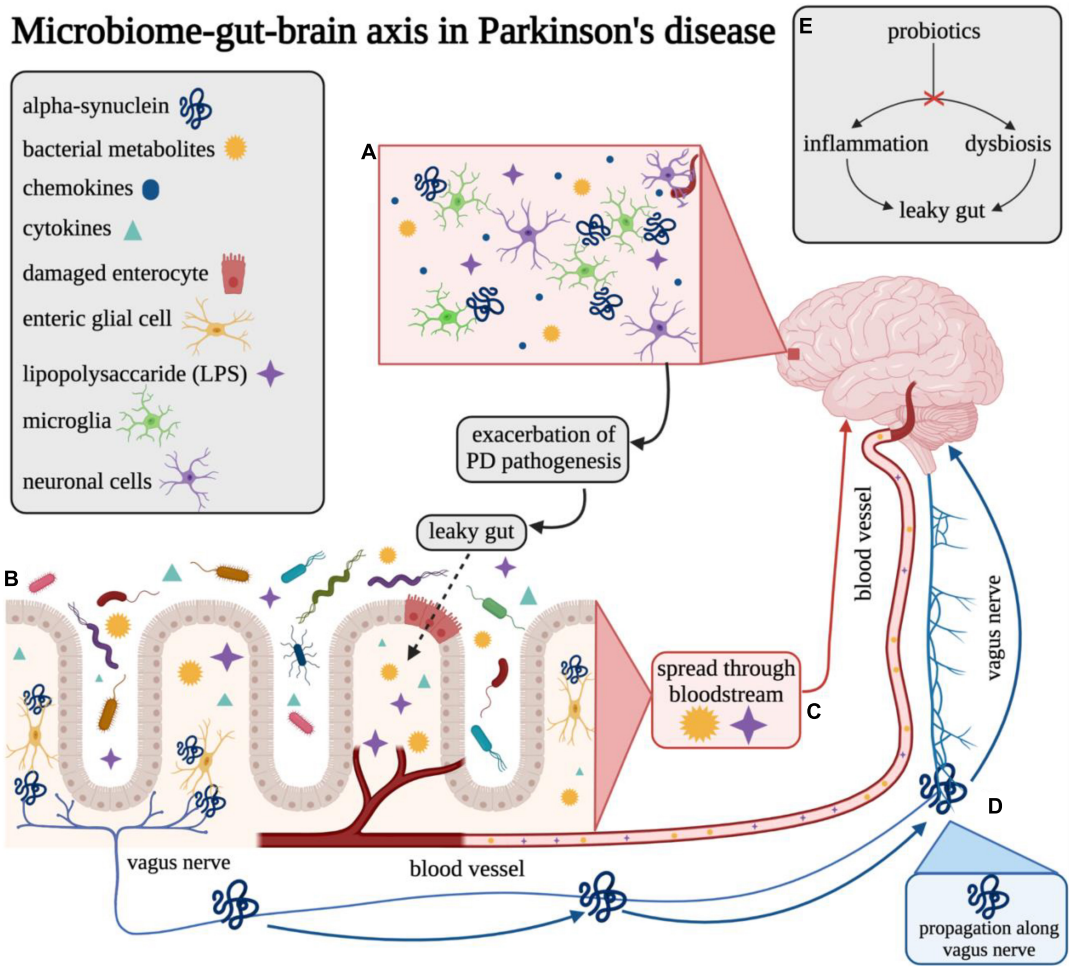

FIGURE 1 | Overview of the gut–brain axis in Parkinson's disease. Visual overview of the proposed microbiome–gut–brain axis in Parkinson's disease. **(A)** LPS and other bacterial metabolites may be able to enter the brain across the blood–brain barrier (BBB) and may elicit release of various chemokines/cytokines that promote an inflammatory response in Parkinson's disease. **(B)** Microbes in the gut lumen can promote inflammatory pathways and cause damage to enterocytes which may lead to compromised gut epithelial barrier integrity ("leaky gut"). **(C)** Bacterial metabolites, such as LPS, can translocate from the gut lumen to the bloodstream across the compromised gut barrier and cause possible systemic and neuroinflammation in the brain. **(D)** Misfolded α-synuclein may be induced by microbes at the intersection of the gut lumen and ENS and may be propagated to neurons in the brain through the vagus nerve. **(E)** Probiotic interventions are thought to reverse dysbiosis through altering the composition of the microbiome. This change is also purported to result in reduction of inflammation and improvement of gut epithelial barrier integrity, thereby preventing or reducing microbial translocation. This figure was created with BioRender.com.

such as hypersalivation, dysphagia, delayed gastric emptying, and gastroparesis. In a study involving a transgenic mouse model of PD, an amyloid precursor protein inhibitor, Posiphen, was injected into the mice to inhibit the misfolding of α-syn (Kuo et al., 2019). Results of this study demonstrated normalized distal colon motility following treatment, an important factor associated with maintenance of a balanced microbiome – presenting a potential novel early intervention for PD, given the frequent early onset of GI symptoms related to gut motility such as constipation, which warrants further investigation. However, a recent study reported the sensitivity associated with the presence of α-syn in colon tissue biopsies of PD patients to be approximately 14% (8/57) (Chahine et al., 2020). A meta-analysis of 21 studies estimated the sensitivity and specificity of α-syn in the colon tissue to be approximately 57 and 82%, with an approximate pooled odds ratio of 10 compared to non-PD controls (Bu et al., 2019). Though presence of α-syn in the gut likely contributes to development and progression, it is neither necessary nor sufficient for onset of PD pathology and is not an adequate predictor of future disease.

Notably, Braak et al. (2003) and Hawkes et al. (2007) proposed potential involvement of the GI tract in the development and progression of PD. The authors hypothesized that an unknown pathogen may enter the body through the nasal cavity, and such a neurotropic pathogen could potentially induce conformational changes in normal α-syn molecules, thereby resulting in their aggregation. The pathogen could then enter via the gastric mucosa and subsequently the central nervous system (CNS) via retrograde transport. Braak and colleagues hypothesized that PD α-synucleinopathy begins in the periphery, gains access to

the CNS via retrograde transport along vulnerable neuronal projections within the ENS, and ascends caudo-rostrally from the lower brainstem in various distinct stages (Braak et al., 2003; Hawkes et al., 2007; Visanji et al., 2013). This hypothesis is unique in that it proposes a potential outside source (e.g., pathogen) as the catalyst for the onset of PD pathology as opposed to an inherent or internally driven mechanism. However, this hypothesis and its related staging system has been historically criticized for its lack of applicability to patients outside of a particular subset of those with the sporadic form of PD. Evidence supports some aspects of this hypothesis such as the presence of α-syn aggregates in the vagus nerve and their ability to spread from the ENS to the CNS (Musgrove et al., 2019; **Figure 1D**) as well as the related prevention of this propagation following vagotomy in animal models of PD (Kim et al., 2019). Further, novel experimental models of PD have been developed through direct initial inoculation of α-syn in the GI tract that subsequently induces synucleinopathy pathology in the CNS (Uemura et al., 2018; Kim et al., 2019; Challis et al., 2020). Nevertheless, recent results from a whole-body autopsy series of PD, incidental Lewy body disease, and otherwise healthy control participants (N = 187) failed to demonstrate a single case in which there was Lewy pathology in the gut without Lewy pathology also present in the brain (Beach et al., 2021). Therefore, further research is warranted to establish whether Lewy body pathology may originate in the gut independently from that of the brain, and whether this pathology in the gut is associated with clinically significant differences in disease-state or health outcomes related to PD compared to those without gut involvement to determine its potential utility as a novel intervention method.

MICROBIOTA COMPOSITION AND METABOLITES ASSOCIATED WITH PARKINSON'S DISEASE AND SYMPTOM SEVERITY

The microbiome is the collection of all individual microbes (bacteria, fungi, viruses, and single-celled organisms), forming complex microbiota, and their genomes within a particular anatomic site (Ursell et al., 2012). It is dynamic across the lifespan and is influenced by a multitude of factors including, age (Badal et al., 2020), lifestyle factors including diet (Frame et al., 2020) and exercise patterns (Bycura et al., 2021), and medication use (Vich Vila et al., 2020). The GI tract in mammals contains a complex and symbiotic ecosystem with diverse interactions between the GI epithelium, immune cells, and commensal microbes. The gut microbiota are thought to be key mediators in the bi-directional communication between the gut and the brain along the gut–brain axis that influence neurodevelopment, brain function, and behavior (Yang et al., 2019). An undesirable shift in microbiota composition, termed dysbiosis, has been associated with both GI and metabolic diseases such as inflammatory bowel disease (Greenblum et al., 2012), obesity (Turnbaugh et al., 2006), and diabetes (Qin et al., 2012). Gut dysbiosis has also been observed in various neurological and psychiatric conditions including autism spectrum disorder (Golubeva et al., 2017), major depression (Foster and Neufeld, 2013), PD (Scheperjans et al., 2015a), and Alzheimer's disease (Vogt et al., 2017). Certain gut microbiota composition dynamics have been associated with specific diseases or conditions across a broad population (e.g., increased relative abundance of the *Akkermansia* genera over time among individuals with PD across multiple geographic locations (Finland, Germany, Japan, Russia, and United States) (Nishiwaki et al., 2020b). However, defining a generalizable "normal" or "healthy" gut microbiome across the lifespan remains an important challenge due to both natural interindividual differences and intraindividual changes in microbiota composition over time.

A study analyzing 190 colonic tissue samples from participants with and without GI-related conditions (e.g., Crohn's disease, ulcerative colitis, and controls being treated for cancer) estimated the average gut microbiome to be comprised of up to 35,000 species of bacterial and archaeal species (Frank et al., 2007); approximately 90% appearing to belong to the phyla Bacteroidetes and Firmicutes (Rinninella et al., 2019). Commensal gut microbiota play an important role in maintaining homeostasis and preventing certain pathologies, partly through production of vital micronutrients and fermentation of otherwise non-digestible polysaccharides into SCFAs (e.g., acetate, propionate, and butyrate) (Wells et al., 2017). SCFAs serve as a source of energy for host tissue, function as signaling molecules to regulate the expression of tight junction proteins of the intestinal epithelial barrier and play a role in the modulation of host immunity (Mathewson et al., 2016). Additional work has demonstrated that butyrate plays a role in regulating immune response via expansion of Treg cell populations (Arpaia et al., 2013; Furusawa et al., 2013), which is suggestive of a role for SCFA in ameliorating pro-inflammatory responses of immune cells to antigenic stimuli. Certain microbes, such as *Faecalibacterium prausnitzii*, help maintain homeostasis through production of SCFAs (butyrate) and modulation of downstream inflammatory pathways through potential induction of a tolerogenic cytokine profile (e.g., low secretion of proinflammatory and elevated secretion of anti-inflammatory cytokines) (Rossi et al., 2015; Lenoir et al., 2020). A recent microbiome-wide association study using two large datasets (N = 333; N = 507) observed an elevation of opportunistic pathogens, while potentially beneficial bacteria able to metabolize carbohydrates (precursors for SCFAs) among individuals with PD, were depleted in this same population (Wallen et al., 2020). Further, compared to conventionally colonized controls, GF mice (mice raised under sterile conditions or depleted of their intestinal microbiota through administration of oral broad-spectrum antibiotics) exhibit substantial physiological alterations, such as increased BBB permeability, that are relevant to neurodegenerative disorders such as PD. Depletion of the gut microbiota through administration of antibiotics was associated with statistically significant improvement in various motor symptoms (duration and severity of dyskinesia, duration of medication "off" state, functional impact of motor functions, and complexity of motor fluctuations) among 14 individuals with PD (Baizabal-Carvallo et al., 2021).

Non-motor symptoms, including GI disturbances such as constipation, have often been found to considerably precede the onset of hallmark neurological features of PD (Klingelhoefer and Reichmann, 2015). Further, over 80% of PD patients report some level of GI dysfunction throughout the course of their disease (Pfeiffer, 2011). Further, according to the Honolulu Heart Program study, males between 51 and 75 years of age who passed less than one bowel movement per day were 2.7 times more likely to develop PD compared to males of the same age range who passed at least one bowel movement per day (Abbott et al., 2001). Although research on the relationship between the GI system and neurodegenerative diseases is still in its early stages, several promising hypotheses have been developed. The gut microbiome is thought to play a role, in at least a subset of PD patients, in both the initiation and progression of PD pathology and symptomology (Gorecki et al., 2019; Yang et al., 2019; Liu et al., 2020). Studies have shown a correlation between the increased occurrence of neurodegenerative diseases and conditions known to be associated with disrupted gut microbiota diversity such as insomnia, REM sleep behavior disorder (RBD), and constipation, with approximately 40–65% of RBD patients developing a neurodegenerative disorder within 10 years of RBD diagnosis (Postuma et al., 2009; Heinzel et al., 2021). Moreover, in a study of 172 RBD patients, 94% of those with associated comorbid neurodegenerative disease had a form of synucleinopathy (Boeve et al., 2013). Gut microbiota may modify the association between RBD and PD given the evidence from a recent study where there was an increase in the relative abundance of genus *Akkermansia*, a genus of mucin-metabolizing bacteria (Earley et al., 2019) that are commonly elevated among individuals with PD (Romano et al., 2021), in individuals with RBD (Heintz-Buschart et al., 2018; Nishiwaki et al., 2020a).

Colonic motility follows a circadian rhythm similar to that of the suprachiasmatic nucleus in the brain and can be become desynchronized due to changes in a variety of external stimuli including light exposure, food intake patterns, and exercise (Duboc et al., 2020). Sleep disturbances are also known to be associated with potentially pathological mechanisms (e.g., proinflammatory pathway expression, stress on the endoplasmic reticulum, abnormal proteostasis, impaired glymphatic clearance, nocturnal cerebral hypoxia, and altered modulation of specific neural circuits) that may increase the risk for PD (Bohnen and Hu, 2019). As pathological α-syn has been observed to propagate along the brain's inherent neural networks (e.g., network-spread hypothesis), sleep disturbances that affect related neural networks may influence either the development or transmission of α-syn aggregates in the brain (Yau et al., 2018). Therefore, sleep-related disorders may be indirectly associated with risk of PD and may result from pathological mechanisms associated with sleep disturbance, with maladaptive shifts in the microbiota being both a possible product of and a risk factor for this potential pathway (e.g., positive feedback loop).

Various studies over the past few decades have observed distinct differences in microbiota composition between individuals with and without PD, as well as between individuals with different PD phenotypes of symptomology (Heintz-Buschart et al., 2018; Heinzel et al., 2021). A recent study observed differences in the microbiota (elevated *Akkermansia*, *Eggerthella*, and *Synergistetes*; depleted *Prevotella*) between A53T transgenic monkeys with early-stage PD and control monkeys (Yan et al., 2021a). These results extend upon commonly observed microbiota differences between human PD patients and healthy controls to an earlier timepoint of disease progression. Generally, studies have found that those with PD have a higher relative abundance of bacteria from the genera *Akkermansia*, *Lactobacillus*, and *Bifidobacterium,* and lower relative abundances of *Prevotella*, *Faecalibacterium*, *Bacteroidetes*, and *Blautia* genera (Keshavarzian et al., 2015; Scheperjans et al., 2015a; Li et al., 2017; Barichella et al., 2019). These differences in relative abundance have been linked to outcomes associated with the disease and could be considered dysbiosis (a deleterious microbiota composition characterized by the loss or gain of bacteria that promote health or disease, respectively (Wilkins et al., 2019) in this population. Specifically, *Lactobacillus*, *Enterococcus*, *Escherichia*, and *Proteus* genera have been positively associated, and *Blautia*, *Faecalibacterium*, and *Ruminococcus* have been negatively associated with the Unified Parkinson's Disease Rating Scale (UPDRS – a scale that measures intellectual function, mood, behavior, ability to perform activities of daily living, and motor functionality and complications), respectively (Li et al., 2017; Barichella et al., 2019). Members of the *Lachnospiraceae* family have been found to be negatively associated with postural instability and gait disturbances (Barichella et al., 2019) and those of *Enterobacteriaceae* have been positively linked to general symptom severity (Scheperjans et al., 2015a). Further, inflammatory biomarkers and SCFAs in the stool are both inversely associated with microbial alpha diversity in the gut, with some bacterial taxa being directly correlated with SCFA levels (↓SCFAs: *Akkermansia*, *Escherichia/Shigella*, *Flavonifractor*, *Intestinimonas*, *Phascolarctobacterium*, *Sporobacter*; ↑SCFAs: *Butyricicoccus*, *Clostridium* sensu stricto, *Roseburia*). However, it should be noted that these relationships are correlational and are likely influenced by a multitude of both disease-specific and non-specific internal and external factors such as medication type and dose, changes in physical activity and diet, and changes in gut transit time related to PD pathology. Additionally, it is not yet known whether these relationships are simply a product of physiological alterations, either due to changes in external factors associated with the disease (e.g., behavioral or diet changes, use of medications, etc.) or pathological disease progression, or whether they may be indicative of the microbiota being a true contributing factor of PD. Given the complex bidirectional relationship between the microbiome and host health in addition to the heterogeneity in clinical phenotypes of PD (Markello et al., 2021), is possible that microbiota dynamics are both a product PD pathology as well as potential drivers for the onset and progression of the disease.

The influence of gut microbiota on its host is determined both by composition and abundance, which are influenced by a variety of internal and external factors, including host genetics, age, dietary and lifestyle habits, and antibiotic and other medication use (Hasan and Yang, 2019). Evidence suggests that gut microbiota indicative of good health have

the ability to positively regulate neuroimmune response in the CNS, and that bacterial dysbiosis (e.g., maladaptive shifts in the microbiota composition and/or abundance) could promote a neuroinflammatory response, increasing the risk for the development of neurodegenerative diseases (Sampson and Mazmanian, 2015). Dysbiosis may lead to compromised gut barrier integrity, subsequent microbial translocation (**Figure 1C**), and disruption of the neuroendocrine system through the production of LPS and upregulation of proinflammatory cytokines (Mulak and Bonaz, 2015). Certain bacteria can specifically signal enteric dopaminergic neurons through neuronal uptake of secreted bacterial amyloids and neurotransmitters, thereby influencing intestinal mobility and secretion through indirect action on vagal afferent fibers (Bonaz et al., 2018). The presence of bacterial overgrowth and dysbiosis may be detected by increased urinary indoxyl sulfate and possibly low abundance of fecal Prevotellaceae (Cassani et al., 2015; Scheperjans et al., 2015a), a bacterial family often found to be depleted in PD and inversely correlated with motor score severity on part 3 of the UPDRS (Gerhardt and Mohajeri, 2018). However, the metabolic capabilities of *Prevotellaceae*, and which specific members of this bacterial family may play a role in PD, are not well understood.

MICROBIOTA, BARRIER PERMEABILITY, AND PARKINSON'S DISEASE PATHOLOGY

The relationship between certain gut microbiota and increased intestinal barrier permeability is noteworthy as compromised barrier integrity has recently been observed in PD patients and could potentially contribute to the misfolding of α-syn proteins (Forsyth et al., 2011; Clairembault et al., 2015). One study found increased levels of fecal calprotectin zonulin and alpha-1-antitrypsin in PD patients compared to age-matched controls, indicating increased intestinal inflammation and disrupted barrier function in this population (Schwiertz et al., 2018). In another study involving sigmoid biopsies of patients with PD, investigators found decreased expression of tight junction proteins ZO-1 and occludin, as well as irregular distribution of these proteins in the tissue biopsies, in PD patients compared to non-PD controls (Clairembault et al., 2015). To our knowledge, only four studies have investigated intestinal permeability in PD compared to matched controls, three of which used a non-invasive orally administered sugar probe as opposed to direct colonic tissue biopsies. Notably, one of these studies found intestinal permeability in PD patients to be significantly correlated with intestinal expression of α-syn, presence of *Escherichia coli* in the gut, and increased levels of LPS-binding proteins in serum (Forsyth et al., 2011). Gut microbiota not only mediate intestinal inflammation and permeability within the GI tract, but may also influence expression of α-syn within the brain due to changes in permeability of the BBB (Braniste et al., 2014; Fröhlich et al., 2016).

Despite all the evidence pointing to the potential link between gut bacteria, inflammation, and PD, the underlying molecular mechanisms remain elusive. PINK1 (kinase) mutations are implicated in PD etiology and PINK1 likely plays a role in immune function and is instrumental in mitochondrial dynamics (Gonçalves and Morais, 2021). Matheoud et al. (2016) reported that PINK1 can suppress antigen presentation derived from degraded mitochondria induced by LPS exposure. Recently, Matheoud et al. (2019) also proposed that in the absence of PINK1, mitochondrial antigen presentation resulting from intestinal infection and insult can lead to dopamine neuron dysfunction (Herrick and Tansey, 2019; Matheoud et al., 2019). These results are consistent with findings where motor deficits in PD patients worsen during peripheral infections (Umemura et al., 2014). Taken together, these results support the notion that PINK1 is a repressor of the immune system and that intestinal infection may be a trigger for PD, which supports the role of the gut–brain axis in this disease (Houser and Tansey, 2017).

POTENTIAL MECHANISMS UNDERLYING GUT–BRAIN AXIS COMMUNICATION IN PARKINSON'S DISEASE

The bidirectional pathway between the gut and the brain has been of interest for several decades and a variety of methods have been utilized to investigate this axis including infection studies, germ-free (GF) animal models, and intervention studies (e.g., prebiotics, probiotics, antibiotics) (Bravo et al., 2012). It is thought that commensal bacteria can both indirectly and directly influence PD pathology through the circulatory and nervous systems, both enteric and central. The neural communication for the GI tract involves a multi-tier network beginning with the myenteric submucosal plexus and enteric glial cells. Most notably, catecholaminergic neurons are most tightly juxtaposed to the lumen of the gut (Chesné et al., 2019). The vagal nerve directly innervates the myenteric plexus, and these neurons lead to prevertebral ganglia within the spinal cord and finally to higher brain centers. Terminals of the vagal afferent neurons, which are positioned within the gut mucosa, directly convey information to the brain and have been shown to be responsive to LPS (de La Serre et al., 2015), an endotoxin produced by Gram-negative bacteria. High levels of LPS have been shown to activate vagal afferent neurons, resulting in hypophagia (reduction in food intake and eating behavior) and weight loss – common non-motor symptoms of PD (Gakis et al., 2009). In PD, certain microbiota compositions (e.g., dysbiosis) have been found to stimulate the production of inflammatory cytokines and LPS, leading to intestinal epithelial damage and compromised barrier integrity (van IJzendoorn and Derkinderen, 2019). Increased intestinal permeability results in microbial translocation and the introduction of bacterial-derived toxins and host-derived inflammatory cytokines (TNF, IL-6, IL-1) into the blood stream (**Figure 1C**), providing an avenue for direct interaction with the nervous system mediated by compromised integrity of the BBB (Obrenovich, 2018). These cytokines have been found to be significantly elevated in the serum of individuals with PD

compared to healthy controls and may correlate with symptom severity and progression of disease (Brodacki et al., 2008; Rentzos et al., 2009; Eidson et al., 2017; Houser and Tansey, 2017; Kim et al., 2018; Rathnayake et al., 2019).

Studies involving the maturation of GF animals have provided evidence supporting the theory that the gut microbiota are essential for the appropriate development of both the ENS and CNS (Stilling et al., 2014). Further, the complete absence of bacterial colonization in the gut of these GF animals has been associated with distinct alterations in the functionality of the CNS and ENS including delayed gastric emptying, increased intestinal transit time, reduced migrating myoelectric complex periods (normal cyclic motility patterns which occur in the stomach and small intestines during periods of fasting), enlarged cecum, altered gene expression related to regulation of neurotransmitters, muscular contractile proteins, and brain-derived neurotrophic factor (BDNF) (Carabotti et al., 2015). Animals raised in GF conditions have also demonstrated increased BBB permeability through decreased expression of tight junction proteins, potentially due to a lack of bacterial-derived SCFAs, capable of improvement following colonization with commensal microbiota (Ma et al., 2019). Taken together, these studies suggest the inherent presence of a bidirectional pathway between the gut and the brain, mediated by the microbiota, is vital for proper nervous system maturation and function.

THE VAGUS NERVE AS A CONNECTION BETWEEN THE GUT AND BRAIN

The vagus nerve is a critical component in the connection between the gut and the brain, through either direct interaction with the microbiota or indirect interaction mediated through microbiota communication with enteroendocrine cells (**Figure 1B**) and subsequent signaling to afferent neurons via hormone production (e.g., cholecystokinin, 5-hydroxytryptamine, peptide YY, etc.) (Bellono et al., 2017). Several retrospective studies have explored the possible link between history of vagotomy (removal of a portion of the vagus nerve thereby severing the neural connection between the gut and the brain) and the risk for developing PD. One large retrospective cohort study involving over 14,000 previously vagotomized patients observed a decreased risk, though not reaching significance, for developing PD in patients who had a specific type of vagotomy, known as a truncal vagotomy (division of the anterior and posterior vagal trunks) (Svensson et al., 2015; Liu et al., 2017). However, another similar study including 9,430 vagotomized patients and matched controls over a cumulative 7.3 million person-year follow-up period produced conflicting results revealing no association between vagotomy and risk for PD (Liu et al., 2017). Further, other recent studies have suggested systemic brain-to-gut communication that bypasses the vagus nerve. Arotcarena et al. (2020) found evidence of either caudo-rostral or rostro-caudal spread of pathological α-syn in a non-human primate model of PD with no pathological lesions observed in the vagal nerve. An additional study using a rat model of PD found evidence of pathological changes originating from nigral overexpression of α-syn (e.g., significant neuronal loss in ileal submucosal plexus, increased glial expression in myenteric plexus, alterations in gut microbiome composition and bile acid metabolism), further supporting the brain-to-gut propagation that may be entirely independent of the vagus nerve (O'Donovan et al., 2020). Additional research is necessary to definitively characterize the potential modes of pathological α-syn propagation in PD to inform potential future screening or therapeutic options.

GUT MICROBIOTA AND EFFICACY OF IMMUNOTHERAPIES AND MEDICATIONS FOR PARKINSON'S DISEASE

While immunotherapy is now frequently used for the treatment of cancer, there has been recent interest in the potential utility of this approach for PD, and other neurodegenerative diseases known to be mediated by host immune function. Such immunotherapy targets elevation of regulatory T cell counts and upregulation of regulatory T cell function, which are both commonly significantly diminished among individuals with PD (Williams-Gray et al., 2018; Yan et al., 2021b). Sulzer et al. (2017) showed that in PD, there is T-cell recognition of defined epitopes, which are derived from α-syn, presented by certain Major Histocompatibility Complex (MHC) alleles. This is capable of driving both helper and cytotoxic T-cell responses in PD patients. Further, T cells that are specifically reactive to α-syn have been associated with preclinical and early stages of PD (Lindestam Arlehamn et al., 2020). The metabolites of certain gut bacteria, particularly butyrate, have recently been observed to improve efficacy of certain cancer therapeutics through upregulation of CD8+ T cells within the microenvironment, both *in vitro* and *in vivo* (He et al., 2021). Additionally, a recent study observed an inverse association between microbial diversity (artificially depleted with an antibiotic cocktail) and intratumor specific immune responses derived from injection with a neoantigen cancer vaccine (Lione et al., 2021). However, the mechanisms underlying the relationship between gut microbiota dynamics and adaptive immune response in animal models of disease are yet to be determined. The results of these recent studies support the hypothesis that microbiota and associated metabolites modify therapeutic efficacy and health outcomes through modulation of the host immune system.

Considering the connection between microbiota and host immune function, we conducted a study to evaluate gut microbiota dynamics associated with a novel adoptive cellular therapy intervention using α-syn-specific T cells and observed distinct clustering of the microbiota between animals in the treatment and control (injection with a novel adoptive cellular therapy or saline placebo) groups post-baseline, respectively (Klann et al., 2020). Further, specific bacterial taxa found to be differentially abundant among the animals in the treatment arm compared to the control arm, such as members of the *Odoribacter*

genus, are known to produce butyrate (SCFA known to play a role in immune modulation and gut epithelial barrier integrity). These results suggest that the gut microbiota are associated with immunotherapy in the context of PD. However, whether observed microbiota dynamics influence outcomes related to the immunotherapy or are conversely a product of the therapy and/or progression of PD pathology is not clear.

Gut microbiota have also been found to influence the ability of the host to uptake certain orally delivered pharmaceuticals due to their functional capabilities and metabolic processes. More specifically, recent research suggests that gut microbiota composition contributes to the variability of L-dopa efficacy among PD patients as certain bacterial species (*Enterococcus faecalis* and *Eggerthella lenta A2*) are known to metabolize this compound prior to crossing the BBB, rendering it inactive and ineffective (Rekdal et al., 2019). Further, common PD drugs, such as levodopa and LD-carbidopa intestinal gel, have been found to induce changes in both the composition and functional metabolic capacity of the gut microbiota (Melis et al., 2021). In a study including 107 PD patients categorized into groups by pharmaceutical regimen (levodopa: $N = 46$, LD-carbidopa intestinal gel: $N = 38$, antiparkinsonian medication naïve: $N = 23$), differences in microbiota composition were observed both between medication groups and the medication naïve group (Melis et al., 2021). Among those in the LD-carbidopa intestinal gel group, the relative abundance of *Enterobacteriaceae*, *Escherichia*, and *Serratia* was increased compared to the levodopa group, and *Proteobacteria* and *Enterobacteriaceae* were found to be in higher abundance and *Firmicutes*, *Lachnospiraceae*, and *Blautia* were found to be reduced in relative abundance compared to the naïve group. *Blautia* and *Lachnospiraceae* were also observed to be reduced among the levodopa group compared to the naïve group. Further, both medication groups were associated with a metabolic profile linked to intestinal inflammation. This evidence suggests a complex relationship between the gut microbiota and PD treatment outcomes in which the overall composition is influenced by common PD pharmaceuticals while specific taxa are also directly involved in the metabolism and uptake of these same pharmaceuticals. The microbes known to metabolize PD pharmaceuticals and decrease their efficacy may represent a promising target for methods aiming to improve patient outcomes related to certain medications. Further, consistent alterations in the gut microbiota composition in response to certain PD medications may inform the development of a potential probiotic supplement to counteract any maladaptive changes linked to worsened symptom severity or disease progression. A recent study including 197 PD cases and 130 controls revealed distinct and statistically significant microbial signatures associated with catechol-*O*-methyltransferase-inhibitors, anticholinergics, and carbidopa/levodopa medications (Hill-Burns et al., 2017). Therefore, the influence of certain medications on the commensal microbiota is also an important factor and potential confounder of the relationship between the microbiome and PD. A homogeneous medication regimen (e.g., medication type, dosage, length of time using medication, etc.) among PD patients would be ideal for valid interindividual comparisons for microbiome studies in this population. Additionally, bacteria previously determined to be associate with PD medication should be considered when characterizing changes in the microbiota related to PD pathology.

MICROBIOTA-TARGETED INTERVENTIONS FOR PARKINSON'S DISEASE

Supplementation with pre- and probiotics during adolescence could improve resiliency toward the development neurodegenerative disorders through modulation of various biomolecules known to reduce inflammation and promote neurogenesis (ferulic acid), suppression TNF production and decrease TLR signaling (histamines), reduction of reactive oxygen species accumulation, and improvement of synaptic plasticity (ghrelin) (Yahfoufi et al., 2020). In the context of PD, probiotics are thought to potentially improve symptoms through altering composition of the gut microbiota to reverse dysbiosis and disrupt pathways related to inflammation (Castelli et al., 2020) and microbial translocation or "leaky gut" (**Figure 1E**; Ghyselinck et al., 2021). Recently, it has been found that, in a *C. elegans* model of synucleinopathy, supplementation with the *Bacillus subtilis* probiotic strain PXN21 actively cleared pre-existing α-syn aggregates as well as conferred protection against the formation of additional aggregations; however, the researchers note that this protection was likely partially mediated by model-specific DAF-16 gene expression (Goya et al., 2020). While there are limited human clinical trials investigating probiotic supplementation for the treatment or management of PD, a few pilot studies have been conducted for the treatment of specific PD-related symptoms, such as constipation, in this population. Recent clinical trials suggest that probiotics can be useful in ameliorating GI issues such as constipation in patients with PD (Cassani et al., 2011; Barichella et al., 2016; Tan et al., 2021), and this is a growing area of research. One recent randomized, double-blind placebo-controlled trial of 120 participants with PD and Rome-III-confirmed constipation was conducted to assess the potential use of probiotics to improve constipation (Barichella et al., 2016). Participants consumed a fermented milk beverage, containing prebiotic fiber fructooligosaccharides (FOS) and 250 × 109 colony forming units (CFUs) of a probiotic combination (*Streptococcus salivarius* subsp. *thermophilus*, *Enterococcus faecium*, *Lactobacillus rhamnosus GG*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii* subsp. *bulgaricus*, and *B. breve and B. animalis* subsp. *lactis*), or a nutritionally similar placebo not containing the pre- and probiotic mixture, once daily for 4 weeks. The authors found that the fermented milk, fortified with pre- and probiotics, effectively increased the number of complete bowel movements, total number of bowel movements, and improved stool consistency (as measured by the Bristol Stool Scale) among individuals with PD. Participants in the treatment arm of this study also reported a decreased need for the use of laxatives, medications frequently used to decrease the severity of rigidity and increase bowel movement frequency

(Augustin et al., 2016). Another randomized double-blind placebo-controlled trial investigated the impact of probiotic supplementation on movement and metabolic parameters in PD (Tamtaji et al., 2019). In this study, participants were randomized to receive either a probiotic combination (*L. acidophilus*, *B. bifidum*, *L. reuteri*, and *L. fermentum*, each 2 × 109 CFUs) or a placebo once daily for 12 weeks. Individuals in the probiotic combination arm experienced an average reduction of approximately five points on the UPDRS compared to an average increase of approximately four points experienced by the placebo arm. However, results did not include changes in subcomponents of the MDS-UPDRS; therefore, a concise conclusion related to the decrease in motor versus non-motor symptoms cannot be determined. While the direction of the association is promising, considering the UPDRS range of 0–195 points, a five-point reduction may not equate to a clinically or biologically relevant change. However, those in the probiotic arm also demonstrated significant reductions in free radicals and oxidative stress [known risk factors for α-syn and other protein misfolding (Scudamore and Ciossek, 2018)], C-reactive protein (proinflammatory biomarker), insulin concentrations, and insulin resistance compared to those in the placebo arm. Another potential mechanism for regulating gut microbiota is through the hypothalamic–pituitary–adrenal (HPA) axis, an important pathway in the study of PD due to its involvement in immune responses to stress and inflammation. A study using GF mice found that inoculation with *Bifidobacterium infantis* effectively counteracted the stress-induced upregulation of the HPA axis (implemented through exposure to acute restraint stress), potentially through undetermined neural- or cytokine-mediated pathways (Miraglia and Colla, 2019). Such regulatory pathways highlight the potential influence of the microbiome on the modulation of stress and production of inflammatory hormones, such as cortisol. Therefore, probiotics may be a promising option to improve common symptoms of PD while also reducing levels of systemic inflammation through modulation of inflammatory pathways.

Individuals with PD also commonly experience comorbid affective disorders such as anxiety, depression, and apathy (Aminian and Strafella, 2013). While no studies specific to PD have investigated treatment of these concurrent conditions by targeting the gut–brain axis, others have been conducted for different neurological disorders. In one such study, the probiotic *Bifidobacterium breve* strain A1 was administered to patients with schizophrenia and resulted in a 25% average decrease in symptoms related to anxiety and depression (Okubo et al., 2019). Similar results were found in a study evaluating the impact of prebiotics in children with Autism Spectrum Disorder (Grimaldi et al., 2018). It is hypothesized that similar treatment methods may be beneficial in reducing psychological manifestations (e.g., depression, anxiety, apathy, psychosis, impulsive or compulsive behaviors, etc.) associated with PD. While the underlying mechanisms of this potential association are not well understood, several pathways have been proposed. Certain microbial metabolites directly interact with enteroendocrine cells (**Figure 1B**) and trigger the release of various endocrinologically active compounds including cholecystokinin and glucagon-like peptide 1, which may initiate changes in host physiology and behavior (Noonan et al., 2020). For example, glucagon-like peptide 1 has also been found to have a preventive effect on the progression of Alzheimer's disease pathology through suppression of TNF levels and protection against synaptic dysfunction related to LPS in a murine model (Kim et al., 2020). Similarly, cholecystokinin is thought to activate neurons in the hindbrain and intestinal myenteric plexus and is necessary component for long-chain fatty acids [compounds associated with gut motility in a rat model (Zhao et al., 2018)] to interact with vagal afferent nerves (Breit et al., 2018).

Other intervention methods targeting the microbiome–gut–brain axis to improve symptoms related to PD include antibiotics, fecal microbiota transplantation, and dietary interventions. While the use of antibiotics as a potential intervention is undoubtedly associated with adverse effects (e.g., propagation of antibiotic resistance, induction of further dysbiosis due to a potential decrease in microbial diversity), there has been recent interest concerning the effects of certain antibiotics, such as anti-inflammatory or antioxidant properties associated with Doxycycline (Sultan et al., 2013; Santa-Cecília et al., 2019), in neurodegenerative disorders to potentially restore balance in microbiome composition (Gazerani, 2019). Further, Minocycline has been linked to neuroprotective effects in an MPTP mouse model of PD through its ability to cross the BBB and prevent the loss of nigrostriatal dopaminergic neurons (Du et al., 2001). It has also been suggested to influence the gut–brain axis through modulation of toll-like receptor 4 (TLR4), an important transmembrane protein present in the intestinal epithelium known to be activated by LPS derived from Gram-negative bacteria (Velloso et al., 2015). Other studies have suggested that increased abundance of particular microbiota, such as *Escherichia coli* (Forsyth et al., 2011) and *Ralstonia* species (Keshavarzian et al., 2015), is associated with ENS inflammation and increased levels of endotoxins and proinflammatory cytokines (e.g., TNF, IFN-gamma, interleukins, and activation of enteric glial cells) (Yang et al., 2019), which may be diminished through the use of either antibiotics or bacteriophages.

Fecal microbiota transplantation, a technique used to transfer feces from a healthy donor to the GI tract of a recipient, has been used to restore the commensal gut microbiota and has proven effective for treating recurrent infections with *Clostridium difficile*. This technique has also been evaluated for use in the treatment of other conditions unrelated to the GI tract including multiple sclerosis (Makkawi et al., 2018) autism (Kang et al., 2019), and amyotrophic lateral sclerosis (ALS) (Mandrioli et al., 2019), and has recently been found to alleviate symptoms associated with Alzheimer's disease in APP/PS1 transgenic mice (Sun et al., 2019). Lastly, dietary and lifestyle interventions have been of interest as a potential complementary intervention for improvement of PD symptoms. Recent evidence suggests that various lifestyle factors, including caffeine consumption and following a Western diet (e.g., high caloric intake, high levels of saturated and omega-6 fatty acids, excessive salt and sugar intake, etc.), may alter the gut microbiota and therefore mediate both the risk of developing PD and the clinical progression of previously diagnosed PD (Scheperjans et al., 2015b; Jackson et al., 2019).

Another potential method of interest in preventing or delaying the onset of PD is the utilization of molecular mimicry, a concept explaining the structural similarities in secreted proteins between microorganisms and their respective hosts. Some researchers hypothesize that α-syn may be recognized as a microbe-associated molecular pattern (MAMP) mimicking bacterial amyloids as α-syn has been observed to bind to toll-like receptor 2 (TLR2; a pattern recognition receptor important for pathogen recognition) on microglia (Kim et al., 2013). Interestingly, a recent study in Thy1-αSyn mice found that gut bacterial amyloid proteins, chiefly expression by curli proteins derived from *Escherichia coli*, promote the aggregation of α-syn in both the gut and the brain, resulting in behavioral deficits, intestinal dysfunction, and motor impairments (Sampson et al., 2020). In another study, a mixture of SCFAs was injected into α-syn transgenic mice leading to neuroinflammation, damage to dopaminergic neurons, and motor dysfunction (Miraglia and Colla, 2019). These mice were then treated with minocycline, which targets TNF activation, therefore reducing aggregation of misfolded α-syn and improving motor function. Although anti-TNF drugs have been used for several decades as a treatment for inflammatory diseases such as inflammatory bowel syndrome, they have just recently been applied to neurodegenerative disorders per exploration of their potential role within the gut–brain axis (Parameswaran and Patial, 2010). Another such study among individuals with inflammatory bowel syndrome (144,018 individuals with IBD and 720,090 matched controls) reported a 78% reduction in the incidence of PD among the population when anti-TNF therapy was previously utilized to

TABLE 1 | Summary of recent pre- and probiotic studies related to Parkinson's disease in humans.

Study design	Sample size	Pre/probiotic	Frequency of use	Main findings	Author and year
Randomized controlled trial	40 PD patients in probiotic arm	65 mL fermented milk drink containing 6.5 × 109 CFU of *Lactobacillus casei* Shirota daily	Once per day for 5 weeks	↑ Number of days with normal stool consistency; ↓ number of days feeling bloated, abdominal pain, and incomplete colon emptying	Cassani et al., 2011
Double-blind, randomized, placebo-controlled trial	34 PD patients in probiotic arm; 38 PD patients in placebo arm	10 billion CFU of *Lactobacillus acidophilus*, *L. gasseri*, *L. reuteri*, *L. rhamnosus*, *Bifidobacterium bifidum*, *B. longum*, *Enterococcus faecalis*, *E. faecium*	Once per day for 4 weeks	↑ Number of spontaneous bowel movements, improvement of stool consistency and quality of life related to constipation	Tan et al., 2021
Double-blind, randomized, placebo-controlled trial	80 PD patients in pre/probiotic mixture arm; 40 PD patients in placebo arm	250×10^9 CFU of *Streptococcus salivarius* subsp. *thermophilus*, *Enterococcus faecium*, *Lactobacillus rhamnosus GG*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii* subsp. *bulgaricus*, *Bifidobacterium* (*breve* and *animalis* subsp. *lactis*)	Once per day for 4 weeks	↑ Number of complete bowel movements per week	Barichella et al., 2016
Double-blind, randomized, placebo-controlled trial	30 PD patients in probiotic arm; 30 PD patients in placebo arm	2×10^9 CFU/g each of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri*, and *Lactobacillus fermentum*	Once per day for 12 weeks	↓ MDS-UPDRS, C-reactive protein, insulin, and malondialdehyde levels; ↑ glutathione levels, insulin sensitivity	Tamtaji et al., 2019
Randomized placebo-controlled trial	40 total PD patients, all increased water (2 L/day) and fiber intake (20–25 g/day); 20 patients in trimebutine arm; 20 patients in probiotic arm	60 mg of *Lactobacillus acidophilus* and *Bifidobacterium infantis*	Trimebutine: 200 mg, 3 times per day for 3 months Probiotic mixture: 2 times per day for 3 months	Trimebutine: ↓ abdominal pain, bloating, constipation, incomplete defecation Probiotic mixture: ↓ abdominal pain and bloating	Georgescu et al., 2016
Double-blind, randomized, placebo-controlled trial	25 PD patients in probiotic arm; 25 PD patients in placebo arm	2×10^9 CFU each of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *L. reuteri*, and *Lactobacillus fermentum*	Once per day for 12 weeks	↓ Expression of IL-1, IL-8, TNF (inflammatory cytokines); ↑ expression of TGF-β and PPAR-γ (immunoregulation factors)	Borzabadi et al., 2018
Randomized placebo-controlled trial	22 PD patients in probiotic arm; 26 PD patients in placebo arm	Hexbio®: 30×10^9 CFU (107 mg each) of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Bifidobacterium infantis*, and *Bifidobacterium longum;* 2% fructo-oligosaccharide prebiotic	2 times per day for 8 weeks	↑ Average bowel opening frequency; ↓ gut transit time	Ibrahim et al., 2020

TABLE 2 | Summary of recent pre- and probiotic studies in animal models of Parkinson's disease.

Study design	Sample size	Pre/probiotic	Frequency of use	Main findings	Author and year
Randomized controlled trial	15 male C57BL/6 mice with 6-OHDA lesions; 15 male C57BL/6 mice with no lesions	270 μl of SLAB51 (*Streptococcus thermophilus*, *Bifidobacterium longum*, *B. breve*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*)	Once per day for 2 weeks	Counteraction of 6-OHDA-induced effects; ↓ neuroinflammation	Castelli et al., 2020, p. 5
Randomized placebo-controlled trial	10 MitoPark PD mice in treatment group; 10 MitoPark PD mice in placebo group	10^{10} CFU of *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Lactobacillus rhamnosus*, *Lactobacillus rhamnosus GG*, *Lactobacillus plantarum LP28*, *Lactococcus lactis* subsp. *lactis*	Once per day for 16 weeks	↑ Motor coordination, preservation of TH+ cells in SNpc; ↓ gait instability	Hsieh et al., 2020
Randomized placebo-controlled trial	10 male Wistar rats in probiotic and 6-OHDA arm; 10 male Wistar rats in 6-OHDA arm; 10 male Wistar rats in placebo/control arm;	2×10^9 CFUs each of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri*, *Lactobacillus fermentum*	Once per day for 2 weeks	↑ Rotational behavior and cognitive function; ↓ lipid peroxidation and neuronal damage	Alipour Nosrani et al., 2021
Randomized placebo-controlled trial	8 groups (7 male C57BL/6 mice each): treatment and placebo groups for each individual probiotic strain, treatment and placebo groups for 3-strain mixture	$8 \pm 2 \times 10^8$ CFU/mL each of *Lactobacillus plantarum CRL 2130*, *Streptococcus thermophilus CRL 807*, *Streptococcus thermophilus CRL 808*	Once per day for 3 weeks	↑ Motor skills, TH+ cell counts, IL-10 counts in serum/brain tissue; ↓ IL-6 and TNF counts in serum	Perez Visñuk et al., 2020
Randomized placebo-controlled trial	26 C57Bl6/J male mice in probiotic group; 26 C57Bl6/J male mice in dextran sodium sulfate group; 26 C57Bl6/J male mice in placebo group	VSL#3®: 5.4×10^9 CFU of *Streptococcus thermophilus*, *Bifidobacterium breve*, *B. lactis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, *L. helveticus*	Once per day for 4 weeks	↓ LPS- and paraquat-induced weight loss	Dwyer et al., 2021
Randomized placebo-controlled trial	6 groups (12 male C57BL/6 mice each): MPTP only, probiotic (10^7 CFU) prior to MPTP, probiotic (10^7 CFU) and MPTP simultaneously, probiotic (10^9 CFU) prior to MPTP, probiotic (10^9 CFU) and MPTP simultaneously, placebo group (no MPTP or probiotics)	10^7 or 10^9 CFUs of engineered probiotic (MG1363-pMG36e-GLP-1) that continually expresses GLP-1 (Chen et al., 2018)	Once per day for 1 week (pre- treatment groups); Once per day for 14 days (treatment for entire study period)	↓ MPTP-induced locomotor impairments, microglia and astrocyte activation, expression of inflammatory cytokines, enteric *Enterobacteriaceae*; ↑ TH+ neurons, enteric *Lactobacillus* and *Akkermansia*	Fang et al., 2019
Randomized placebo-controlled trial	5 male C57BL/6 mice in probiotic and saline group; 5 male C57BL/6 mice in probiotic and MPTP group; 5 male C57BL/6 mice in placebo and saline group; 5 male C57BL/6 mice in placebo and MPTP group;	Novarex®: 2×10^6 CFU of *Lactobacillus rhamnosus* (L-GG), *Bifidobacterium animalis* (BB-12), *Lactobacillus acidophilus* (LA-5) in a commercial mixture	Once per day for 30 days	↓ MPTP-induced neurotoxicity of dopaminergic neurons, expression of MAO B and MPP^+ in striatum, glial activation, behavioral impairments ↑ butyrate levels in brain, expression of BDNF and GDNF in SN	Srivastav et al., 2019
Randomized placebo-controlled trial	6 groups (146 male C57BL/6 mice total): placebo/saline only ($N = 36$), saline and probiotic ($N = 32$), saline and non-viable probiotic ($N = 5$), MPTP only ($N = 36$), MPTP and probiotic ($N = 32$), MPTP and non-viable probiotic ($N = 5$)	10^9 CFUs of *Bifidobacterium breve* strain A1 [MCC1274] (*B. breve* A1)	Once per day for 4 days	↑ Hippocampal synaptic plasticity, expression of postsynaptic density protein-95 and synaptophysin, CA1 spine density; ↓ expression of neuropsin (OPN5), fear response	Ishii et al., 2021
Randomized placebo-controlled trial	6 groups (35 male Sprague-Dawley rats total): non-PD and placebo only ($N = 5$), untreated PD ($N = 5$), L-DOPA treated PD ($N = 5$), probiotic treated PD ($N = 5$), nutrient media treated PD ($N = 5$), probiotic and nutrient media treated PD ($N = 5$)	10^{11} CFUs of *Lactobacillus salivarius* subsp. *salicinius* AP-32	Once per day for 8 weeks	↓ Dopaminergic neuron loss, loss of TH+ cells in striatum and SNc, weight loss ↑ locomotor speed and stride length, mitochondrial function, antioxidative enzyme activity (GPx and SOD), fecal SCFA concentration	Nurrahma et al., 2021
Randomized placebo-controlled trial	12 adult male Sprague-Dawley rats in probiotic group; 10 adult male Sprague-Dawley rats in 6-OHDA and probiotic group; 9 adult male Sprague-Dawley rats in 6-OHDA and placebo group;	10^9 CFUs of *Lactobacillus rhamnosus* HA-114	Once per day for 6 weeks	↓ Hippocampal-dependent cognitive deficits	Xie and Prasad, 2020
Randomized placebo-controlled trial	10 male C57BL/6 mice in MPTP group; 10 male C57BL/6 mice in MPTP and probiotic group; 10 male C57BL/6 mice in control/placebo group	5×10^8 CFU of *Clostridium butyricum* strain WZMC1016	Once per day for 4 weeks	↓ Motor deficits, dopaminergic neuron loss, synaptic dysfunction, microglial activation; ↑ levels of colonic GLP-1 and GPR41/43, expression of cerebral GLP-1R	Sun et al., 2021

treat symptoms related to the inflammatory bowel syndrome compared to those who were never exposed to anti-TNF therapy (Peter et al., 2018). Prevention or attenuation of inflammation in both the gut and the brain, through potential pathways, such as those outlined above, may confer protection against the development or progression of neurodegenerative disorders such as PD. Nevertheless, the currently FDA-approved anti-TNF inhibitors are also immunosuppressive which warrants caution when considering this approach in elderly populations. Results from several recent human and murine studies strongly suggest that gut microbiota may be modified, through the supplementation of certain pre- and probiotic combinations, to improve various clinical endpoints associated with PD [e.g., improvement in GI-related symptoms and motor coordination, decreased levels of inflammatory biomarkers and microglia activation, decreased MDS-UPDRS score, etc. (**Tables 1**, **2**)].

While these results are promising, further research is needed to evaluate both the feasibility and potential efficacy of utilizing long-term pre- and probiotic interventions. It is pertinent, however, to consider the potential importance of a neuroprotective microbiome across the lifespan to confer resilience against the development of future neurodegenerative disorders. Another important component for delaying onset and slowing progression of neurodegenerative disorders is early detection. The early identification of PD by specific subtype or phenotype would provide an opportunity for individualized treatment to target specific disease components, such as underlying dysbiosis of the gut microbiome. For example, a study of two Taiwanese PD populations, tremor and non-tremor dominant, found *Bacteroides* species to be more abundant in the non-tremor PD group, correlated with plasma levels of TNF-α and severity of motor symptoms defined by UPDRS part III, as well as an overall decreased abundance of *Prevotella* species in both groups of PD patients compared to non-PD controls (Lin et al., 2019). By evaluating the composition of gut microbiota in early stages of disease onset, it may be possible to differentiate between clinical phenotypes and therefore improve and expand upon current treatment strategies.

DISCUSSION AND FUTURE PERSPECTIVES

As the prevalence of PD is projected to continue to increase worldwide over the next several decades (Marras et al., 2018), it is imperative to elucidate the distinct mechanisms and pathological origins underlying this disease. There is an increased interest in understanding the potential role, if any, of the gut–brain axis in the course of disease. It is a continuously progressing field of study, which is complicated by the multitude of factors mediating the potential influence of the gut microbiome on PD including diet and lifestyle habits, levels of inflammation, presence of comorbidities, and use of supplements or medications, etc. While this avenue of research is promising, further research is needed to ascertain the role and magnitude of bidirectionality, as well as underlying mechanisms of the gut–brain axis. The evidence presented in this review suggests there may be additional pathways apart from the vagal nerve route in which α-syn aggregates may be initiated along the gut–brain axis, including immune system mediators, gut-related hormones, and microbiota-derived signaling molecules. The aggregation and propagation of enterically derived α-syn is likely indicative of an early pathological stage that may later initiate the hallmark motor and non-motor symptoms of PD. The exact role of the microbiota in α-syn-related pathology has yet to be determined, including whether enterically derived pathological α-syn is a product of early subclinical changes in brain physiology related to PD or rather deleterious shifts in the gut microbiota prior to the onset of PD pathology, or perhaps a combination of both. Further, additional research in human subjects with consistent results are needed to verify and support promising findings from murine model studies. A better understanding of the role of the microbiome–gut–brain axis in PD will inform the conception and development of novel therapeutic interventions and diagnostic tools to ultimately improve patient outcomes. Further, future research may also consider evaluating the feasibility of PD interventions targeting the gut microbiota in the context of efficacy, acceptability, and adherence.

AUTHOR CONTRIBUTIONS

VM and VV-M contributed to the conception and design of the manuscript. AR-Z, AS, MF, VV-M, and VM contributed to interpreting the relevant literature. AG compiled and summarized relevant literature. EK drafted and revised the manuscript. UD wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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The Effect of Long-Term or Repeated Use of Antibiotics in Children and Adolescents on Cognitive Impairment in Middle-Aged and Older Person(s) Adults: A Cohort Study

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Objectives: We evaluated the effects of long-term/recurrent use of antibiotics in childhood on developing cognitive impairment in middle and old age from UK Biobank Database.

Methods: UK Biobank recruited participants aged 37–73 years. Cognitive impairment was ascertained by fluid intelligence questionnaire. Primary outcome was the occurrence of cognitive impairment in middle and old age. Multivariate logistic regression models were used to explore the relationship between long-term/recurrent use of antibiotics and cognitive impairment.

Results: Over 3.8–10.8 years' follow-up, 4,781 of the 35,921 participants developed cognitive impairment. The odds of cognitive impairment in middle and old age among long-term/recurrent use of antibiotics in childhood were increased by 18% compared with their counterparts (adjusted odd ratio 1.18, 95% confidence interval 1.08–1.29, $p < 0.01$). The effect of long-term/recurrent use of antibiotics in childhood on cognitive impairment was homogeneous across different categories of various subgroup variables such as sex, age, APOE4, ethnic groups, income before tax, smoking status, alcohol status, BMI, hypertension and diabetes but the effect of long-term/recurrent use of antibiotics in childhood was modified by the educational qualification (p-value for interaction <0.05).

Conclusion: Long-term/recurrent use of antibiotics in childhood may increase the risk of cognitive impairment in middle and old age.

Keywords: antibiotics, children, adolescents, cognitive impairment, middle-aged and older person(s), cohort study

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INTRODUCTION

Cognitive impairment is the nomenclature of one or more cognitive impairment areas, ranging from complete cognition to mild cognitive impairment and finally dementia (Courtney, 2013; Ljubenkov and Geschwind, 2016; Rajji, 2018). Cognitive impairment is a serious health problem with high incidence, high disability rate and increasing global costs (Sacuiu, 2016). It is estimated that about 47 million people worldwide suffered from dementia in 2015, and the figure is expected to double every 20 years (Fiest et al., 2016; Livingston et al., 2017). This number will reach 66 million by 2030 and 115 million by 2050 (Fiest et al., 2016; Livingston et al., 2017). A growing body of evidence shows that age, cerebrovascular disease, diabetes, mental illness, etc., are the risk factors of cognitive impairment (Baumgart et al., 2015; Bellou et al., 2017).

Adolescence is a critical period of development, marking the transition from childhood to adulthood. It is in the last stage of development before adulthood that the brain is highly responsive to certain environmental cues (Paus et al., 2008). Compared with adults, the microbiota composition of adolescence is generally simpler and less stable, which is highly diverse and stable (Fouhy et al., 2012; Borre et al., 2014). These differences may be due to the relatively immature intestinal flora during adolescence, making it more susceptible to infection (Fouhy et al., 2012; McVey Neufeld et al., 2016). Therefore, significant changes in the composition of the gut microbiota may lead to the occurrence of neurodegenerative disorders (Verdu, 2006; McVey Neufeld et al., 2016). The use of antibiotics is an important reason for changes in the intestinal flora.

Antibiotics are usually used to remove or prevent bacterial colonization in the human body, and not to target specific types of bacteria (Hadar et al., 2018). Studies have found that antibiotic treatment impairs gut microbiota-brain communication and even cognitive impairment (Fröhlich et al., 2016). Slykerman et al. (2017) identified an association between antibiotic treatment in the first year of life and cognitive, behavior and emotion in childhood. However, there are limited clinical data on the effects of long-term/recurrent use of antibiotics as child or teenager (LRUAC) on cognitive impairment in middle and old age. Thus, the relationship between the LRUAC and the potential risk of developing dementia remains unknown. In the present study, we used a national population data bank in the United Kingdom to explore the associations between LRUAC and cognitive impairment in middle and old age.

MATERIALS AND METHODS

Study Design and Population

We used the UK Biobank Database to perform the population-based retrospective cohort study with a representative sample of 502,505 participants. The cohorts, with subjects aged 37–73 years, were established from 2006 to 2010 across England, Scotland and Wales. At baseline (between 2006 and 2010), a detailed assessment of their characteristics and a completed cognitive testing were made. The participants were followed until 2014–2015. The study was approved by the North-West Multicenter Research Ethics Committee. The research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and participants gave informed consent. The data were anonymized and no additional ethical approval was required for the present analyses.

Assessment of Cognitive Impairment

Fluid intelligence (FI) is defined as the ability to reason and to solve new problems independently of previously acquired knowledge, and is related to working memory (Salthouse and Pink, 2008), attention (Cochrane et al., 2019), executive functions (Lyall et al., 2016). FI is used as a tool to evaluate cognitive function (Kindermann et al., 2012; Kendall et al., 2019; Tank et al., 2022). Cognitive impairment was defined as >1 standard deviation (SD) reduction in FI score (Folley et al., 2019). FI score sums the number of correct answers out of 13 verbal and numerical reasoning items that participants were required to answer within 2 min. Baseline assessment of FI was carried out with touch-screen questionnaire in 2006–2010 in UK Biobank Assessment Centre, and follow-up assessment of FI was completed online follow-up remotely in 2014–2015. Primary outcome is the cognitive impairment in middle and old age.

Assessment of Long-Term/Recurrent Use of Antibiotics as Child or Teenager

Long-term/recurrent use of antibiotics as child or teenager was recorded as "During childhood or as a teenager did you receive long-term or recurrent courses (3 or more per year) of antibiotics (for example for tonsillitis or acne)?" The above information was collected in 2017–2018.

Assessment of Covariates

To control for confounding factors, we included the following baseline variables (2006–2010) as covariates in the analysis: age, sex, educational qualification, apoe4, ethnicity, income before tax, smoking status, alcohol status, Body Mass Index (BMI, classified as underweight: BMI ≤18.5, normal weight: BMI 18.5–24.9, overweight: BMI 25–29.9, and obesity: BMI ≥30.0). History of hypertension was originated from the question: "Has a doctor ever told you that you have had any of the following conditions?" And answers were: 1. Heart attack, 2. Angina, 3. Stroke, 4. High blood pressure, −7. None of the above, −3. "Prefer not to answer." History of diabetes was derived from the question "Has a doctor ever told you that you have diabetes?"

Statistical Analyses

Using the analysis of Chi-square tests or Fisher's exact test, we compared the proportion of cognitive impairment by sociodemographic factors (e.g., gender, age, educational qualification, ethnicity, and income level), and clinical conditions and medical histories (e.g., Apoe4, smoking history, drinking history, BMI, history of hypertension, and diabetes). Using multivariate logistic regression models, we calculated adjusted odds ratios (ORs) and 95% confidence intervals (CIs) between LRUAC and non- LRUAC controlling

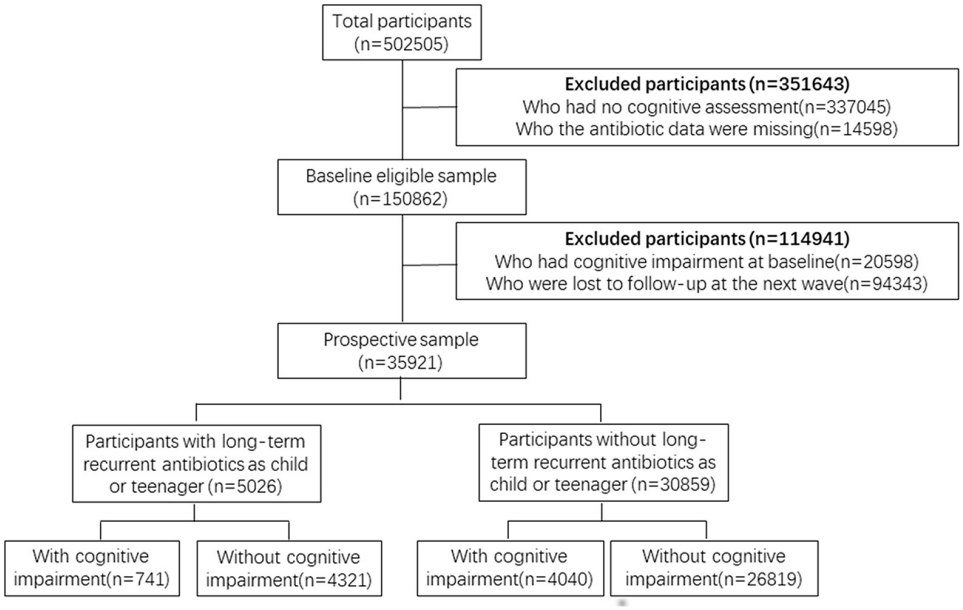

FIGURE 1 | Flow chart of participant inclusion and grouping.

for gender, age, qualification, apoe4, ethnicity, income level, smoking history, drinking history, BMI, history of hypertension, history of diabetes.

P-values less than 0.05 were considered statistically significant. Missing data in covariates were imputed using multiple imputation method (mice package, $m = 5$, method = "rf," seed = 12). Sensitivity analysis was performed based on the complete cases without multiple imputation for missing values in covariates. Analyses were conducted with RStudio (Version 1.4.1717, PBC).

RESULTS

A total of 502,505 consecutive cases from UK Biobank were evaluated. 466,584 participants were excluded from the analysis because they had no cognitive assessment, they had cognitive impairment at baseline, they were lost to follow-up at the next wave or the antibiotic data were missing. After exclusions, a total of 35,921 participants without cognitive impairment remained in the study, which included 30,859 participants without long-term recurrent antibiotics as child or teenager. During an average 5.2 years' follow-up (min 3.8, max 10.8 years), 741 (14.7%) participants with LRUAC and 4040 (13.1%) participants without LRUAC were identified cognitive impairment. **Figure 1** shows the process of participant selection.

Table 1 presented potentially modifiable risk factors for cognitive impairment, included LRUAC, female, aged, low qualification, Ethnic groups, low income before tax, current smoking, non-alcohol, higher BMI, hypertension and diabetes.

Table 2 displayed the relationship of LRUAC and cognitive impairment with logistic regression model. Compared with those without LRUAC, participants with LRUAC had a substantially higher odds for cognitive impairment by 20% (OR = 1.20; 95% CI, 1.10–1.31) after adjusting for Model 1 (age, sex, educational qualification, apoe4, and ethnic). The increased odds was slightly attenuated to 18% (OR = 1.18, 95% CI 1.08∼1.29) after adjusting for Model 2 (Model 1 plus income before tax, smoking status, alcohol status, BMI, hypertension, diabetes). The OR was 1.24 (95% CI 1.09–1.41) based on the complete cases without multiple imputation for missing values (Model 3) (**Table 2**, left). Other than that, after adjusting for Model 2, 60–70 years old, black, Asian, other ethnic group, current smoking increased the risk for cognitive impairment. While male, higher qualification and income, previous alcohol reduced the risk for cognitive impairment.

We also evaluated the risk of cognitive impairment after LRUAC in each variable subgroup and found that the effect of long-term/recurrent use of antibiotics in childhood on cognitive impairment was homogeneous across different categories of subgroup variables such as sex, age, APOE4, ethnic groups, income before tax, smoking status, alcohol status, BMI, hypertension and diabetes but the effect of LRUAC was modified by higher qualification (p-value for interaction <0.05, **Table 3**), with the decreased effect of LRUAC on cognitive impairment being found only among participants with a higher qualification of College or University degree; NVQ or HND or HNC or equivalent.

DISCUSSION

In this population-based study, we found LRUAC was associated with higher risk of cognition impairment in middle and

TABLE 1 | Demographic and clinical characteristics of patients at baseline.

Characteristics		Overall population (*n* = 35921)	Cognitive impairment		P
			yes	no	
LRUAC,%	Yes	35921	741 (14.6)	4321 (85.4)	**
	No		4040 (13.1)	26819 (86.9)	
Sex,%	Female	35921	2750 (13.6)	17462 (86.4)	
	Male		2031 (12.9)	13678 (87.1)	
Age,%	<60	35921	2434 (11.7)	18398 (88.3)	***
	60–70		2329 (15.5)	12665 (84.5)	
	> = 70		18 (18.9)	77 (81.1)	
Qualification,%	None of the below	35838	649 (37.2)	1098 (62.8)	***
	A levels/AS levels or equivalent; O levels/GCSEs or equivalent; CSEs or equivalent; Other professional qualifications		2759 (11.6)	21052 (88.4)	
	College or University degree; NVQ or HND or HNC or equivalent		1348 (13.1)	8932 (86.9)	
Apoe4,%	Yes	29926	1113 (13.5)	7130 (86.5)	–
	No		2826 (13.2)	18821 (86.8)	
Ethnic groups,%	White	35921	4421 (12.8)	30231 (87.2)	**
	Black		131 (26.3)	368 (73.7)	
	Asian		155 (34.1)	299 (65.9)	
	Other ethnic group		74 (23.4)	242 (76.6)	
Income before tax,%	Less than 18,000	32862	878 (21.5)	3201 (78.5)	***
	18,000 –51,999		2327 (13.8)	14580 (86.2)	
	52,000–100,000		788 (8.9)	8049 (91.1)	
	Greater than 100,000		223 (7.3)	2816 (92.7)	
Smoking status,%	Current	35868	381 (16.3)	1954 (83.7)	***
	Previous		1759 (13.9)	10897 (86.1)	
	Non		2635 (12.6)	18242 (87.4)	
Alcohol status,%	Current	35910	4468 (13.1)	29541 (86.9)	***
	Previous		126 (13.5)	813 (86.5)	
	Non		184 (19.2)	778 (80.8)	
BMI,%	Obesity	35261	1033 (15.1)	5828 (84.9)	***
	Overweight		1962 (13.5)	12567 (86.5)	
	Normal weight		1674 (12.3)	11982 (87.7)	
	Underweight		27 (12.6)	188 (87.4)	
Hypertension,%	Yes	35883	1219 (15.4)	6704 (84.6)	***
	No		3559 (12.7)	24401 (87.3)	
Diabetes,%	Yes	35882	203 (17.6)	951 (82.4)	***
	No		4569 (13.2)	30159 (86.8)	
Follow-up time (y)		35921	5.19 ± 1.02	5.29 ± 1.14	***

*LRUAC: long-term/recurrent use of antibiotics in childhood. ⁻P > 0.05, **P < 0.01, and *** P < 0.001.*

old age, independently of age, sex, educational qualification, apoe4, ethnic, income before tax, smoking status, alcohol status, Body Mass Index (BMI), history of hypertension, and history of diabetes.

Antibiotics changed the world and saved the lives of many patients who would have died of infection (Hadar et al., 2018). In fact, more than 10% of European children use antibiotics each year (Sturkenboom et al., 2008). In the United States, antibiotics account for 25% of all prescriptions for pediatricians (Finkelstein et al., 2003). However, a large body of evidence has begun to suggest that such frequent use of antibiotics can also come at a price. There is indeed evidence that antibiotic treatment has significantly changed the microflora composition of adults and infants (Francino, 2015; Azad et al., 2016; Yassour et al., 2016). Even short-term antibiotic treatment can have a long-term effect on the composition of microflora (Isaac et al., 2017). Stable microflora composition plays a role in regulating the immune system, hormone secretion and response, and metabolism (Spor et al., 2011; Neuman et al., 2015; Blaser, 2016; Shamriz et al., 2016). Therefore, changes in the composition of microflora caused by antibiotics may lead to disease. Exposure to antibiotics in young children is associated with an increased risk of excessive weight gain, asthma, allergies and autoimmune diseases, such as inflammatory bowel disease (IBD) (Cho et al., 2012; Cox et al., 2014; Bokulich et al., 2016; Korpela et al., 2016; Turta and Rautava, 2016). The impact of antibiotic use on future

TABLE 2 | Association between long-term/recurrent use of antibiotics and cognitive impairment and fluid intelligence (FI) difference.

	Cognitive impairment								
	Model 1 (*n* = 35921)			Model 2 (*n* = 35921)			Model 3 (*n* = 35921)		
	OR	95%CI	P	OR	95%CI	P	OR	95%CI	P
LRUAC (vs no)	1.20	1.10,1.31	**	1.18	1.08,1.29	**	1.24	1.09,1.41	**
Gender (vs. female)	0.93	0.87,0.98	*	0.89	0.83,0.95	***	0.94	0.87,1.02	
Age (year) (vs.<60)									
60–70	1.48	1.39,1.57	***	1.28	1.20,1.37	***	1.08	1.00,1.17	
> = 70	1.89	1.12,3.16	*	1.50	0.88,2.54		1.44	0.75,2.58	
Apoe4 (vs no)	1.02	0.95,1.10		1.03	0.95,1.11		1.05	0.96,1.14	
Ethnic groups (vs White)									
Black	2.68	2.18,3.28	***	2.69	2.19,3.30	***	2.53	1.99,3.22	***
Asian	3.90	3.20,4.76	***	4.05	3.30,4.96	***	4.10	3.22,5.21	***
Other ethnic group	2.17	1.67,2.82	***	2.17	1.66,2.83	***	2.35	1.71,3.26	***
Qualification (vs None of the below)									
A levels/AS levels or equivalent; O levels/GCSEs or equivalent; CSEs or equivalent; Other professional qualifications				0.26	0.24,0.30	***	0.31	0.27,0.36	***
College or University degree; NVQ or HND or HNC or equivalent				0.23	0.21,0.26	***	0.28	0.25,0.33	***
Income before tax (vs Less than 18,000)									
18,000–51,999				0.68	0.62,0.75	***	0.67	0.61,0.74	***
52,000–100,000				0.46	0.41,0.52	***	0.45	0.39,0.51	***
Greater than 100,000				0.38	0.32,0.44	***	0.35	0.29,0.42	***
Smoking status (vs Non)									
Current				1.26	1.12,1.43	***	1.21	1.04,1.40	***
Previous				1.06	0.99,1.13		1.07	0.98,1.15	
Alcohol status (vs Non)									
Current				0.88	0.74,1.05		0.89	0.72,1.11	
Previous				0.75	0.58,0.97	*	0.77	0.56,1.04	
BMI (vs Underweight)									
Obesity				1.17	0.77,1.78		1.17	0.73,2.01	
Overweight				1.11	0.73,1.69		1.20	0.74,2.05	
Normal weight				1.06	0.69,1.60		1.28	0.79,2.20	
Hypertension (vs no)				1.07	0.99,1.16		1.10	1.01,1.21	*
Diabetes (vs no)				1.32	0.95,1.32		1.05	0.86,1.28	

*Analyses were adjusted for Model 1 (age, sex, qualification, apoe4, and ethnic groups); Model 2 (Model 1 plus income before tax, smoking status, alcohol status, BMI, hypertension, and diabetes); Model 3 (Model 2 based on completed cases without multiple imputation for missing values). LRUAC: long-term/recurrent use of antibiotics in childhood. *P < 0.05, **P < 0.01, and ***P < 0.001.*

health and disease has caused widespread concern (Diamond et al., 2011; Diaz Heijtz et al., 2011).

Although no direct clinical studies prove that antibiotics are associated with cognition impairment, there are several cohort studies reveal effects of antibiotics on central nervous system diseases. Antibiotic exposure in the first 2 years of life increase risk of attention-deficit/hyperactivity disorder (ADHD) with average 8.8 years follow-up (Aversa et al., 2021). Even prenatal antibiotic exposure is associated with increased risk of ADHD (Hamad et al., 2020), cerebral palsy or epilepsy (Meeraus et al., 2015) in the child. Quinolone or d cephalosporins increase stroke risk slightly in older person(s) in 2 years (Luchsinger et al., 2001). In this study, we found LRUAC's effect on cognition impairment more than 30 years later in middle and old age. These studies suggest that antibiotics could cause long-term brain damage. In this study, age, sex, apoe4, income before tax, smoking status, alcohol status, BMI, hypertension, and diabetes had no interaction with LRUAC but qualification (college or university degree; NVQ or HND or HNC or equivalent) had a quantitative interaction with LRUAC on cognitive impairment: the effect of LRUAC on cognitive impairment diminished in participants with high qualification, which is consistent with a previous study (Ko et al., 2021).

There are some animal studies exhibiting the effects of antibiotics on cognition and the mechanics in the brain. Lach's study shows that microbiota depletion with antibiotic treatment during the adolescent period cause enduring neurobehavioral disfunction, with obvious change of gene expression in the amygdala (Lach et al., 2020). Study by Wang et al. (2015) showed that rats taking ampicillin increased serum corticosterone, increased anxiety-like behavior, and impaired spatial memory. Further support to this notion is the fact that antibiotics,

TABLE 3 | Long-term/recurrent use of antibiotics and risk of cognitive impairment: Subgroup analysis.

	Number of subjects	OR,95%CI	P	OR,95%CI from interaction	P for interaction
Sex					
Female	20212	1.26,1.13–1.40	**	0.84,0.69–1.02	
Male	15709	1.04,0.88–1.22		1	
Age					
<60	20832	1.19,1.07–1.33	***	1	
60–70	14994	1.16,1.00–1.34	*	0.93,0.76–1.13	
> = 70	95	1.32,0.11–14.8		0.93,0.14–5.94	
Qualification					
None of the below	1747	1.62,1.19–2.19	**	1	
A levels/AS levels or equivalent; O levels/GCSEs or equivalent; CSEs or equivalent; Other professional qualifications	23811	1.16,1.04–1.30	**	0.73,0.53–1.03	
College or University degree; NVQ or HND or HNC or equivalent	10280	1.11,0.94–1.31		0.69,0.48–0.98	*
Apoe4					
No	26068	1.21,1.09–1.35	***	1	
Yes	9853	1.10,0.90–1.35		0.90,0.71–1.14	
Ethnic groups					
White	34652	1.20,1.10–1.31	***	1	
Black	499	0.83,0.46–1.50		0.78,0.44–1.38	
Asian	454	0.89,0.45–1.77		0.67,0.34–1.31	
Others	316	1.32,0.60–2.86		1.27,0.61–2.63	
Income before tax					
Less than 18,000	4525	1.34,1.07–1.68	*	1	
18,000–51,999	18568	1.10,0.96–1.26		0.85,0.65–1.12	
52,000–100,000	9555	1.16,0.94–1.43		0.91,0.68–1.23	
Greater than 100,000	3273	1.66,1.15–2.40	**	1.22,0.78–1.92	
Smoking status					
Non	20901	1.20,1.06–1.35	**	1	
Previous	12681	1.20,1.04–1.40	*	0.99,0.82–1.20	
Current	2339	0.99,0.72–1.36		0.86,0.61–1.21	
Alcohol status					
Non	963	1.23,0.76–1.99		1	
Previous	940	0.80,0.47–1.37		0.69,0.34–1.41	
Current	34018	1.20,1.09–1.31	***	0.95,0.58–1.56	
BMI					
Obesity	6979	1.20,1.01–1.44	*	1	
Overweight	14829	1.17,1.01–1.36	*	0.84,0.69–1.02	
Normal weight	13896	1.18,1.02–1.37	*	0.84,0.69–1.02	
Underweight	217	1.41,0.37–5.31		0.84,0.69–1.02	
Hypertension					
No	27991	1.21,1.10–1.34	***	1	
Yes	7930	1.09,0.91–1.31	0.43	0.95,0.76–1.17	
Diabetes					
No	34762	1.20,1.10–1.31	***	1	
Yes	1159	0.74,0.44–1.25		0.61,0.36–1.03	

*Analyses were adjusted for age, sex, qualification, apoe4, ethnic group, income before tax, smoking status, alcohol status, BMI, hypertension, and diabetes. *P < 0.05, **P < 0.01, and ***P < 0.001.*

such as streptozotocin, have been used to induce sporadic AD forms in animal models, thereby affecting learning and memory performance (Cattaneo et al., 2017). Antibiotic treatment from early adolescence significantly decreased hippocampal BDNF and enhanced learning and memory impairments in a mouse model of multiple sclerosis (Zeraati et al., 2019), and prevented the development of anxiety- and depression-related behaviors, oxidative stress and hypothalamic-pituitary-adrenal axis dysregulation in AD mice (Mosaferi et al., 2021). The evidence suggests that the use of antibiotic cocktails in adolescent

mice can lead to permanent changes in intestinal microflora and an increase in pro-inflammatory cytokines (Desbonnet et al., 2014, 2015; Cattaneo et al., 2017). In humans, some antibiotics, cefepime, can cross the blood-brain barrier, leading to changes in mental state, loss of consciousness, myoclonus and insanity (Payne et al., 2017). fMRI study also shows that antibiotic influences insular cortex functional connectivity that could involve cognitive flexibility and memory processing (Sometti et al., 2021).

However, controversial results have been obtained in some clinical and animal trials. Studies have shown that antibiotics also have beneficial effects on Alzheimer's disease. Through the triple eradication of antibiotics (omeprazole, clarithromycin, and amoxicillin) to eliminate Helicobacter pylori and other pathogens, the cognitive function parameters of patients with AD were positively improved (Kountouras et al., 2009). The possible mechanism is that some antibiotics can have a beneficial effect on Alzheimer's disease by reducing neuroinflammation caused by biological disorders. Antibiotics have been shown to reduce inflammation and improve cognitive impairment in AD animal models. The use of rifampicin in the animal model of AD can reduce the levels of A β and inflammatory cytokines in the brain (Yulug et al., 2018). Minocycline has a similar effect on Aβ and reduces the activation of microglia in rodent AD model (Budni et al., 2016). In 2004, patients with suspected Alzheimer's disease and mild to moderate dementia who took a combination of doxycycline and rifampicin significantly improved the cognitive subscale (SADAScog) of the Standard Alzheimer's Disease Assessment scale at 6 months of age (Loeb et al., 2004). In contrast, in 2013, a multicenter, double-blind, randomized, 2 × 2 factor-controlled trial of mild to moderate AD patients showed no significant effect on cognitive ability after 12 months of treatment with doxycycline or rifampicin alone or in combination (Molloy et al., 2013). Similarly, in 1999, d-cycloserine was found to effectively improve cognitive impairment in patients with AD (Tsai et al., 1999), but these positive effects were not found in subsequent cochrane review (Laake and Oeksengaard, 2002).

The strengths of this study are the large sample size, the cohort study with long follow-up time, and the robust result for the effect of LRUAC on cognitive impairment. Also, the study has some limitations. Firstly, the records of LRUAC were collected during 2017–2018, which may be subject to recall bias. The cognition normal participants were more likely to recall they did not expose to LRUAC, which might exaggerate the effect of LRUAC. Secondly, the FI score was collected from different ways: basic collection in UK Biobank Assessment Centre during 2006–2010 and follow-up collection online remotely during 2014–2015, which would cause measurement bias toward the score. Lastly, this was an observational study and the observed results may still be subject to possible unobserved confounding factors.

In this population-based retrospective cohort study, we observed the impact of the LRUAC on the development of the cognitive impairment. We hope that our results could provide useful clues for assessing the lifelong effects of inappropriate use of antibiotics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the North-West Multi-center Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZLiu: full access to the UK Biobank data and statistical analysis. HZ and DW: concept and design. ZLiu, SW, XC, and LL: drafting of the manuscript. All authors acquisition, analysis, or interpretation of data and critical revision of the manuscript for important intellectual content.

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REVIEW
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Enteric Nervous System: The Bridge Between the Gut Microbiota and Neurological Disorders

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The gastrointestinal (GI) tract plays an essential role in food digestion, absorption, and the mucosal immune system; it is also inhabited by a huge range of microbes. The GI tract is densely innervated by a network of 200–600 million neurons that comprise the enteric nervous system (ENS). This system cooperates with intestinal microbes, the intestinal immune system, and endocrine systems; it forms a complex network that is required to maintain a stable intestinal microenvironment. Understanding how gut microbes influence the ENS and central nervous system (CNS) has been a significant research subject over the past decade. Moreover, accumulating evidence from animal and clinical studies has revealed that gut microbiota play important roles in various neurological diseases. However, the causal relationship between microbial changes and neurological disorders currently remains unproven. This review aims to summarize the possible contributions of GI microbiota to the ENS and CNS. It also provides new insights into furthering our current understanding of neurological disorders.

Keywords: gastrointestinal microbiota, enteric nervous system, central nervous system, neurological disorders, microbiota-gut-brain axis

Abbreviations: 5-HT, 5-hydroxytryptamine (or serotonin); AChE, acetylcholine-esterase; AD, Alzheimer's disease; Adrb2, encoding b2 adrenergic receptors [AR]; AHR ligands, hydrocarbon receptor ligands; ALS, amyotrophic lateral sclerosis; ANS, autonomic nervous system; ASD, autism spectrum disorder; BBB, blood brain barrier; BMP2, bone morphogenetic protein 2; BMPR, BMP receptor; CagA, cytotoxin -associated gene A; CD, Crohn disease; CNS, central nervous system; CSF1, colony stimulatory factor1; DCs, dendritic cells; DRG, dorsal root ganglia; EA, esophageal achalasia; ECCs, enterochromaffin cells; EGCs, enteric glial cells; ENS, enteric nervous system; FD, functional dyspepsia; FFAR2 (GPR43), free fatty acid receptor 2; FFAR3 (GPR41), free fatty acid receptor 3; FGIDs, functional gastrointestinal disorders; FMT, fecal microbiota transplantation; GBS, Guillain-Barré syndrome; GDNF, glial cell-derived neurotrophic factor; GF, germfree; GI, gastrointestinal; GIP, gastric inhibitory polypeptide; GLP1, glucagon-like peptide-1; GPBAR1, protein-coupled bile acid receptor 1 (TGR5); *H. pylori*, *Helicobacter pylori*; HAEC, Hirschsprung-associated enterocolitis; HBP, D-glycero-β-D-manno-heptose-1,7-biphosphate; HSCR, Hirschsprung disease; HDACs, histone deacetylases; HPA axis, hypothalamic-pituitary-adrenal axis; IBS, irritable bowel syndrome; ICCs, interstitial cells of Cajal; IECs, intestinal epithelial cells; IPANs, intrinsic primary afferent neurons; LpMs, lamina propria macrophages; LPS, lipopolysaccharide; MAMPs, microbe-associated molecular patterns; MG, myasthenia gravis; MGB axis, microbiota-gut-brain axis; MMs, muscularis macrophages; MS, multiple sclerosis; MVs, microvesicles; MyD88, myeloid differentiation primary response 88; NE, norepinephrine; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; PD, Parkinson's disease; PGN, peptidoglycan; PN, peripheral neuropathy; PNS, peripheral nervous system; PRRs, pattern-recognition receptors; PSA, polysaccharide A; PYY, peptide YY; RSD, resistant starch diet; SERT, serotonin-selective reuptake transporter; SP, substance P; TGFβ1, transforming growth factor β1; TLRs, toll-like receptors; TPH1, tryptophan hydroxylase 1; UC, ulcerative colitis; VacA, vacuolating cytotoxin A; VZV, varicella-zoster virus.

INTRODUCTION

Incidences of neurological disorders gradually increase with age in humans, accompanied by poor prognoses and associated social burdens. Drokhlyansky et al. (2020) found that disease-related genes were dysregulated with aging, and that the enteric nervous system (ENS) expressed risk genes for neuropathic, inflammatory, and extraintestinal diseases. This suggests that there are neuronal contributions to such diseases (Drokhlyansky et al., 2020). The microbiota-gut-brain (MGB) axis provides a new understanding of the essence of these diseases; however, to date research has been limited to analysis of the correlations between microbiota and different mechanisms; it lacks support from more favorable evidence.

The gastrointestinal (GI) tract harbors a considerable number of commensal microorganisms, including bacteria, viruses, fungi, and protozoans, which frequently vary depending on the host's diet, drug intervention, and diseases. The bacteria in the GI tract are relatively stable; they comprise four main phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. The ENS can be reasonably regarded as the bridge between the intestinal microbiota and the nervous system. The ENS is considered to be a separate branch of the autonomic nervous system (ANS); it is located throughout the length of the GI tract and is arranged into two ganglionated plexuses: the submucosal plexus (Meissner's plexus) and the myenteric plexus (Auerbach's Plexus), which comprise nitrergic and cholinergic enteric neurons. The ENS also hosts an abundance of calretinin- or neuropeptide-expressing neurons, as well as catecholaminergic and inhibitory gamma-aminobutyric acid (GABA) neurons (Qu et al., 2008). The ENS forms a complete sensorimotor reflex circuit consisting of intrinsic primary afferent neurons (IPANs), interneurons, and motor neurons within the gut wall; this circuit is responsible for the coordination of gut functions such as motility, peristalsis, and intestinal mucosal immunity (Wehrwein et al., 2016; Allaire et al., 2018). Interestingly, Smith-Edwards et al. (2021) found that sympathetic neurons can regulate the activity of neurons and non-neuronal cells differentially in the proximal and distal colon, thereby promoting distinct changes in motility patterns.

Given the proximity of the GI microbiota to the ENS, gut microbes can influence its development and function—either directly or indirectly. These complex interactions involve signaling that is initiated by microbe- or host-derived components or metabolites; they eventually affect enteric nervous excitability and GI function.

The cell bodies of the sensory vagal fibers that relay sensory information from the ENS to the central nervous system (CNS) reside in the nodose/jugular ganglia and the dorsal root ganglia (DRG). These vagal fibers mostly synapse bilaterally on the nucleus tractus solitarius (NTS). The individual components of the MGB axis communicate with each other bi-directionally (either antagonistically or synergistically) within the ANS. The ANS, in combination with the hypothalamic-pituitary-adrenal (HPA) axis and neuroendocrine signaling, can induce CNS-modulated changes in the gut (Mayer et al., 2015). The enteric immune and enteric endocrine systems also influence ENS activity and neurochemistry.

The GI tract incessantly monitors the dynamic intestinal microenvironment, where enteroendocrine and immune cells can facilitate signaling from the gut lumen to the ENS. During this process, neuropods have been proposed to transduce signals from enteroendocrine cells to sensory neurons (Bohórquez et al., 2015). Enteric neurons, mucosal immune cells [e.g., muscularis macrophages (MMs)], 5-hydroxytryptamine (5-HT) from enteroendocrine cells, molecules from gut microbes [e.g., lipopolysaccharides (LPSs) and short chain fatty acids (SCFAs)], and diet ingredients, all contribute to gut sensation, secretion, and motility.

As a bidirectional link, the ENS connects the GI microbiota with many of the host's systems. It is widely believed that the gut microbiota are critically important for the appropriate development and maintenance of ENS function. Given that disorders of the ENS correlate with CNS diseases, exploring the mechanisms of gut microbe-ENS interactions could provide a new perspective for furthering our understanding of neurological disorders; it could also help develop novel therapeutic strategies.

INFLUENCE OF GI MICROBIOTA ON THE ENS

The gut exposes the host to the external environment; it has a mucosal surface of approximately 32 m^2 (Helander and Fändriks, 2014) and interfaces intimately with commensal bacteria and the molecules they produce, including microbe-derived components and metabolites such as microbe-associated molecular patterns (MAMPs), neurotransmitters, and hormones. A general reduction in the myenteric plexus density has been observed in both the jejunum and the ileum of postnatal germ-free (GF) mice, but not in the duodenum; this suggests that early exposure to gut microbes is essential for the postnatal development of the ENS (Collins et al., 2014).

Direct Interactions Between GI Microbiota and the ENS

Most commensal microbes are separated from the gut epithelial surface by a layer of mucin; hence, they are unlikely to directly interact with the GI tissue. Furthermore, very few gut bacteria directly contact the epithelium or live in the adherent mucus layer (Derrien et al., 2011; Johansson et al., 2011). Therefore, there are several methods of communication between bacteria and their hosts, including the production of molecules as a consequence of the degradation of microbiota constituents and the synthesis and secretion of molecules by bacteria.

Microbe-Derived Components

Microbe-associated molecular patterns are molecular signatures that are highly conserved across whole classes of microbes but are absent in the host, such as LPS or polysaccharide A (PSA) on the surface of gram-negative bacteria or peptidoglycan (PGN) on the surface of gram-positive bacteria (Medzhitov, 2007; Boller and Felix, 2009). Each MAMP is detected by specific surface-localized

receptors that are termed pattern recognition receptors (PRRs), such as transmembrane, surface, or endosome toll-like receptors (TLRs); they are expressed in myenteric neurons, enteric glial cells (EGCs), and innate immune cells.

TLR3 and TLR7, which recognize viral RNA, and TLR4, which recognizes LPS, have been demonstrated in the ENS of the murine intestine and human ileum, both of which innervate Peyer's patches. Moreover, TLR4 is abundantly expressed in the murine distal large bowel (Barajon et al., 2009; Boller and Felix, 2009), while TLR4–/– mice are characterized by a reduced number of nitrergic inhibitory neurons and abnormal intestinal motility. These characteristics have also been observed in GF mice, antibiotics-treated mice, and mice with the ENS-specific deletion of myeloid differentiation primary response 88 (MyD88) (Anitha et al., 2012; Hung et al., 2019, 2020). MyD88, as an adaptor molecule, is essential for TLR-mediated signal transduction. TLR4 is also expressed in enteric stem cell niches, and intense interplay has been observed between LPS and Wnt signaling during enteric stem cell regulation (Schuster et al., 2014; Di Liddo et al., 2015). Therefore, the LPS-mediated activation of TLR4 appears to promote the survival of enteric neurons and regulate gut motility (Anitha et al., 2012), suggesting that the TLR4 pathway plays a critical role in the development of ENS.

TLR2 is expressed in the enteric neurons, EGCs, and smooth muscle cells of the intestinal wall; it detects gram-negative bacterial PSA and gram-positive bacterial PGN and lipoprotein. A phenotype similar to that observed in TLR4–/– mice, the reduction of nitrergic neurons, and acetylcholine-esterase (AChE) fibers in the myenteric ganglia have all been observed in TLR2–/– mice, accompanied by gut dysmotility (Brun et al., 2013). Carbachol has been shown to induce a strong tetrodotoxin-dependent chloride secretion, as shown by short-circuit current increment in wild type (WT) mice, but not in TLR2–/– mice (Brun et al., 2013). Furthermore, TLR2 signaling has been demonstrated to affect the composition of neurons in the ENS and intestinal contractility (Brun et al., 2013).

These observations show that TLR pathways allow microbes to access the ENS. Thus, bacterial or viral components may directly activate the ENS. Nevertheless, the mechanisms by which TLR ligands control enteric neurogenesis require further investigation.

Microbe-Derived Metabolites

The most extensively studied gut microbe-derived metabolites are SCFAs, which are bacterial fermentation products that originate from starch and fiber; more than 95% of SCFAs consist of acetate, propionate, and butyrate. A wide range of pre-clinical evidence supports the involvement of SCFAs as modulators of not only colonic function but also multiple inflammatory and metabolic processes (Erny et al., 2017; Gill et al., 2018; Krautkramer et al., 2020). SCFAs also majorly contribute directly to the integrity of the intestinal epithelial barrier by regulating mucus production and the expression of tight junction proteins. Therefore, SCFAs have been implicated as the first line of defense between gut microbes and the permeability of the host's intestinal barrier.

Short chain fatty acids can activate several G protein-coupled receptors (GPCRs), of which free fatty acid receptors 2 and 3 (FFAR2 and FFAR3, also referred to as GPR43 and GPR41, respectively) have been studied the most extensively. SCFAs can activate the peripheral nervous system (PNS), in which FFAR3 has been detected in the enteric neural plexus, autonomic and somatic sensory ganglia, and portal nerve (Nøhr et al., 2015; Kaji et al., 2016). In the gut, FFAR3 is expressed on nitrergic and cholinergic enteric neurons.

All SCFAs inhibit the activity of histone deacetylases (HDACs); butyrate has been reported to present the most potent activity (Cleophas et al., 2016). Furthermore, acetate can be converted to acetyl-CoA, which increases the acetylation of histone (Soliman et al., 2012). SCFAs have also been shown to influence the neurochemical phenotype of the ENS of an adult rat. Butyrate can increase the proportion of myenteric cholinergic neurons by inhibiting HDACs but not that of nitrergic neurons. Both the application of butyrate and the adoption of a resistant starch diet (RSD) have been found to increase the colonic concentrations of butyrate and enhance gut motility (Soret et al., 2010). Therefore, SCFAs might be involved in maintaining ENS homeostasis.

Polyamines are metabolites of gut microbes that play essential roles in stress responses, inflammation, and neuronal signaling in the CNS and ENS. Moreover, polyamines are required for the expression of TLR2; hence, they modulate the integrity of the intestinal epithelial barrier (Chen et al., 2007).

Microbe-Derived Neurochemicals

Microbial species can produce an array of neurotransmitters, such as GABA, norepinephrine (NE), 5-HT, dopamine, and acetylcholine. They can target intestinal and extraintestinal immune/neuronal elements. Therefore, the study of microbe-derived neurochemicals, a field referred to as "Microbial endocrinology" (Lyte, 2011, 2014), is becoming essential for advancing our knowledge of interactions between gut microbes and the ENS. This suggests that the bacterial production of neurotransmitters may be a primary molecular mechanism of bacteria-neuron communication.

Indirect Interactions Between GI Microbiota and the ENS

Bacterial products such as MAMPs, SCFAs, and microvesicles (MVs) interact with the enteric immune system, enteric epithelium, smooth muscle, and endocrine system. All of these systems contribute to the maintenance of intestinal immune innate tolerance, the development of the adaptive immune system, and the homeostasis of the intestinal microenvironment. In turn, molecular mediators that are secreted by gut-resident immune cells and enteroendocrine cells in the intestinal epithelium can be detected by corresponding receptors in the ENS; they can also affect enteric function (Kamada et al., 2013; La Fata et al., 2018). Moreover, intestinal stromal cells, such as interstitial cells of Cajal (ICCs) and EGCs, might participate in the interactions between gut microbes and the ENS.

Enteric Immune System

Gastrointestinal microorganisms activate resident immune cells within the gut; they may signal to enteric neurons through immune mediators (Maynard et al., 2012; Sampson and Mazmanian, 2015; Rooks and Garrett, 2016). Indeed, the dysbiosis of intestinal flora, uncontrolled immune responses to pathogenic stimuli, and adaptive changes in the ENS represent the main factors in the pathogenesis of several GI disorders (Vindigni et al., 2016; Zhang et al., 2017).

A distinct population of macrophages that is distributed throughout the intestinal muscularis externa regulates the peristaltic activity of the colon under a steady state. MMs display a tissue-protective phenotype, whereas lamina propria macrophages (LpMs) preferentially express a pro-inflammatory phenotype. MMs express high levels of Adrb2 [Adrenoceptor Beta 2, which encodes b2 adrenergic receptors (ARs)], which is the most significantly differentially expressed gene between MM and LpM populations (Chang et al., 2014). Adrb2 is essential for NE signaling and MMs reside close to enteric neurons labeled with the calcium indicator GCaMP3. MMs have been suggested to interact with active neurons in the gut muscularis. MMs change the patterns of smooth muscle contractions by secreting bone morphogenetic protein 2 (BMP2), which activates the BMP receptor (BMPR) that is expressed by enteric neurons. In turn, enteric neurons secrete colony stimulatory factor1 (CSF1), which is required for the development of MMs as a growth factor. Finally, LPS from gut microbes regulate the expression of BMP2 by MMs and the expression of CSF1 by enteric neurons. Therefore, the cross-talk between the ENS and the muscular macrophages is dependent on LPS from intestinal bacteria (Muller et al., 2014; Gabanyi et al., 2016).

In addition to being expressed in enteric neurons, EGCs, and the smooth muscle cells of the intestinal wall, TLR2 is also expressed in dendritic cells (DCs) (Wang et al., 2006). As an immunodominant component of the *Bacteroides fragilis* capsule, PSA can interact with TLR2 on DCs. The application of live *B. fragilis* or pure PSA can evoke action potentials in IPANs within approximately 5 s. This leads to an increased excitability of IPANS. However, mutant *B. fragilis* that are devoid of PSA were observed to fail to elicit such a response regarding the activity of IPANs (Mao et al., 2013). In contrast, LPS were not observed to affect the activity of IPANs. Based on the latency of the neuronal response, PSA may first act on gut-resident immune cells, which would then activate IPANs via the release of an intermediate mediator (Ochoa-Repáraz et al., 2010; Mao et al., 2013).

As noted previously, SCFAs could prevent the activity of IPANs from affecting the host's gene expression. The inhibition of HDACs enhances the histone acetylation of gene regulatory elements and increases gene transcription (Falkenberg and Johnstone, 2014). Epigenetic regulation by butyrate has been demonstrated in colonic T cells and macrophages within the immune system (Arpaia et al., 2013; Furusawa et al., 2013; Chang et al., 2014). In contrast, however, little is known about the epigenetic modification of ENS by SCFAs. In the future, therefore, it is essential to determine which microbe-derived SCFAs modulate the epigenetic status of genes that are expressed in neurons and investigate the role of SCFAs in enteric neurogenesis.

As microbe-derived molecules, formyl peptides, D-glycero-β-D-manno-heptose-1,7-biphosphate (HBP), and hydrocarbon receptor ligands (AHR ligands) are also necessary for enteric immune homeostasis. Therefore, it is expected that these molecules may have an effect on ENS development.

Enteric Endocrine System

As a neurotransmitter or hormone, 5-HT (also known as serotonin) modulates intestinal secretion and motility. Approximately 90% of the body's 5-HT is produced by enterochromaffin cells (ECCs), which are a type of enteroendocrine cell that are present in the epithelium of the gut. 5-HT is synthesized by the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1), before being stored in secretory granules. TPH2 also produces a much smaller fraction of the total 5-HT in the neurons of the myenteric plexus (Walther et al., 2003). Reigstad et al. (2015) observed that SCFAs, but not LPS, can promote TPH1 transcription in human ECCs. In turn, the activation of both submucosal and myenteric 5-HT4R-expressing neurons has been noted (Yano et al., 2015). Thus, gut bacteria that act through SCFAs are essential determinants of enteric 5-HT production and homeostasis, which in turn both affect the activity of the ENS. In addition to being biosynthesized, extracellular 5-HT within the gut is also regulated by serotonin-selective reuptake transporter (SERT), which is expressed in intestinal epithelial cells (IECs) (Bian et al., 2007) and is regulated by microbe-derived factors, such as the TLR ligand LPS (Bian et al., 2007).

Gut microbes regulate both bile acid synthesis and the production of secondary bile acids (Bian et al., 2007; Tremaroli and Bäckhed, 2012). Secondary bile acids can activate TGR5 (also known as protein-coupled bile acid receptor 1, GPBAR1), which is highly expressed in enteric neurons and enteroendocrine L cells (Poole et al., 2010). TGR5 signaling has been suggested to play a vital role in intestinal propulsive activity; the TGR5-dependent enhancement of peristalsis may also be partly mediated by the production of 5-HT (Alemi et al., 2013).

Other than 5-HT, SCFAs can also directly stimulate enteroendocrine cells [ECCs or enteroendocrine L cells (Panaro et al., 2014)] to produce several neuropeptides, such as peptide YY (PYY), neuropeptide Y (NPY), cholecystokinin, glucagon-like peptide-1 (GLP1), GLP2, gastric inhibitory polypeptide (GIP), and substance P (SP); these neuropeptides can influence GI functions and affect the development of the ENS.

Enteric Stromal Cells

The ENS not only comprises a broad range of neuronal subtypes but also harbors enteric stromal cells, including EGCs and ICCs. ICCs are fibroblast-like interstitial cells that regulate the pattern and frequency of peristaltic contractions (Sanders et al., 2014); they act as pacemakers in the vicinity of smooth muscle cells.

Enteric glial cells differ significantly from the Schwann cells seen in the PNS in that they do not form basal laminae. Furthermore, they show many similarities to CNS glia, in both morphology and function. Unlike enteric neurons, whose cell bodies are restricted to the ganglia in the muscularis propria and submucosa, EGCs are distributed throughout the gut wall,

including in the lamina propria of the mucosa. EGCs are associated with both submucosal and myenteric neurons, the terminals of which run to the epithelial basement membrane and blood capillaries. EGCs coordinate signal propagation between myenteric neurons and epithelial cells, thus regulating gut motility, as well as the secretory and absorptive functions of the intestinal epithelium. In addition, EGCs maintain the integrity of epithelial barrier; they also participate in the development of the ENS by releasing specific mediators, e.g., glial cell-derived neurotrophic factor (GDNF), and by transforming growth factor β1 (TGFβ1) (Neunlist et al., 2014).

Kabouridis et al. (2015) demonstrated that EGCs in the submucous microenvironment are highly dependent on stimulation by the commensal bacteria that regulate the initial colonization of EGCs. Moreover, the mechanisms that underlie the interplay between gut microbes and EGCs rely on TLRs, particularly on TLR2 and TLR4, which are expressed in EGCs. Indeed, GF mice have been observed to show a marked decrease in the density of mucosal EGCs; this decrease could be restored upon recolonization (Kabouridis et al., 2015). Furthermore, the expressions of GDNF and glial markers (e.g., GFAP and S100β) have been observed to be significantly reduced in the myenteric plexus of TLR2−/− mice, while the administration of GDNF can rescue the ENS deficit. Thus, in this context, the microbe-TLR2 pathway promotes the functional maturation of EGCs and affects the development and homeostasis of the ENS via mesenchyme-derived neurotrophic factors (Turco et al., 2014).

It has been suggested that the molecular phenotype of EGCs exhibits a certain degree of plasticity. However, it is currently unclear as to whether this apparent plasticity of EGCs is manifested in all stages of adult life (Boesmans et al., 2015; Nagy and Goldstein, 2017).

Epithelial Cells

As membrane vesicles from the GI microbiota, MVs facilitate the movement of signals into the intestinal microenvironment. A recent study has identified that MVs indirectly communicate with IPANs in the myenteric plexus through unknown signals generated in the epithelium (Al-Nedawi et al., 2015). In this study, *Lactobacillus rhamnosus* and MVs formed by *L. rhamnosus* increased the number of action potentials recorded in preparations with an intact epithelium. However, no effect was observed when MVs were directly applied to IPANs of the myenteric plexus. Therefore, it appears that mucosal elements from epithelial cells are required to transduce the MV effect to the ENS.

NEUROLOGICAL DISORDERS OF THE GUT

As a "second brain," the ENS could be affected by neurodegeneration and other systemic diseases, similarly to the CNS. In addition, abnormalities in the ENS have been associated with severe GI disorders such as Hirschsprung disease (HSCR), esophageal achalasia (EA), gastroparesis, and neuropathic intestinal pseudo-obstruction. They have also been linked to a series of systemic diseases, which can manifest as intestinal innervation deficiencies; they have also been found to correlate with extra-GI diseases.

Many animal studies have observed a correlation between host aging and senescence-like phenotypic changes in the ENS. Host aging-related ENS degeneration includes dystrophic or degenerating nerve fibers; the alteration of the morphology of the enteric ganglia; and the accumulation of lipofuscin α-synuclein, hyperphosphorylated tau protein, and reactive oxygen species in aged enteric neurons (Thrasivoulou et al., 2006; Abalo et al., 2007; Phillips et al., 2009; Gamage et al., 2013). Aging-related alterations of the gut microbiota may instigate intestinal inflammation, which may eventually result in above aging-related ENS degeneration. Moreover, elderly and younger populations have shown differences in the compositions of their gut microbes (Maynard and Weinkove, 2018). It is inferred that the hallmarks of successful aging may be a balance among core microbiota as well as between pro- and anti-inflammatory activity (Badal et al., 2020).

Deficiency of Intestinal Innervation

In the ENS, a complicated and timed migration of enteric neural crest derived progenitor cells leads to well-developed and interconnected networks within the gut wall. A lack of migration or differentiation of these enteric neural crest derived cells can result in the aganglionic innervation of the GI tract, which manifests as HSCR or EA.

The premature differentiation of progenitors into neurons or glial cells impairs the colonization of neural crest-derived cells in the distal bowel, resulting in missing enteric neurons at variable lengths in the intestine. Therefore, patients with HSCR exhibit intestinal obstructions. Patients with HSCR also exhibit altered gut microbial compositions, characterized by high levels of Bacteroidetes, Firmicutes, and Proteobacteria (Li et al., 2016). Pierre et al. (2014) demonstrated that a loss of the ENS results in defective intestinal motility, leading to intestinal bacterial overgrowth and increased abundances of pro-inflammatory bacterial lineages. Hirschsprung-associated enterocolitis (HAEC) has been shown to be closely related to the disturbance of the intestinal bacteria; the most abundant phylum detected in patients with HAEC and HAEC remission (HAEC-R) is Proteobacteria, followed by Firmicutes (Li et al., 2016). Additionally, Li et al. (2016) also observed that in each patient with HSCR, specimens from different intestinal sites differed significantly, but were more similar in each patient with HAEC and HAEC-R. Hirschsprung disease occurs through a complex process that involves multiple genetic mutations and disorders of the intestinal microenvironment. The causality that impacts the development of the ENS by gut microbes thus needs to be further investigated regarding the pathogenesis of Hirschsprung disease.

Esophageal achalasia is a motility disorder that is characterized by the failure of the lower esophageal sphincter (LES) to relax, and by esophageal dysmotility. The pathogenesis of neurogenic theory is widely accepted; it involves the selective degeneration of inhibitory neurons in the esophageal myenteric plexus (Pressman and Behar, 2017). The etiology of EA currently remains elusive, but several factors have been mentioned, including

genetic predisposition, viral infection, and autoimmunity. Viral infections in individuals with a genetic predisposition may induce autoimmunity and the degeneration of inhibitory neurons in the esophageal myenteric plexus. Thus, viral infections may play an essential role in the pathogenesis of EA. However, more studies are needed in this regard.

Correlation With Neurological or Systemic Diseases

Gastrointestinal function can be compromised by neurological diseases of the CNS, or by neuronal deficits induced by systemic diseases. For example, patients with stroke, Parkinson's disease (PD), multiple sclerosis (MS), Guillain-Barré syndrome (GBS), amyotrophic lateral sclerosis (ALS), myasthenia gravis (MG), and other neurological diseases of the CNS often also experience digestive symptoms such as neurogenic dysphagia, abdominal distention, and constipation. These symptoms may arise from defects in any component of the MGB axis. Although neurological diseases in the CNS are considered as a potential influence on the GI tract, the underlying mechanisms still need to be studied further.

The ENS is vulnerable to neurological or systemic diseases. GI function may also contribute to neurological disorders inside or outside of the gut. GI dysfunction, e.g., malabsorption, celiac disease, ulcerative colitis (UC), and Crohn's disease (CD), may lead to neurological symptoms. For example, the malabsorption of nicotinamide leads to neurological deficits and dementia (Fricker et al., 2018), while celiac disease has been shown to lead to neurological manifestations in up to 10% of all patients. The most common neurological symptoms are cerebellar ataxia, MS, peripheral neuropathy (PN), and other less described clinical conditions (Casella et al., 2016). PN in the form of ataxic and inflammatory demyelinating polyneuropathy has been found in inflammatory bowel disease (IBD) but is usually more severe in CD (Gondim Fde et al., 2015).

NEURODEGENERATIVE DISEASES IN THE CNS

Gut microbe-derived metabolites (e.g., SCFAs) can stimulate ECCs to produce neuropeptides or neurotransmitters, which, in turn, can diffuse into the bloodstream and reach the CNS. Besides, microbe-derived components can activate intestinal or circulating immune cells that can migrate to the CNS via the brain lymphatic network (Louveau et al., 2015; Rooks and Garrett, 2016). In addition, gut microbes also impact the ENS-vagus pathway (Furness, 2012). Therefore, bacterial molecules and neural-immune-endocrine pathways present a dynamic communication network in the MGB axis.

Short chain fatty acids produced by gut microbes ensure the integrity of the blood–brain barrier (BBB) by upregulating tight junction proteins; they also regulate the maturation and activation of microglial cells (Erny et al., 2015; Gao et al., 2021). In addition, SCFAs have also been implicated in the alleviation of stress-related disorders (van de Wouw et al., 2018). These examples reinforce the role of gut microbes in the MGB axis. Thus, it is reasonable to consider that part of the etiology of neurodegenerative diseases of the CNS may involve gut microbes.

The human ENS system changes gradually with aging, accompanied by the occurrence of neurodegenerative diseases. PD is characterized by the selective degeneration of dopaminergic neurons in the substantia nigra and Lewy bodies (abnormal depositions of α-synuclein) in the surviving dopaminergic neurons; this results in motor symptoms. A high percentage of patients with PD are characterized by abnormal GI motility and constipation. In fact, GI symptoms often appear before motor symptoms. Lewy bodies have been observed in the enteric neurons of patients with PD, which precede the development of motor symptoms by several years (Braak and Del Tredici, 2008). The ENS has been suggested to be an initial site of α-synuclein aggregations; these aggregations can subsequently spread to the brain through vagus nerve fibers. Indeed, patients undergoing vagotomies exhibit a reduced risk of developing PD (Svensson et al., 2015).

Additionally, Scheperjans et al. (2015) observed that the relative abundance of Enterobacteriaceae was positively associated with the severity of postural instability and gait difficulty, suggesting that intestinal bacteria are altered in PD, and are related to motor phenotypes. GFAP has been demonstrated to be overexpressed and hypophosphorylated in the EGCs of patients with PD, compared to those of healthy subjects and patients with atypical parkinsonism (Clairembault et al., 2014). Thus, EGC dysregulation might be associated with PD development. Besides, it has been found that shifts of the phage/bacteria ratio in lactic acid bacteria (produce dopamine and regulate intestinal permeability) are major factors in PD pathogenesis (Tetz and Tetz, 2018). The depletion of Lactococcus spp. in the PD patients was most likely due to the increase of lytic c2-like and 936-like lactococcal phages (Tetz et al., 2018). According to these studies, intestinal pathology could be an early and potential biomarker of PD. Nevertheless, it remains unclear as to whether the changes observed in gut microbiota contribute to the pathogenesis or consequences of PD.

In contrast with PD, few studies have investigated the enteric manifestations of Alzheimer's disease (AD), despite it being the most common neurodegenerative disorder. AD is characterized by Aβ plaques and hyperphosphorylated tau protein at various stages, and ultimately in widespread cortical regions. A variety of taxa have been observed to be altered in patients with AD, with decreased levels of Firmicutes and Bifidobacterium, and increased levels of Bacteroidetes (Vogt et al., 2017). These alterations in gut microbes have been shown to correlate strongly with the pathological loads of Aβ and phosphorylated tau protein in patients with AD. Harach et al. observed that Aβ pathology was markedly diminished in GF mice with AD mutations (APP/PS1 model) in comparison to conventional AD animals (Harach et al., 2017). Moreover, early antibiotic treatment in AD mice has been shown to reduce Aβ deposition later in life (Minter et al., 2017). In addition, the treatment of AD mice with a *Bifidobacterium breve* strain can prevent Aβ-induced cognitive deficits, partially restore memory function, and improve inflammatory status (Kobayashi et al., 2017). However, another study showed no improvement in cognition for patients with severe AD after the

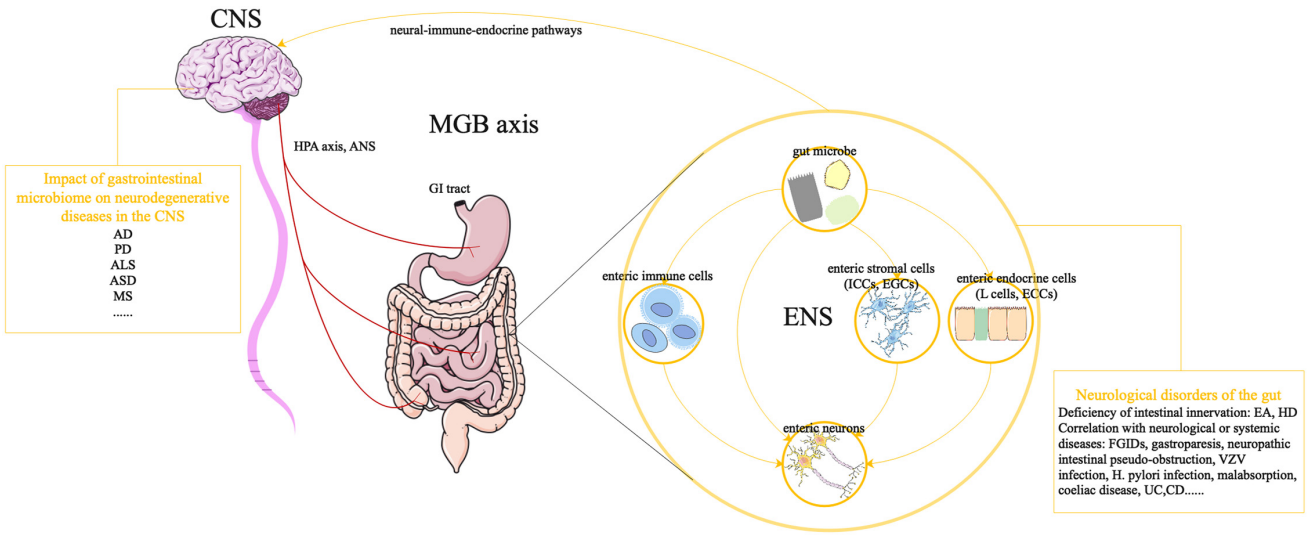

FIGURE 1 | Effects of GI microbiota on the ENS and MGB axis. The ANS, in combination with the HPA axis and neuroendocrine signaling, can induce CNS-modulated changes in the gut. Meanwhile, bacterial molecules and neural-immune-endocrine pathways impact the development of the CNS, giving rise to a dynamic communication network in the MGB axis. The development and function of the ENS are controlled by GI microbiota, through direct or indirect mechanisms. Bacterial molecules can pass through the intestinal epithelial barrier to directly interact with enteric plexuses or act on non-neuronal intermediary cells (e.g., enteric immune cells, ICCs, EGCs, EGCs, and enteric L cells), whose products can be detected by enteric neurons. Abnormalities in the ENS are associated with life-threatening GI disorders, which can be manifested as intestinal innervation deficiencies (e.g., EA, HSCR) and can be correlated with neurological or systemic diseases (gastroparesis and neuropathic intestinal pseudo-obstruction). In addition, gut microbes might be associated with neurodegenerative CNS diseases, such as AD, PD, ALS, ASD, MS, and other neurological diseases. CNS, central nervous system; ANS, autonomic nervous system; HPA axis, hypothalamic-pituitary-adrenal axis; MGB axis, microbiota-gut-brain axis; AD, Alzheimer's disease; PD, Parkinson's disease, ALS, amyotrophic lateral sclerosis; ASD, autism spectrum disorder; MS, multiple sclerosis; GI, gastrointestinal; ENS, enteric nervous system; ICCs, interstitial cells of Cajal; EGCs, enteric glial cells; ECCs, enterochromaffin cells; EA, esophageal achalasia; HSCR, Hirschsprung disease; FGIDs, functional gastrointestinal disorders; VZV, varicella-zoster virus; *H. pylori*, *Helicobacter pylori*; UC, ulcerative colitis; CD, Crohn's disease.

consumption of a multistrain probiotic (containing *Lactobacillus* and *Bifidobacterium* strains) for 12 weeks (Agahi et al., 2018). Therefore, the translational value of using therapeutics to target the MGB axis for patients with AD remains unclear.

Huntington's disease is a hereditary neurodegenerative disease that can cause progressive motor decline, cognitive dysfunction, and neuropsychiatric symptoms (Walker, 2007). Patients with Huntington's disease also suffer from a range of GI disturbances, including diarrhea, nutrient deficiencies, and unintended weight loss (Djoussé et al., 2002; van der Burg et al., 2017). Kong et al. (2020) observed gut dysbiosis in R6/1 transgenic mice (a Huntington's disease model), which was associated with body weight impairment and motor deficits.

Autism spectrum disorder (ASD) is one of the most common neurodevelopmental disorders worldwide (Maenner et al., 2020). ASD is characterized by stereotypical behaviors and deficits in social communication. In addition, patients with ASD often experience GI problems, which tend to correlate strongly with the severity of autism, and with increased irritability, anxiety, and social withdrawal (Gorrindo et al., 2012). Patients with autism have been reported to exhibit altered gut microbial diversity and increased abundances of *Clostridia* and *Candida* (Li et al., 2017; Luna et al., 2017). Animal models of ASD have also shown dysbiosis (Sgritta et al., 2019). Moreover, social impairments in these models were restored via the manipulation of microbiota (Hsiao et al., 2013; Sgritta et al., 2019).

Intestinal microbes are known to impact the development of the CNS through the brain-gut axis; they may even modulate it throughout the host's life span. Further exploration of the modulation of gut microbiota and associated metabolic dysfunction may lead to the development of novel therapeutic approaches, such as enviromimetics, for a wide range of neurological and psychiatric disorders (Gubert et al., 2020).

PROSPECTIVE AND CONCLUSION

As the second-largest genome in the human body, the intestinal microbiota have been the focus of significant attention regarding their regulation of the host and the external environment. New target molecules from the microbiota have been found and applied in practice. Despite a growing body of research, the MGB axis remains an obstacle regarding our understanding of its causal relationship, and regarding how the intestinal microbiota play a regulatory role in the systemic ENS, beyond the local intestinal tract. The ENS is a vital, irreplaceable bridge that connects intestinal microbiota with multiple systems. This review identified the following three aspects as being worthy of further study: (1) exploring how the microbiota act on the intestinal nervous system and its mechanism; (2) determining how the intestinal nervous system regulates intestinal function, thus affecting the intestinal microbiota; and (3) exploring how

intestinal nerves regulate the physiological function of other nervous systems (such as the CNS). Meanwhile, it is noteworthy that multiple species differences should be carefully considered when applying findings from mouse ENS research to human GI studies (May-Zhang et al., 2021).

An accumulating amount of evidence suggests that bacterial molecules can pass the intestinal epithelial barrier to interact with enteric plexuses directly; they can also act on non-neuronal intermediary cells, whose products can be detected by enteric neurons. Gut microbes may also be involved in the development and maintenance of the CNS. Extensive metagenomic studies have determined that neurological diseases of the gut and brain are associated with the alteration of intestinal bacteria. Prebiotics, probiotics, and fecal microbiota transplantations (FMTs) have been used to manage certain neurological disorders to modulate gut microbes. In addition, increasing numbers of clinical trials have suggested that such treatments can provide beneficial effects to afflictions such as UC and *Clostridium difficile* infections (Paramsothy et al., 2017, 2019; Weingarden and Vaughn, 2017; Costello et al., 2019). Moreover, the therapeutic potential of microbiota-targeted interventions has also been emphasized in anti-aging medicine (Vaiserman et al., 2017).

Therefore, in the future it will be essential to shift from simply conductive correlative analyses, and instead move toward prospective longitudinal studies, causative and mechanistic analyses, and larger scale trials of potential therapeutic approaches. Understanding the underlying mechanisms of gut microbe-ENS interactions could help to generate novel therapeutic applications for microbes regarding neurodegenerative diseases (**Figure 1**).

AUTHOR CONTRIBUTIONS

P-HZ, CZ, and Q-LL created the concept and designed the project. Z-HG and YZ drafted the manuscript. All authors contributed substantially to all aspects of the article and revised versions.

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A Bibliometric Review on Gut Microbiome and Alzheimer's Disease Between 2012 and 2021

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Research on the microbiome has drawn an increasing amount of attention over the past decade. Even more so for its association with disease. Neurodegenerative diseases, such as Alzheimer's disease (AD) have been a subject of study for a long time with slow success in improving diagnostic accuracy or identifying a possibility for treatment. In this work, we analyze past and current research on microbiome and its positive impact on AD treatment and diagnosis. We present a bibliometric analysis from 2012 to 2021 with data retrieved on September 2, 2021, from the Scopus database. The query includes "Gut AND (Microbiota OR Microbiome) AND Alzheimer*" within the article title, abstract, and keywords for all kinds of documents in the database. Compared with 2016, the number of publications (NPs) on the subject doubled by 2017. Moreover, we observe an exponential growth through 2020, and with the data presented, it is almost certain that it will continue this trend and grow even further in the upcoming years. We identify key journals interested in the subject and discuss the articles with most citations, analyzing trends and topics for future research, such as the ability to diagnose the disease and complement the cognitive test with other clinical biomarkers. According to the test, mild cognitive impairment (MCI) is normally considered an initial stage for AD. This test, combined with the role of the gut microbiome in early stages of the disease, may improve the diagnostic accuracy. Based on our findings, there is emerging evidence that microbiota, perhaps more specifically gut microbiota, plays a key role in the pathogenesis of diseases, such as AD.

Keywords: Alzheimer's disease, bibliometric analysis, gut microbiome, gut microbiota, microbiota-gut-brain axis, trend topics

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INTRODUCTION

In November of 1906, during the 37th Meeting of the Society of Southwest German Psychiatrists in Tubingen, Germany—Alois Alzheimer first described the disease that bears his name (Lage, 2006; Valleix, 2007). A year later, the article entitled "About a peculiar disease of the cerebral cortex" was published (Stelzmann et al., 1995; Strassnig and Ganguli, 2005). Since then, the Alzheimer's disease (AD) hallmark pathology has been extensively studied, leading to a variety of explanations. One such explanation, and perhaps the most widely accepted to date, is that AD

develops as a result of an accumulation of amyloid-β (Aβ) protein fragments outside neurons. These fragments in turn may contribute to cell death by interfering with neuron-to-neuron communication at synapses, and as hyperphosphorylated tau tangles within neurons, blocking the transport of nutrients and other essential molecules inside neurons (Alzheimer's Association, 2019). Finding an explanation for these pathologies could mean finding a treatment, however, a comprehensive understanding of the etiopathogenesis has not yet been elucidated, directly impacting the failure of several clinical trials (Tobore, 2019). Among several hypotheses that have been proposed to explain the causes of AD, we have: the amyloid hypothesis, calcium homeostasis hypothesis, cholinergic hypothesis, inflammatory hypothesis, lymphatic system hypothesis, metal ion hypothesis, mitochondrial cascade hypothesis, neurovascular hypothesis, and tau propagation hypothesis (Liu et al., 2019). Thus far, they have not yielded a definite treatment or prevention strategy, but they have led to novel approaches that could potentially explain AD.

Similar to the relationship between the gut microbiome and neurodegenerative diseases, the human gut microbiome is the diverse collection of microorganisms (e.g., bacteria, archaea, and fungi) that reside in the gastrointestinal tract (Fung et al., 2017). In fact, there are studies that report that the microbiota may be the etiopathogenesis of amyloidosis in the brains of subjects with AD, since bacterial infection induces Aβ peptide oligometry and the gut microbiota can produce its own peptides (Pistollato et al., 2016).

It is important to keep track of the advances and the contributions from clinicians and basic scientists so that improved diagnosis and better treatments can be developed. In this study, we summarize through a bibliometric approach the most recent, highly cited research, the countries with the highest contribution, and in general, a landscape of published work on the relationship between the gut microbiome and AD during the last decade. Bibliometric analyses are tools that allow for a statistical examination of scientific publications. They mainly help in defining past and future trends on certain research topics (Kokol et al., 2021). They also allow researchers to perform a more in-depth analysis of the collaborations between authors, countries, and understand the impact of scientific publications within the research community (Kutluk and Danis, 2021). While several bibliometric analyses have been published regarding AD (Sorensen, 2009; Song et al., 2015; Serrano-Pozo et al., 2017; Dong et al., 2019; Schilder et al., 2020), none of them tackles the topic of the relationship between the disease and the gut microbiome. Since this is one of the most novel and strong theories in AD pathology development, it is of utter importance to find future trends that could guide researchers to develop new studies.

MATERIALS AND METHODS

Data Source and Search Strategy

Data were retrieved on 2 September 2021, from the Scopus database. The query used was "Gut AND (Microbiota OR Microbiome) AND Alzheimer*" within the article title, its abstract, and its keywords for all the documents in the database. This means that we queried all publications with the words gut, either microbiota or microbiome, and the term Alzheimer's with any type of ending (e.g., Alzheimer, Alzheimers, and Alzheimer's).

Documents were then filtered by language, including only articles written in English, and then by type, considering not only original articles, but also reviews, conference papers, conference reviews, and letters. A PRISMA 2020 flow diagram (Page et al., 2021) depicting the flow of information through the different phases of the analysis can be found in **Figure 1**. Quantitative and qualitative analyses, such as citation information (e.g., author(s), document title, and year); bibliographical information (e.g., affiliations and publisher); abstract and keywords (e.g., author keywords and index keywords); funding details and other characteristics were extracted using the export document settings in the Scopus database.

Maps Based on Bibliographical Data

All analyses carried out in this study were performed with the help of the open-source R package bibliometrix (Aria and Cuccurullo, 2017).

RESULTS

Main Information

A total of 610 publications on the relationship between the gut microbiome and AD were found between 2012 and 2021, namely, 318 reviews, 288 original articles, 2 letters, 1 conference paper, and 1 conference review. Descriptive information regarding the collection can be consulted in **Table 1** and the year-wise distribution of these publications is shown in **Figure 2**.

Based on the plot of **Figure 2**, it is evident that the productivity of this research topic has increased exponentially. It can be expected that the research field would grow further soon. Hence, to test this claim, we fit an exponential regression model on the 2012–2020 data. The results of the regression analysis used to estimate the 2021 and 2022 publications are shown in **Figure 3**.

According to the regression analysis results, it was estimated that 380 and 688 articles would be published in 2021 and 2022, respectively.

Scientific Sources

Table 2 shows the top 11 sources that generated most of the publications in the study, specifying the number of publications (NPs), total number of citations (TC), h-index, and the year of the first publication for that source (YFP).

We can see that these journals contribute approximately 25% of the scientific knowledge on the matter. It is important to highlight, however, that even though the International Journal of Molecular Sciences, Journal of Alzheimer's Disease, and Nutrients have 25, 23, and 21 publications, respectively (+ 3% each), Scientific Reports amassed the most citations (1,461). Further analysis on the primary focus of the most relevant sources revealed that this is indeed an interdisciplinary research field. For instance, the source with the most publications,

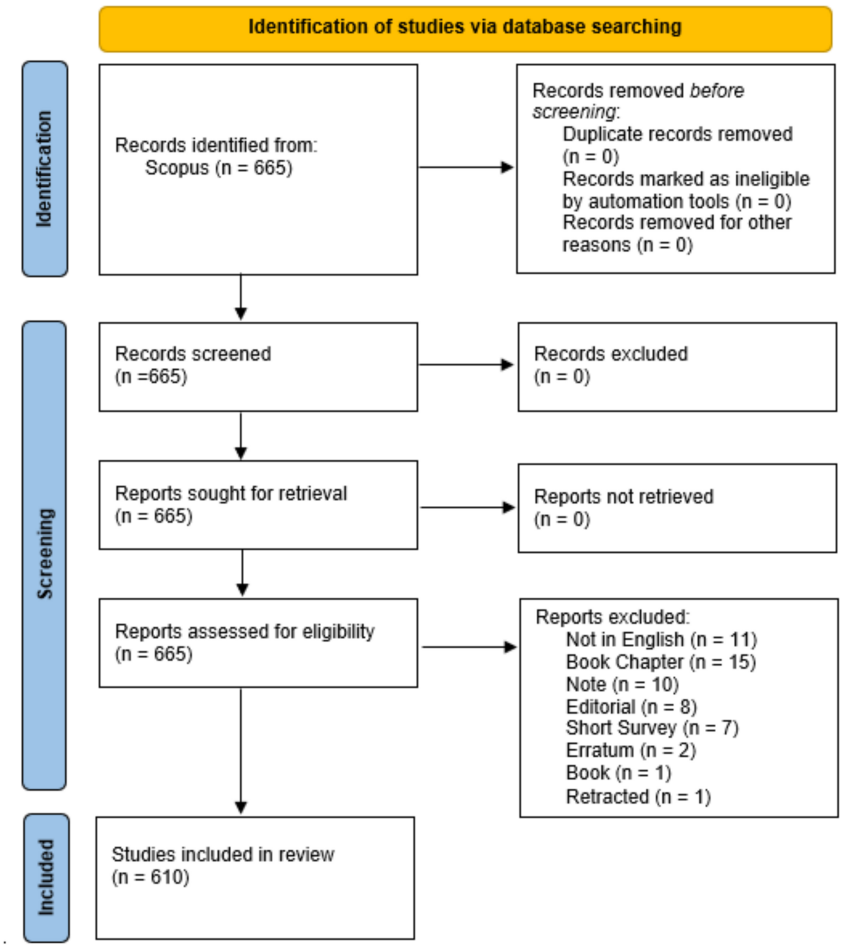

FIGURE 1 | The PRISMA 2020 statement.

TABLE 1 | Summary of descriptive information on the collection found from 2012 to 2021.

Main information	Explanation	Count
Documents	Total number of scientific publications	610
Sources	The frequency distribution of sources as journals	312
Author's keywords	Total number of keywords	1,313
Keywords plus (ID)	Total number of word or phrases that frequently appear in the title of an article's references	4,839
Authors	Total number of authors	2,764
Authors appearances	The author's frequency distribution	3,639
Authors of single-authored documents	The number of single authors per articles	39
Authors of multi-authored documents	The number of authors of multi-authored articles	2,725
Authors per document	The average number of authors in each document	4.53
Co-authors per documents	The average number of co-authors in each document	5.97
Single-authored documents	Total number of single-authored documents	41
Documents per author	Total number of documents per author	0.221
Average citations per document	The average number of quotes in each article	29.34
Collaboration index (CI)	Total number of authors of multi-authored articles/total number of multi-authored articles	4.79

International Journal of Molecular Sciences, provides an advanced forum for all aspects of molecular research in chemistry. The Journal of Alzheimer's Disease has principal concerns on the etiology, pathogenesis, epidemiology, genetics, behavior, treatment, and psychology of AD. Nutrients Journal is related to human nutrition with subject areas, such as

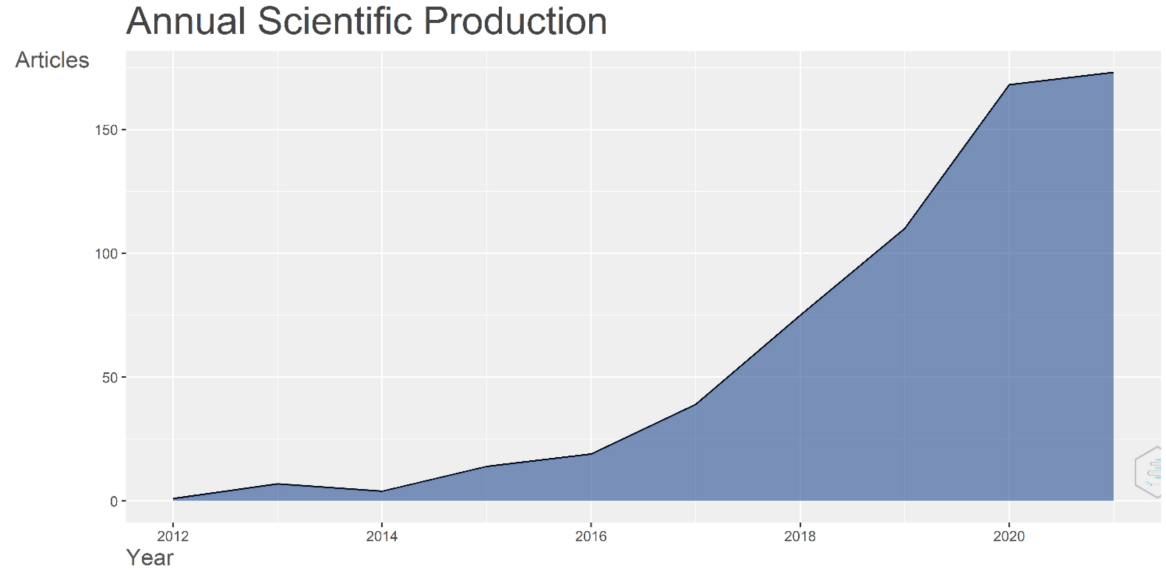

FIGURE 2 | Year-wise distribution of the Scopus documents in 2012–2021.

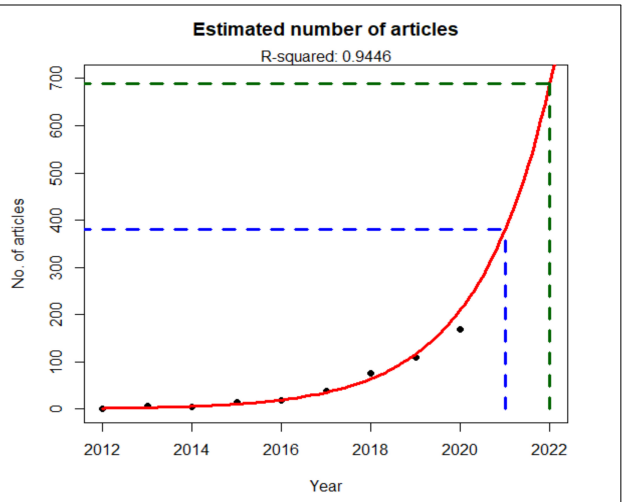

FIGURE 3 | Estimation of publications by year on gut microbiome and AD.

TABLE 2 | A list of journals with the highest number of publications (NPs) on the subject in our collection.

Source	NP (%)	TC	*h*	YFP
International journal of molecular sciences	25 (4.10)	288	10	2015
Journal of alzheimer's disease	23 (3.77)	1,052	12	2015
Nutrients	21 (3.44)	602	10	2016
Scientific reports	14 (2.29)	1,461	11	2016
Frontiers in aging neuroscience	13 (2.13)	227	7	2017
Aging	9 (1.48)	136	7	2018
Pharmacological research	9 (1.48)	325	7	2013
Journal of neuroinflammation	8 (1.31)	342	5	2018
Microorganisms	8 (1.31)	509	5	2018
Molecular nutrition and food research	8 (1.31)	87	5	2018
Progress in neuro-psychopharmacology and biological psychiatry	8 (1.31)	101	5	2019

TABLE 3 | Number of publications of the most productive countries.

Country	NP (%)
United States of America	456 (74.75)
China	446 (73.11)
Italy	226 (37.05)
Spain	82 (13.44)
South Korea	72 (11.80)
Germany	68 (11.15)
Ireland	65 (10.66)
United Kingdom	56 (9.18)
Canada	51 (8.36)
India	47 (7.70)

macronutrients, micronutrients, functional foods, diet-related disorders, and malnutrition. Scientific Reports has a broader scope and publishes original research from all areas of the natural and clinical sciences. As expected, Frontiers in Aging Neuroscience is more focused on the understanding of the underlying mechanisms of central nervous system and aging as well as other age-related neural diseases, but recently, through research topics, which are peer-reviewed article collections around cutting-edge research themes, articles related to the gut microbiome and AD have been published.

Country Scientific Production

The top 10 countries according to the NPs are listed in **Table 3**. The worldwide scenario when it comes to AD and gut microbiome is led by three countries: the United States of America (456), China (446), and Italy (226). It is worth noting that percentages do not add up to 100 because, as it will be

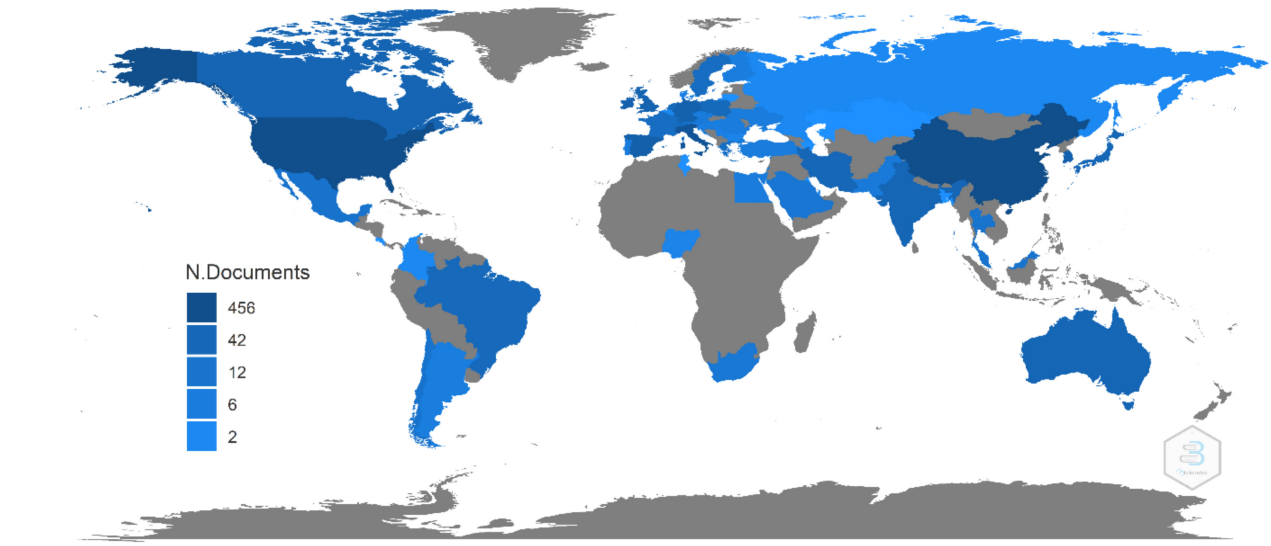

FIGURE 4 | Number of publications per country based on affiliation.

discussed in the next subsection, a publication could have authors with affiliations from different countries. **Figure 4** visually details this metric for all countries.

Several countries played a key role in publishing on the microbiota-gut-brain axis theory for AD. To analyze the international collaboration rate, publications were classified according to the country of affiliation of the corresponding author. Records were classified as either single country publications (SCPs) or multiple country publications (MCPs) and the MCP/NP ratio was calculated. **Figure 5** shows the per-country production stratified by the SCP-MCP class.

Countries with the highest international collaboration rate with at least 14 NPs are Australia, the United Kingdom, and Spain, with an MCP/NP ratio of 57.14, 43.75, and 38.89%, respectively. Nonetheless, work needs to done constructing scientific networks cultivating international collaboration in countries, such as India with only 5.88% of their publications classified as MCP or other Latin-American countries that do not even appear in this list.

Among the top four countries in our collection with 610 articles, the major relationships were United States of America-China 18; United States of America-Canada 6; United States of America-Germany 6; and United States of America-United Kingdom 6 as depicted in **Figure 6**. In this figure, only collaborations with a minimum of three articles were presented. In 2019, at the Lausanne Workshop on AD, the idea of worldwide collaboration was presented. The aim was to develop a global mechanism of action for a better diagnosis and to accelerate research on the drug development that can efficiently treat AD for all people in all places. In 2021, the Davos Alzheimer's Collaborative launched a global partnership with international organizations from governments and the private sector mobilizing the world against AD. Today, it is recognized that we need a global effort to tackle AD. We would expect to see more pink lines in this map in the following years.

Citation Analysis

Out of the 610 articles published in the 2012–2021 period on the gut microbiome and AD, the top ten articles that received the most citations are shown in **Table 4**. It is worth noting that, even though Scientific Reports had the highest number of citations per source, it fell to 4th place in this table.

Trending Topics

Thus far, we have analyzed the state-of-the-art of published research in AD and microbiota. Another important piece of information is to capture trending topics so future research on AD could focus on emerging priorities for this disease. To uncover trending topics, the titles of the articles in the most recent publications were analyzed through trigrams. Results shown in **Figure 7** indicate that trending topics seem to be related to human gut microbiota, short-chain fatty acids, and fecal microbiota transplantation. However, it is also worth mentioning that mild cognitive impairment (MCI), an early stage of memory loss or other cognitive ability loss and a condition that is known to be the prelude to AD, is also trending.

Mild cognitive impairment was a trendy term in 2019 and remains somewhat active, followed by the central nervous system, which does not seem to have the same impact in 2021. There has been an effort to address AD through metabolic changes and even databases, such as the Alzheimer's Disease Neuroimaging Initiative (ADNI) fully devoted to this approach. However, it is clear that human gut microbiota has today's attention and that

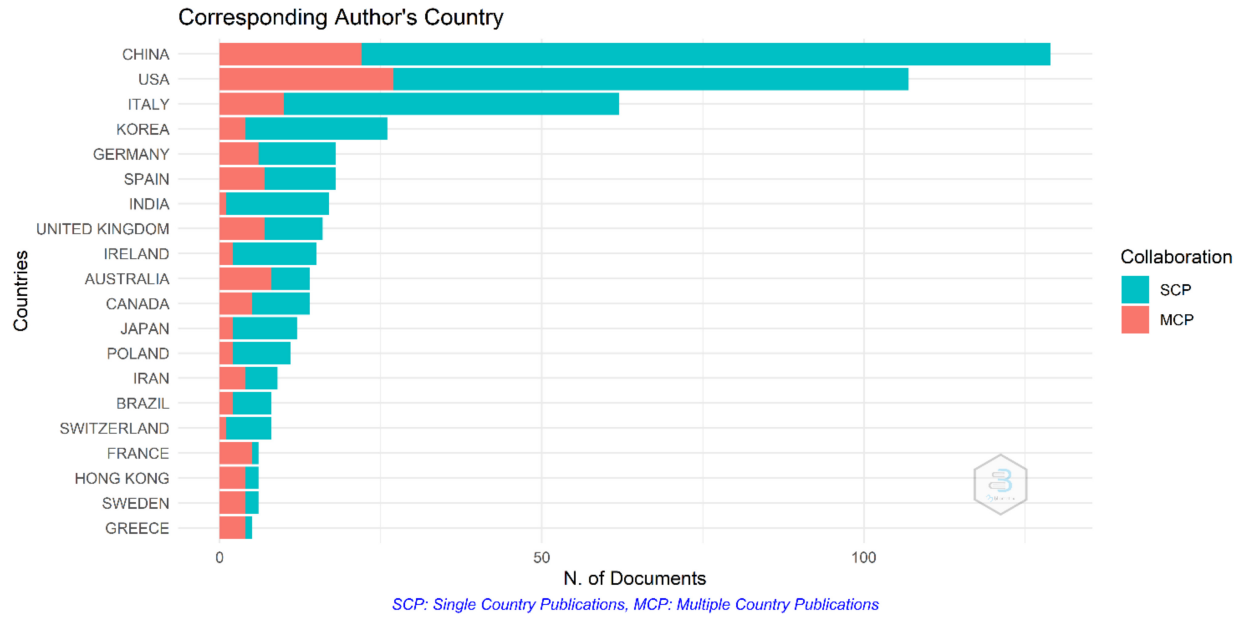

FIGURE 5 | International collaboration by country. Classification goes as multiple country publications (MCPs) in red and at a higher frequency, single country publications (SCPs) in blue.

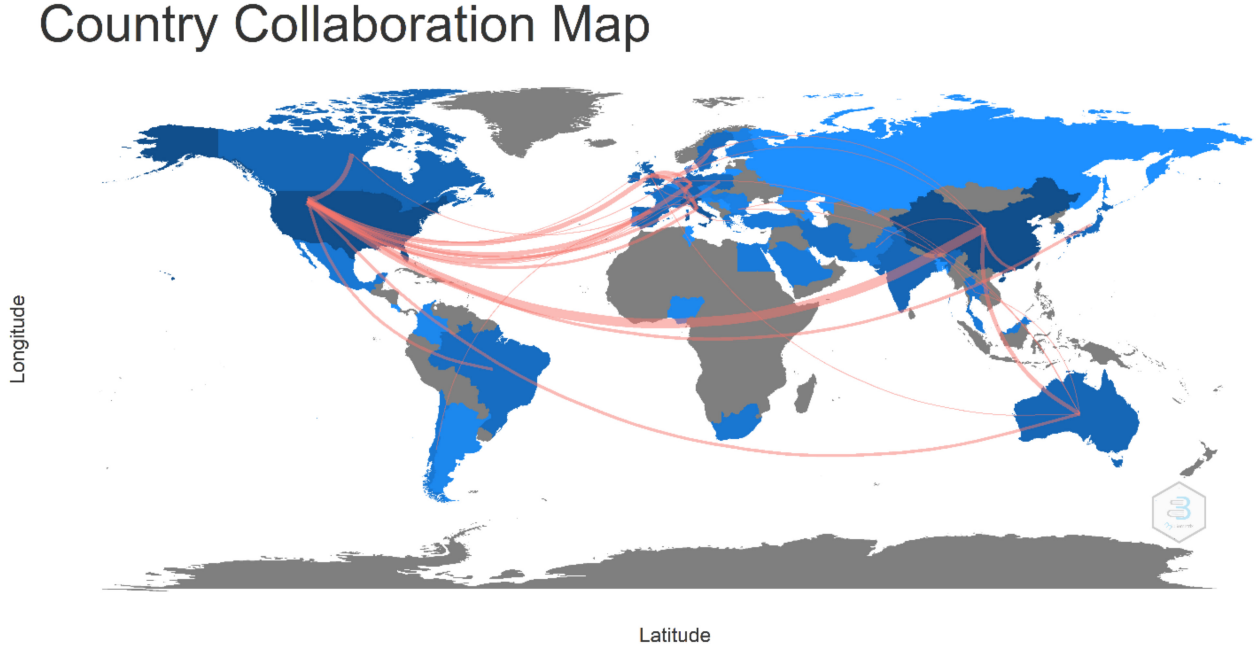

FIGURE 6 | International collaborations among countries. The pink color line represents the connection between countries and its thickness identifies the degree of collaboration. The thicker the line the stronger the collaboration rate.

this can get even trendier due to the success of therapies that use fecal microbiota transplantation.

Conceptual Structure

The conceptual structure represents relations among concepts or words in a set of publications. It is what scientists talk about, the main themes and trends. We performed a factorial analysis—a data reduction technique that serves as a filter of terms that are mostly redundant or of low frequency on the KeyWords Plus. Reduction is achieved by selecting terms that represent a linear combination of other redundant terms through multiple correspondence analysis. **Figure 8** is shown below.

TABLE 4 | Publications with the highest number of citations.

Title	First author	Journal	YP	TC
• Interactions between the microbiota, immune and nervous systems in health and disease	Fung, T. C.	Nature Neuroscience	2017	596
• The microbiota-gut-brain axis	Cryan, J. F.	Physiological reviews	2019	468
• What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases	Rinninella, E.	Microorganisms	2019	453
• Gut microbiome alterations in Alzheimer's disease	Vogt, N. M.	Scientific Reports	2017	439
• Brain-gut interactions in inflammatory bowel disease	Bonaz, B.	Gastroenterology	2013	345
• Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly	Cattaneo, A.	Neurobiology of aging	2017	334
• Microglia in neurodegeneration	Hickman, S.	Nature Neuroscience	2018	320
• Reduction of abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota	Harach, T.	Scientific Reports	2017	279
• Gut instincts: microbiota as a key regulator of brain development, aging and neurodegeneration	Dinan, T. G.	The Journal of Physiology	2017	256
• The gut microbiota and Alzheimer's disease	Jiang, C.	Journal of Alzheimer's Disease	2017	254

YP stands for year of publication.

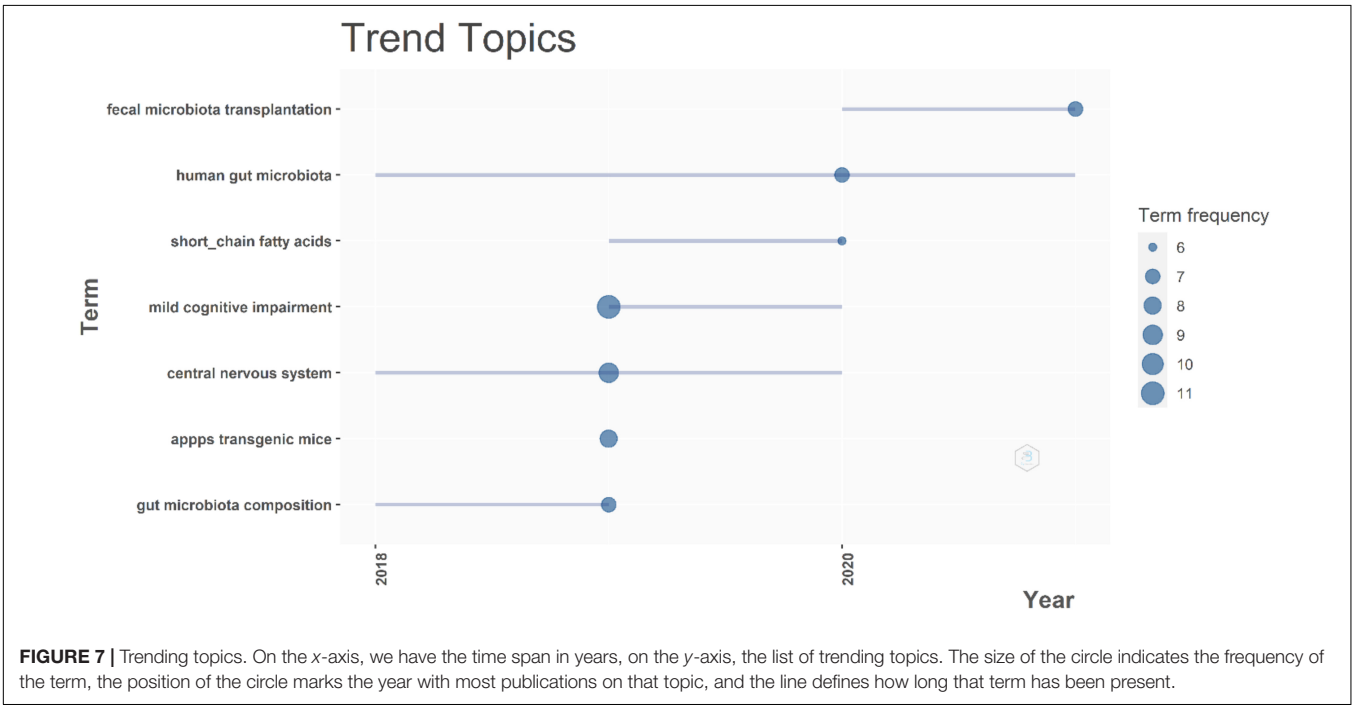

FIGURE 7 | Trending topics. On the *x*-axis, we have the time span in years, on the *y*-axis, the list of trending topics. The size of the circle indicates the frequency of the term, the position of the circle marks the year with most publications on that topic, and the line defines how long that term has been present.

Here, the proximity between words is because a large proportion of articles use them together, and the origin of the map represents the average position of all column profiles and therefore represents the center of the research field (Cuccurullo et al., 2016).

DISCUSSION

Research on microbiome has drawn an increasing amount of attention over the past decade. Even more so for its association with disease. On the other hand, AD has been a subject of study for a longer time with a lack of success in improving diagnostic accuracy or a possibility for treatment. Based on our findings, there is emerging evidence that microbiome—perhaps more specifically the gut microbiome—is a key player in the pathogenesis of diseases, such as AD. In the case of microbiome and AD, a significant number of scientific publications have been published lately. From a selection of 610 scientific contributions in the fields in a Scopus search, we found 52.13% review articles, 47.21% original research articles, and an almost a negligible percentage of conference papers. Compared with 2016, we identified an important increase in 2017 only to double the number of papers in 1 year. Nevertheless, it was just a starting point that led to an exponential growth by 2020 and, with the data presented, it is almost certain that it will continue this trend and grow even further in the upcoming years. Our projections estimate that 2021 will end with almost 400 papers in the subject while this number will almost get to 700 in 2022.

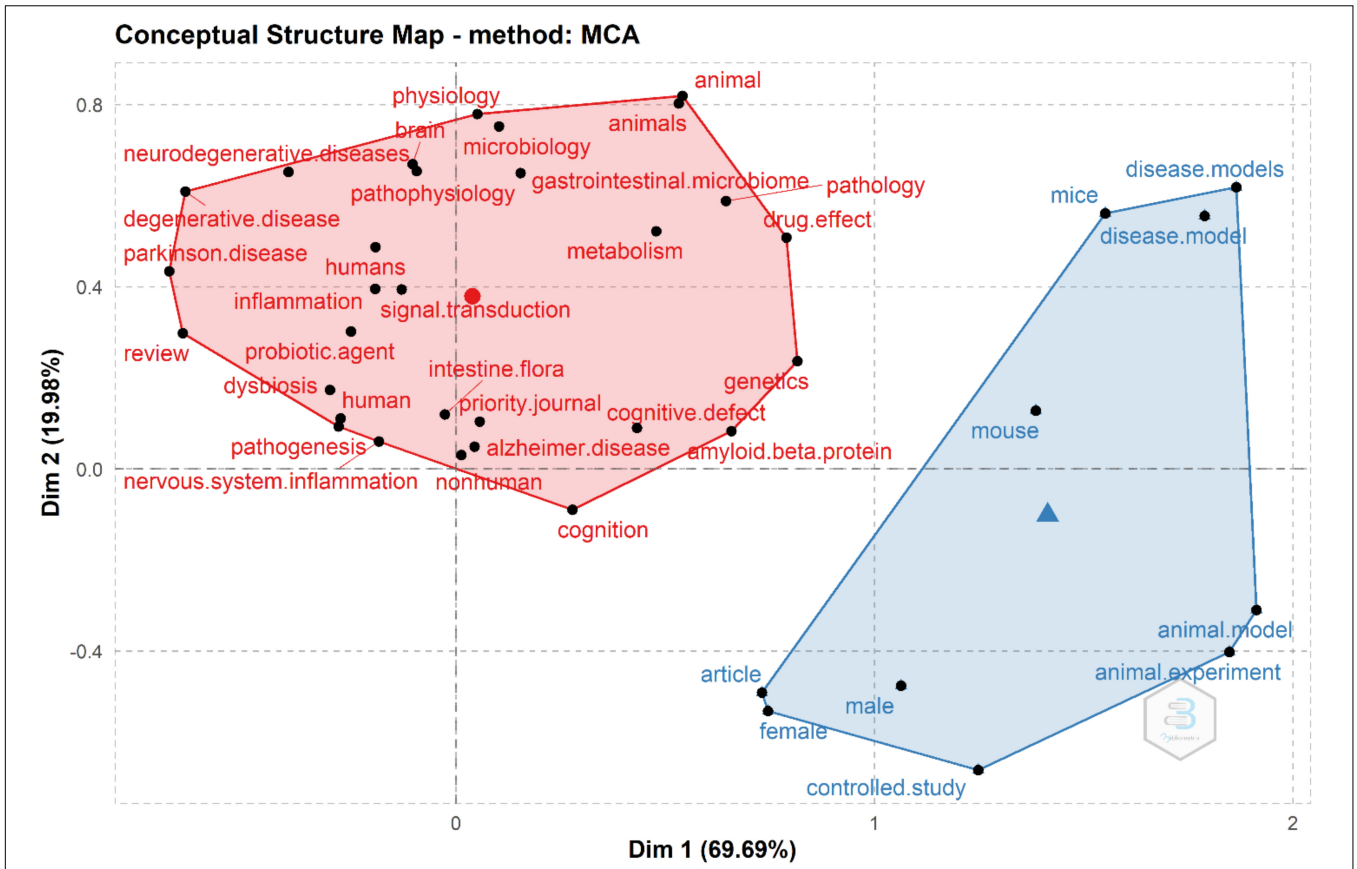

FIGURE 8 | Conceptual structure map. We observe two clusters, the one related with mouse models on the relationship between the gut microbiome and AD, and the other that studies the gut microbiome composition and dysbiosis with or without the impact of other methods in humans like probiotic agents for the restoration of cognition and reduction of amyloid-β fragments.

We also identified key journals in the subject. The International Journal of Molecular Sciences, Q1 and 2020 JCR impact factor of 5.923 and open access covered 4.10% of publications from our search. In terms of citations, Scientific Reports Q1, 2020 JCR impact factor of 4.379, open access, led with 1,461 followed by Journal of Alzheimer's Disease Q1, 2020 JCR impact factor of 4.472 with 1,052. From the top 11 journals, Pharmacological Research is the oldest, with a publication on the matter being 8 years of existence while the other account for less than 5 years. Furthermore, Progress in Neuro-Psychopharmacology and Biological Psychiatry is the newest of these journals with only 2 years of discussion on the topic. The higher h-index among the journals corresponded to the Journal of Alzheimer's Disease (12), followed by Scientific Reports (11), and a tie in third place between International Journal of Molecular Sciences and Nutrients with an h-index of 10 each. Somehow without a surprise, we found that the United States of America and China are the leaders in terms of NPs with 456 and 446 total articles, respectively. Italy has the third place with 226 publications, counts onward an average of approximately 63 for the following seven countries.

It is encouraging to discover that the two most cited papers (587 and 450 cites, respectively) hit right on target "Interactions between the microbiota, immune and nervous systems in health and disease" published in Nature Neuroscience, and "The microbiota-gut-brain axis" in Physiological Reviews. Both have come a long way from the 5th more cited article "Brain-gut interactions in inflammatory bowel disease" published in 2013. While Nature Neuroscience published the most cited paper to date, it is Scientific Reports the journal that amassed most citations with two of the most cited papers in the topic (4th and 8th). We hypothesize that this might be because the latter is fully open access while the former is still in a transition mode but not entirely open access.

While the tendency to publish in this theme will certainly increase in the upcoming years, a bibliometric analysis allows us to explore trending topics (**Figure 7**) that we can find one of the most interesting is "short-chain fatty acids." This corresponds to some novel theories that state the role of these molecules in the development of AD while they are derived as microbial metabolites due to gut dysbiosis (Marizzoni et al., 2020). This trend has just gained interest since 2019.

A major goal in AD research is to be able to diagnose the disease beyond the cognitive test complementing it with other clinical biomarkers. MCI is normally considered an initial stage

for AD. This test, in conjunction with the role of the gut microbiome in early stages of the disease, has the potential to improve the diagnostic accuracy.

Another trend that will most likely increase because its direct association to treatment is "fecal microbiota transplantation." This technique initially developed to treat patients with *Clostridium difficile* infection (CDI) after prolonged antibiotics schemes (Cammarota et al., 2014), however, its use to control gut microbiome dysbiosis has been increasing and it is being examined as a possible treatment for pathologies, such as AD or Parkinson in *in vivo* models (Sun et al., 2019). Furthermore, a case reported by Hazan in 2020 showed cognition improvement in a patients with AD-CDI after a fecal transplant, increasing the Mini-Mental State Examination (MMSE) score from 20 to 29 points within 6 months (Hazan, 2020). Another case was reported this year with similar findings, a 90-year-old woman with AD and severe CDI improved after the fecal transplant; subsequently, this increase in cognitive function was associated with certain bacterial genera, such as *Bacteroides, Bacteroidia, Tannerellaceae,* and *Actinobacteria.* Moreover, the short-chain fatty acids were found to be significantly different between before and after fecal microbiota transplant (Park et al., 2021). Other researchers have characterized the gut microbiome of patients with AD through fecal samples, finding that reductions in *Faecalibacterium* and increases in *Bifidobacterium* were significantly correlated with clinical indicators of the AD (Ling et al., 2021).

Animal models have been used to understand the microbiome role in AD. We observe that in "appps transgenic mice" trend. However, we can soon expect more human-oriented research, particularly in microbiota research. The structure of knowledge analysis shows two main groups. On the one hand, there are murine models of AD, which mainly presents the basic science research focused on transgenic animals and the development of new models, characterized by the words mice, animals, and model. On the other hand, a cluster focused on the role of dysbiosis in the disease within human population. Not only in the role of the microbiota in neurodegenerative diseases, such as AD or Parkinson, but also on the role of probiotics and prebiotics in the management of the disease.

Research overall has been moving toward a better understanding of the microbiome's role in human health. The gut-brain axis has been followed by other terms, such as the most recently described gut-skin axis, which explores the links between the gut composition and different skin processes, such as keratinization and modulation of the cutaneous immune response (Salem et al., 2018). This new field has motivated researchers in several disciplines to study the microbiome and its effects allowing us to steadily comprehend the role the microbiota plays and how to use them to our benefit. AD is not the exception, by September 14, 2021, we found 14 clinical trials registered in clinicaltrials.gov that explore this relationship. Currently, nine out of those are still recruiting participants, three have been completed, one has been terminated, and another is reported as "unknown." While this is a still a small amount, it is indeed the start of the novel research in this area. While some steps have already been taken and some degree of characterization has been achieved, there is still some way to research (Rinninella et al., 2019).

Some gut microorganisms have already been hypothesized to play a role in the pathogenesis of AD, such as *Lactobacillus spp.* beneficial to brain functions leading to an optimistic therapy to improve cognitive function (John et al., 2021). Other researchers correlate periodontal hygiene and microbial composition in the mouth to the development of the disease, suggesting a possible therapeutic in the future (Ryder and Xenoudi, 2021). Metabolic diseases have already addressed the role of prebiotics as modifiable factors in insulin resistance diseases with products, such as grenade juice in some patients with the presence of enzymes as urolithin A (Li et al., 2015). The use of non-antibiotic medications has also been described as a gut microbiome modulator; it has even been tried as a novel therapeutic agent. Research within this subject has shown that atorvastatin, a drug from the statins family, can be used to treat hypercholesterolemia and prevent cardiac disease by modifying the gut microbe composition diminishing proinflammatory species and increasing anti-inflammation ones, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* (Khan et al., 2018). Dietary interventions and the supplementation with probiotics of the studied bacteria or other commensal organisms are known to increase the target inside the gut and have been widely explored in metabolic diseases, cancer (Huang et al., 2019), and neurological diseases, such as multiple sclerosis (Mirza et al., 2020) and epilepsy (Dooling and Costa-Mattioli, 2018).

CONCLUSION

The role of microorganisms in non-infectious diseases has been growing in recent years. A case of pathogenesis of virus in Epstein-Barr or bacteria, such as *Helicobacter pylori* and its association to gastric cancer and gastritis are only two examples. We are now entering an era in which the question is no longer whether or not the microbiome has an impact on human health. Investigations in AD are beginning to open into this field, and we are at the start of an exponential growth in knowledge generation as well as hope for accurate diagnosis and treatment for AD. This work presents a trending analysis of emerging topics addressing AD. Some will not only remain in the upcoming years but may become the future for new drugs and a step forward toward precision medicine. Microbiota composition varies largely from person to person and even more so from population to population, mainly because of diet and lifestyles. Hence, studies focusing merely on microbiota composition may not be sufficient. There should be an increase in longitudinal studies with follow-ups that evaluate treatments and response and in before-and-after alpha and beta diversities of microbes. Research into whether dysbiosis can be treated with fecal transplantation, the use of prebiotics or probiotics. We know AD is highly correlated to metabolism, and in turn, metabolism is driven primarily by the microbiota. We expect this work not only presents a summary of what has been done

so far but it will serve as an insight into the future possibilities of AD treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AT-C, CR-E, and DC-A: conceptualization, investigation, writing original draft preparation, and methodology. AT-C: software and data curation. AT-C, DC-A, and AM-T: validation. AT-C, DC-A, AM-T, and CR-E: formal analysis. AT-C and CR-E: resources. AM-T and CR-E: writing-review and editing. AT-C and AM-T: visualization. CR-E: supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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ORIGINAL RESEARCH
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Absence of Bacteria Permits Fungal Gut-To-Brain Translocation and Invasion in Germfree Mice but Ageing Alone Does Not Drive Pathobiont Expansion in Conventionally Raised Mice

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Age-associated changes in the structure of the intestinal microbiome and in its interaction with the brain via the gut-brain axis are increasingly being implicated in neurological and neurodegenerative diseases. Intestinal microbial dysbiosis and translocation of microbes and microbial products including fungal species into the brain have been implicated in the development of dementias such as Alzheimer's disease. Using germ-free mice, we investigated if the fungal gut commensal, *Candida albicans*, an opportunistic pathogen in humans, can traverse the gastrointestinal barrier and disseminate to brain tissue and whether ageing impacts on the gut mycobiome as a predisposing factor in fungal brain infection. *C. albicans* was detected in different regions of the brain of colonised germ-free mice in both yeast and hyphal cell forms, often in close association with activated (Iba-1$^+$) microglial cells. Using high-throughput ITS1 amplicon sequencing to characterise the faecal gut fungal composition of aged and young SPF mice, we identified several putative gut commensal fungal species with pathobiont potential although their abundance was not significantly different between young and aged mice. Collectively, these results suggest that although some fungal species can travel from the gut to brain where they can induce an inflammatory response, ageing alone is not correlated with significant changes in gut mycobiota composition which could predispose to these events. These results are consistent with a scenario in which significant disruptions to the gut microbiota or intestinal barrier, beyond those which occur with natural ageing, are required to allow fungal escape and brain infection.

Keywords: ***Candida albicans*, gut-brain, ITS1 sequencing, mycobiome, pathobiont, dementia, ageing**

INTRODUCTION

Ageing is the dominant risk factor associated with the development of neurodegenerative dementias. Altered intestinal microbiota structure and function (microbial dysbiosis) with age is considered a contributing factor in the development of age-associated chronic low-grade systemic and tissue inflammation, termed inflammageing (Boulangé et al., 2016;

Fransen et al., 2017; Thevaranjan et al., 2017; Boehme et al., 2021; Parker et al., 2021) which contributes to neuroinflammation and neurodegenerative disease (Scott et al., 2017; Boehme et al., 2019). Whilst bacterial community diversity, composition, and function changes significantly with age in both animal models and in humans (Claesson et al., 2011; Yatsunenko et al., 2012; Langille et al., 2014; Clark et al., 2015; O'Toole and Jeffery, 2015), comparatively little is known about the impact of ageing upon other members of the intestinal microbiota, including viruses, archaea, and fungi. Fungal diversity in the gut microbiome is decreased in adults compared to infants and children, with fungal richness being higher in females than males regardless of age (Strati et al., 2016). However, little is known of the intestinal fungal composition of elderly versus young adults, or whether fungal composition is altered due to ageing *per se*, or results from changes in behaviour and lifestyle, which occur concomitantly with ageing.

Fungi account for a relatively small fraction of the total human faecal microbiota (10^5–10^6 cells/g faecal matter compared with 10^{11} bacterial cells/g) (Huseyin et al., 2017) and for around 0.1% of the faecal microbiota gene content (Qin et al., 2010; Li et al., 2014; Sender et al., 2016). However, this is likely to be an underestimate of the true fungal intestinal load due to the comparatively smaller number of fungal reference genomes currently available, bias in microbiome analyses introduced by extraction and sequencing methods sub-optimal for mycobiome characterisation (Richard and Sokol, 2019), and the issue that faecal sampling is unlikely to accurately reflect fungal load throughout the GI tract and at the epithelial surface.

Fungal pathogens acquired externally to the host, and reactivation of latent infections, can lead to systemic fungal infection, resulting in significant pathology and mortality (Brown et al., 2012). In some circumstances, fungal species within the gut microbiota, which are normally well tolerated, may disseminate via the circulation to other sites including the brain. For example, cryptococcal meningoencephalitis can occur in immunocompromised individuals or those undergoing specific drug treatment, as well as in premature infants of very low birth weight (Gottfredsson and Perfect, 2000). Invasive candidiasis is a potentially life-threatening fungal infection caused by several *Candida* species, the most common being *Candida albicans*, a dimorphic fungus, which is a common human gut commensal (Brown et al., 2012). When able to penetrate the body's barrier sites, *C. albicans* can cause superficial mucosal infections, and in some cases severe systemic sepsis with associated mortality exceeding 70% (Brown et al., 2012; Allert et al., 2018).

Increased risk of developing Alzheimer's disease (AD) has been associated with infections of the central nervous system (CNS), potentially via impacting innate immune mechanisms and/or protein misfolding (Mawanda and Wallace, 2013). Viral, bacterial, and fungal species have been investigated in this context (Hammond et al., 2010; Huang et al., 2014; Fung et al., 2017; Dominy et al., 2019; Tetz et al., 2020); however, no single infectious agent has to date been demonstrated to be causative in AD onset. Fungal antigens from a variety of species have been detected in the serum of AD patients, including *C. albicans* and a number of other *Candida* species (Pisa et al., 2015b; Alonso et al., 2018). In addition, analysis of post-mortem brain tissue from AD patients and healthy controls identified genetic material from multiple fungal species (including *Candida*), fungal proteins, and fungal cell bodies unique to the brains of AD patients (Alonso et al., 2014; Pisa et al., 2015a,b).

Despite these findings, the concept of a brain-associated microbiota remains highly controversial, and there is no compelling evidence of microbial representation in the CNS of normal healthy hosts. More plausible is that microbes, including fungi, escape confinement in the gut or elsewhere and disseminate more widely when barrier sites and/or the immune system have been seriously compromised. In ageing, declining immune function (immunosenescence), inflammageing, intestinal microbial dysbiosis, and the high incidence of co-morbidities create an environment more permissive to microbial translocation to the circulatory system and dissemination to tissues beyond the gastrointestinal tract (GIT).

Animal studies of the mycobiome and fungal infection can control for or eliminate most of the confounding factors which complicate interpretation of fungal changes in ageing human populations. Mice, for example, harbour many of the same fungal taxa which inhabit the human gut, with a characteristic feature of both the murine and human gut mycobiome being the dominance of the Ascomycota and Basidiomycota phyla (Hallen-Adams et al., 2015; Nash et al., 2017; Ward et al., 2018; Doron et al., 2019; James et al., 2020; Mims et al., 2021). *C. albicans* is frequently present in the healthy human gut as a benign commensal (Brown et al., 2012; Nash et al., 2017; James et al., 2020) and can also be found in captive-bred mice (Doron et al., 2019; Mims et al., 2021), although it may be absent in wild murine species (Bendová et al., 2020).

Multiple *Candida* species can colonise mouse models and persist in the GIT (Prieto and Pla, 2015). Systemic dissemination and candidiasis is evident in immunocompromised mice, however, this often requires high initial inoculums for non-*albicans Candida* species (Conti et al., 2014; Segal and Frenkel, 2018). *C. albicans* can also stably colonise mice and has been used to study fungal intestinal colonisation and dissemination in neonatal mice, antibiotic- or chemotherapy-treated adult mice, and germ-free mice (Field et al., 1981; Kinneberg et al., 1999; Mellado et al., 2000; Wiesner et al., 2001; Schofield et al., 2005; Koh et al., 2008; Koh, 2013). When administered intravenously, *C. albicans* can infect the mouse brain and cause localised cerebritis (Wu et al., 2019).

Here we assessed whether fungal cells could traverse the intestinal barrier and disseminate to the brain by colonising C57BL/6 germ-free mice with a human-derived isolate of *C. albicans* by oral gavage, using confocal microscopy to assess fungal cell dissemination throughout the brain (a graphical overview is shown in **Figure 1**). High-throughput amplicon sequencing of the fungal internal transcribed spacer 1 (ITS1) region was used to investigate the effect of ageing on the composition and diversity of the murine gut mycobiome, and to identify potential fungal pathobionts.

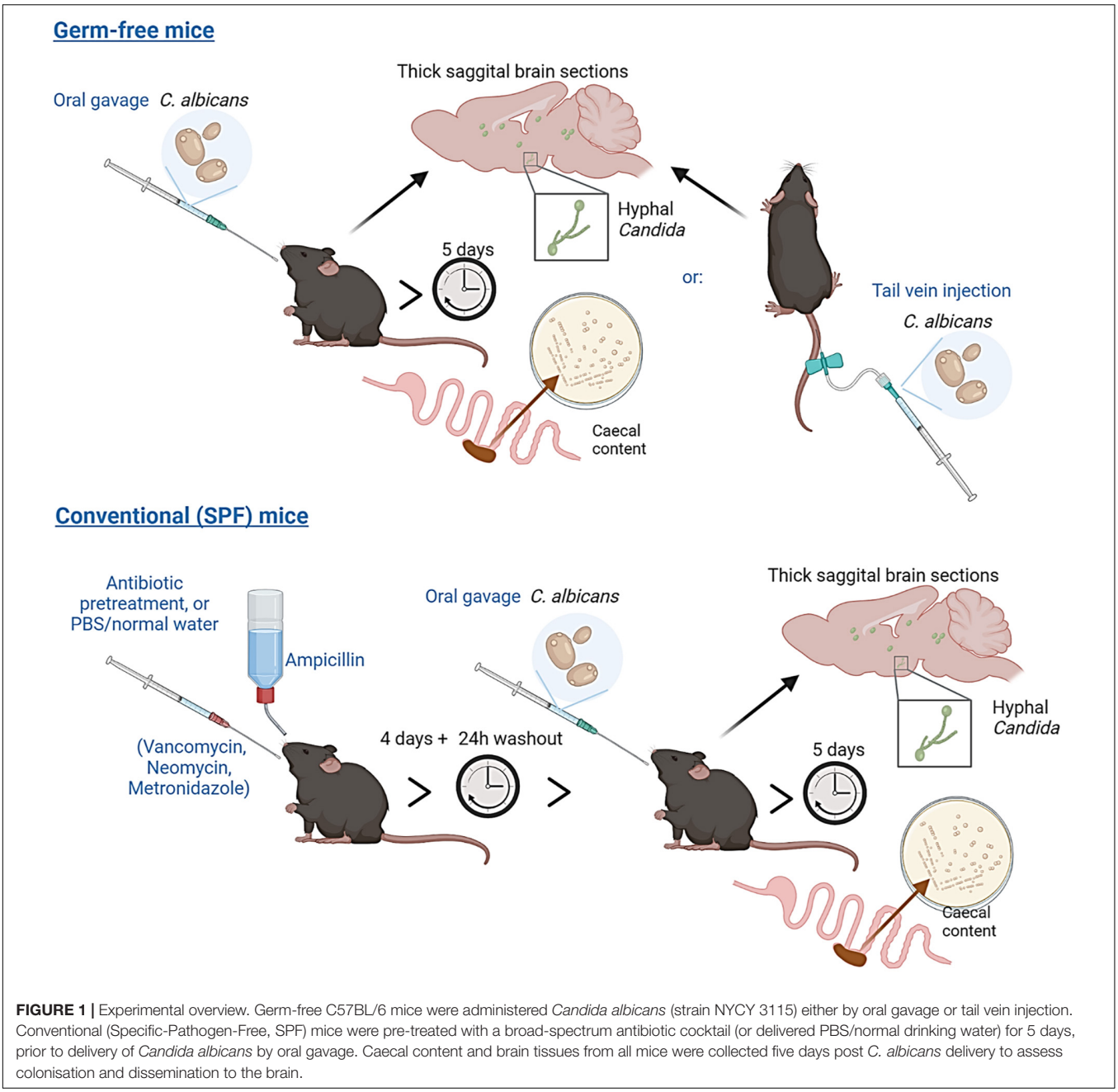

FIGURE 1 | Experimental overview. Germ-free C57BL/6 mice were administered *Candida albicans* (strain NYCY 3115) either by oral gavage or tail vein injection. Conventional (Specific-Pathogen-Free, SPF) mice were pre-treated with a broad-spectrum antibiotic cocktail (or delivered PBS/normal drinking water) for 5 days, prior to delivery of *Candida albicans* by oral gavage. Caecal content and brain tissues from all mice were collected five days post *C. albicans* delivery to assess colonisation and dissemination to the brain.

MATERIALS AND METHODS

Yeast Strain and Growth Conditions

Candida albicans strain (NCYC 3115) is a human clinical isolate from patient faeces collected in a United Kingdom hospital and was provided by the National Collection of Yeast Cultures (Norwich, United Kingdom). For inoculum preparation, stocks were cultured in YM liquid medium (10g/L glucose, 3g/L malt extract, 5g/L peptone, 3g/L yeast extract) at 30°C for 48h with shaking (200 rpm). Cells were collected by low-speed centrifugation (3,000 rpm, 5 min), washed twice in sterile phosphate buffered saline (PBS) and re-suspended in PBS prior to delivery to mice. Fungal colonisation was assessed by measuring CFUs (colony forming units) of *C. albicans* present in the caecum of each mouse. Caecal contents, collected five days post-delivery, were mechanically homogenised in PBS to 100 mg/mL then serially diluted and spread plated onto YM medium. All agar plates were incubated aerobically at 37°C, and colony counts measured after 2 days incubation. Colony morphology was also assessed (and counts determined) by visual inspection, with colonies of differing morphology (morphotypes) selected and stocked for additional phenotyping. YM broth cultures derived from two, post-passaged, colony morphotypes (white and domed vs. darker and flattened) were incubated at 37°C, without

agitation, and examined after 3 days by standard light microscopy for the presence/absence of hyphal and pseudohyphal cells. Species identity was confirmed by standard colony PCR using *C. albicans*-specific primers (Asadzadeh et al., 2018), and by ITS1 sequencing (White et al., 1990; Gardes and Bruns, 1993). Details of all fungal primers used in this study are provided in **Supplementary Figure 4**.

Animal Experiments

All experiments involving animals were performed in accordance with EU and United Kingdom Home Office Legislation and local Animal Welfare and Ethical Review Body approval. Male and female specific pathogen-free (C57BL/6 -SPF) mice aged 3 months or 24 months, and male germ-free (C57BL/6-GF) mice aged 3 months, were maintained in individually ventilated cages (SPF) or in sterile isolators (GF) in adjacent rooms of the Quadram Institute Germ-Free mouse facility within the University of East Anglia Disease Modelling Unit. All mice received autoclaved water and were fed RM3 (SPF) or RM3-(Autoclavable) (GF) diet (Special Diets Services). All mice were maintained under 12-h light-dark cycle. A dose of 2.5×10^5 ($n = 5$) or 5×10^5 ($n = 5$) *C. albicans* cells re-suspended in 200 μL PBS was administered to germ-free animals by oral gavage, whilst a lower dose of 2.5×10^4 cells in 100 μL of PBS was used for tail vein injection control mice. SPF mice ($n = 16$, 8 females and 8 males) were pre-treated for four days with either PBS ($n = 8$) or a cocktail of broad-spectrum antibiotics (VMNA, 0.5 mg/mL vancomycin, 1 mg/mL metronidazole and 1 mg/mL neomycin delivered in 200 μL sterile water by daily oral gavage, and 1 mg/mL ampicillin delivered via drinking water, available *ad libitum*), $n = 8$. Following a 24 h washout period 5×10^5 *C. albicans* cells re-suspended in 200 μL PBS were administered by oral gavage. Mice were then maintained in individually ventilated cages until sacrifice. Brains and caecal content were harvested at day 5 post-inoculation and used for downstream analysis.

Formalin-fixed paraffin-embedded brains were sectioned at 5 μm. Sagittal vibratome sections of 100 μm thickness were prepared from PFA-fixed whole brains embedded in low-melt agarose, a method adapted from Snippert et al. (2011) and were cleared post-staining and prior to mounting using RapiClear (CamBioscience, Cambridge, United Kingdom). *C. albicans* was visualised in sections using a rabbit polyclonal anti-*C. albicans* antibody (NB100-64750 Novus Biologicals, 1:100), and for activated microglia/macrophages using rabbit anti-Iba-1 (ab178846, Abcam, 1:100) for single staining or Abcam ab150167 (1:100) for co-stains. Secondary antibodies used were goat anti-rabbit IgG Alexa Fluor-594 (Invitrogen, 1:100), Goat Anti-Rat Alexa-647 (ab150167, Abcam, 1:500) or donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen, 1:500). Nuclei were stained with Hoechst 33258. Images were collected and analysed using a Zeiss LSM880 confocal microscope and ZEN 2010 software, and FIJI/ImageJ v2.1.0 (Schindelin et al., 2012). *C. albicans* cells were quantified from 100 μm sagittal vibratome sections taken starting from the midline of the left hemisphere of the brain, five sections were taken from each brain sample from *C. albicans*-colonised germ-free mice ($n = 5$ mice), non-colonised control germ-free mice ($n = 3$ mice) and from *C. albicans*-colonised SPF mice receiving either antibiotic or PBS only pre-treatment ($n = 8$ mice/group). Cells were not included in counts if they were obviously within vessels, or were on the periphery of the section and therefore considered to not be truly within the brain tissue.

Genomic DNA Extraction

Faecal pellets were collected from temporarily singly housed SPF mice using sterile picks and sterile RNA-DNA-free microtubes and were stored at −70°C prior to processing and DNA extraction. For fungal DNA amplification, total microbial DNA was extracted from ∼50 mg of faeces from each animal using the QIAamp PowerFecal Pro DNA kit (QIAGEN, Hilden, Germany) and following the manufacturer's protocol. In addition, all samples were homogenised using a FastPrep-24 benchtop instrument (MP Biomedicals, Irvine, CA, United States) at 6.0 m/s for 1 min. Extracted DNA was quantified and quality checked using the Qubit 3.0 fluorometer and associated Qubit dsDNA BR Assay Kit (Thermo Fisher, Waltham, MA, United States). DNA samples were stored at −20°C prior to further analysis.

Internal Transcribed Spacer 1 Amplification and Sequencing

The fungal ITS1 region was amplified from 100 ng of faecal DNA by using the pan-fungal ITS1F and ITS2 primer set (White et al., 1990; Gardes and Bruns, 1993), with each primer modified at the 5' end to include an Illumina adapter tail, using KAPA2G Robust DNA polymerase (Kapa Biosystems, Wilmington, MA, United States). Amplification was performed at 94°C (5 min) with 35 cycles of 92°C (30 s), 55°C (30 s), 72°C (45 s), and a final extension of 72°C (5 min). Amplification reactions were set up in duplicate for each DNA sample, and negative (PCR dH_2O) and positive controls (0.01 ng of *C. albicans* DNA) were included in each PCR run. Following ITS1 PCR, a 0.7x SPRI purification using KAPA Pure Beads (Roche, Wilmington, MA, United States) was performed and the purified DNA was eluted in 20 μl of 10 mM Tris-HCl. In a second PCR, library index primers were added using a Nextera XT Index Kit v2 (Illumina, Cambridge, United Kingdom) and amplification was performed at 95°C (5 min) with 10 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s), and a final extension of 72°C (5 min). Following PCR, libraries were quantified using the Invitrogen™ Quant-iT dsDNA high sensitivity assay kit (Thermo Fisher) and run on a FLUOstar Optima plate reader (BMG Labtech, Aylesbury, United Kingdom). Libraries were pooled following quantification in equal quantities. The final pool was SPRI cleaned using 0.7x KAPA Pure Beads, quantified on a Qubit 3.0 fluorometer and run on a High Sensitivity D1000 ScreenTape (Agilent Inc., Santa Clara, CA, United States) using the Agilent Tapestation 4200 to calculate the final library pool molarity. The pool was run at a final concentration of 8 pM on an Illumina MiSeq instrument using the MiSeq© v3 (2 × 300 bp) Kit (Illumina). All sequencing

was performed at Quadram Institute Bioscience, Norwich. The raw data were analysed locally on the MiSeq instrument using MiSeq reporter.

Mycobiome Characterisation

Illumina MiSeq reads were analysed using the automated pipeline Dadaist2, a dedicated workflow for ITS profiling (Ansorge et al., 2021). The quality profile of the raw reads (in FASTQ format) was assessed using *Fastp* 0.20.0 (Chen et al., 2018), which was also used to remove reads with ambiguous bases. Locus-specific primers were removed using *SeqFu* 1.8 (Telatin et al., 2021). The identification of representative sequences was performed using DADA2 (Callahan et al., 2016), to produce a set of amplicon sequence variants (ASVs), and their taxonomic assignment was determined using the UNITE Fungal ITS database (release 8.2) (Nilsson et al., 2019). The multiple alignment of the representative sequences was performed using ClustalO (Sievers and Higgins, 2021) and the guide tree was produced using FastTree (Price et al., 2009). Data normalization and diversity were produced using the Rhea scripts (Lagkouvardos et al., 2017). The output feature table, taxonomic classification, phylogeny and metadata files were exported and further analysed using MicrobiomeAnalyst (Dhariwal et al., 2017) and the built-in plotting provided by Dadaist2. Every ASV with a zero count in all samples was removed to assess alpha diversity measures.

Statistical Analysis

Three alpha-diversity measures were used to estimate fungal taxa richness (Chao1) as well as taxa richness and evenness (Shannon and Simpson) using MicrobiomeAnalyst (Dhariwal et al., 2017). Data was not rarefied, was scaled by total sum scaling, was non-transformed, and statistical significance was assessed by Student's *t*-test (threshold for significance $P < 0.05$). For comparison of specific taxa, data were CLR-transformed prior to comparison between two groups by *t*-test.

Other Software

Figure 1 was created using BioRender illustration software: https://biorender.com/.

RESULTS

Candida albicans Translocates From Gut to Brain in Monocolonised Germ-Free Mice and Induces an Inflammatory Response in the Brain

Candida albicans (NCYC 3115) was administered by oral gavage to two groups of germ-free adult C57BL/6 mice, in doses of either 2.5×10^5 or 5×10^5 cells. A third group were administered an inoculum of 2.5×10^4 cells by tail vein injection, a dose previously shown to result in fungal translocation to the brain with no lethality (Wu et al., 2019). Control mice received PBS alone by gavage. Both delivery routes, oral or intravenous, resulted in successful colonisation of the GIT, as measured by CFU recovered from caecal content five days post-delivery (**Figure 2A**). Oral administration of 2.5×10^5 cells resulted in caecal counts ranging from 1×10^5 to 1×10^7 CFU, whereas administration of the higher dose of 5×10^5 cells resulted in caecal counts ranging from 6.2×10^6 to 2.2×10^7 CFU. Mice receiving yeast cells intravenously had lower caecal CFU counts of $8 \times 10^5 - 3 \times 10^6$. Caecal content from control mice receiving PBS alone yielded no fungal colonies. Species identity of colonies was confirmed by standard colony PCR using *C. albicans*-specific primers (Asadzadeh et al., 2018).

Two types of post-passage *C. albicans* colonies were cultured from the caecal contents (**Figures 2B–E**), a white and domed morphotype (as per the wild-type), and a darker and flattened morphotype, chromatically and morphologically resembling the previously described Gastrointestinally indUced Transition (GUT) phenotype (Pande et al., 2013). Approximately 66% of colonies recovered from the caecal content of mice in the present study were of this 'GUT'-like phenotype, suggesting substantial adaptation of the administered wild-type *C. albicans* to the C57BL/6 germ-free gut. Cultures derived from this phenotype failed to produce hyphae, either on solid or in liquid media, when grown at 37°C. This was in marked contrast to white phenotype-derived cultures which readily produced hyphae (and pseudohyphae) when grown at this elevated temperature (data not shown).

In mice receiving *C. albicans* orally of either lower (2.5×10^5) or higher dose (5×10^5) inoculum, and in mice receiving the inoculum intravenously, *C. albicans* cells were detected in brain tissue five days post-colonisation by immunostaining with an anti-*C. albicans* antibody (**Figures 3A–F**). Individual *C. albicans* cells and cell clusters were found throughout the brain, in the ventricular spaces, cerebellum, hypothalamus, midbrain and cortex. Clusters and individual *C. albicans* cells were confirmed to be within the plane of the brain tissue by imaging of z-stacks (**Figure 3A**). Individual *C. albicans* cells and cell clusters were frequently found in, or adjacent to, vessels within the brain tissue (**Figure 3B**), and within the ventricular spaces, including the cerebral aqueduct (**Figure 3C**). *Candida albicans* cells were frequently surrounded by Iba-1^+ cells resembling both resident microglia and infiltrating macrophages within or exiting vessels (**Figures 3D,E**), indicating induction of an inflammatory microglial/macrophage response. In one mouse, striking granuloma-like clusters of fungal and Iba-1^+cells were seen in the posterior parietal cortex (**Figure 3D**), which plays a key role in spatial representation of objects for action planning and control. Less frequently hyphae were detected within brain tissue samples (**Figure 3F**) indicating *C. albicans* cells were viable and in an invasive form. No fungal cells or similar microglial clusters were observed in PBS control germ-free mouse brain samples. As mice were not transcardially perfused before brain harvest, we cannot completely rule out that a small number of counted *C. albicans* cells may have been within vessels/capillaries that were sectioned or optically sliced in such a way that we did not identify the vessels. However, the identification of hyphal forms within the brain tissue, and clusters of microglia identified surrounding *C. albicans* cells strongly suggests active invasion of the brain tissue as opposed to circulating yeast

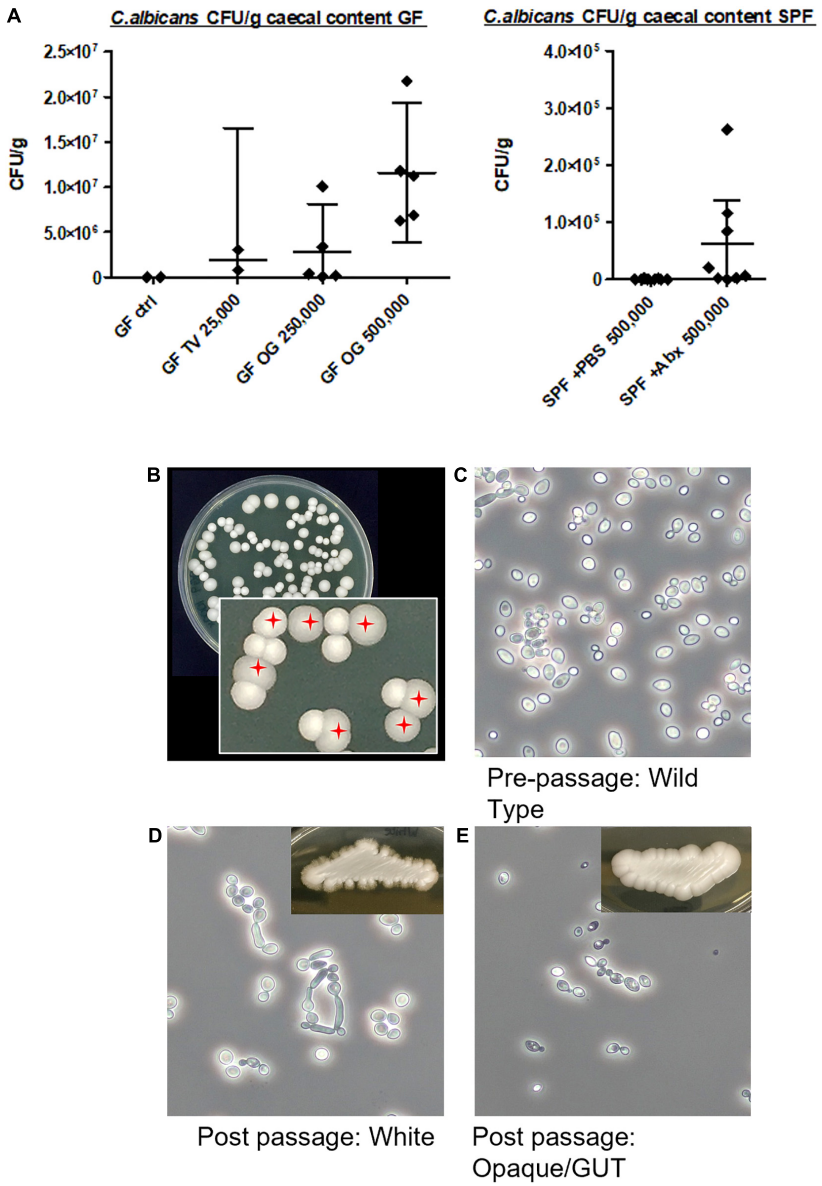

FIGURE 2 | Colonisation of the caecum by *Candida albicans* NCYC 3115 following tail vein injection or oral gavage. **(A)** Colony-forming units (CFU) recovered from caecal content following delivery of *C. albicans* NCYC 3115 to germ-free mice (left) or SPF mice (right). No colonies were present in the caecal content of germ-free control mice administered PBS alone (GF ctrl). TV, tail vein; OG, oral gavage. Numbers on x-axis labels denote amount of *C. albicans* cells administered, error bars denote 95% CI. **(B)** Example YM agar plate with zoom inset showing two phenotypically distinct *C. albicans* colony morphotypes recovered from caecal contents. White and domed morphotype and darker and flattened/Gastrointestinally indUced Transition (GUT) morphotype (red crosshairs). **(C–E)** Photomicrographs of pre-passage wild type cells **(C)**, post-passage white phenotype cells **(D)**, and post-passage darker/GUT phenotype cells **(E)**, all grown at 37°C for 3 days in YM broth.

cells in dissemination form (Gow et al., 2011; Noble et al., 2016).

Short-Term Depletion of Gut Bacteria in Conventional Mice Permits Expansion of *Candida albicans* in the Caecum

To test whether depletion of the gut bacterial community in conventional mice would also allow for fungal expansion and dissemination, we pre-treated SPF mice with a short course of broad-spectrum antibiotics (VMNA), or PBS, prior to *C. albicans* delivery by oral gavage (**Figure 1**). Colony counts from caecal content (**Figure 2A**) showed increased caecal colonisation in antibiotic-pre-treated SPF mice (SPF + Abx) compared with PBS-pre-treated SPF controls, but at much lower levels compared to *C. albicans*-colonised germ-free mice (mean 1.15×10^7 cells/g caecal content in colonised germ-free versus 6.15×10^4 in colonised SPF + Abx). On analysing the brains of the SPF

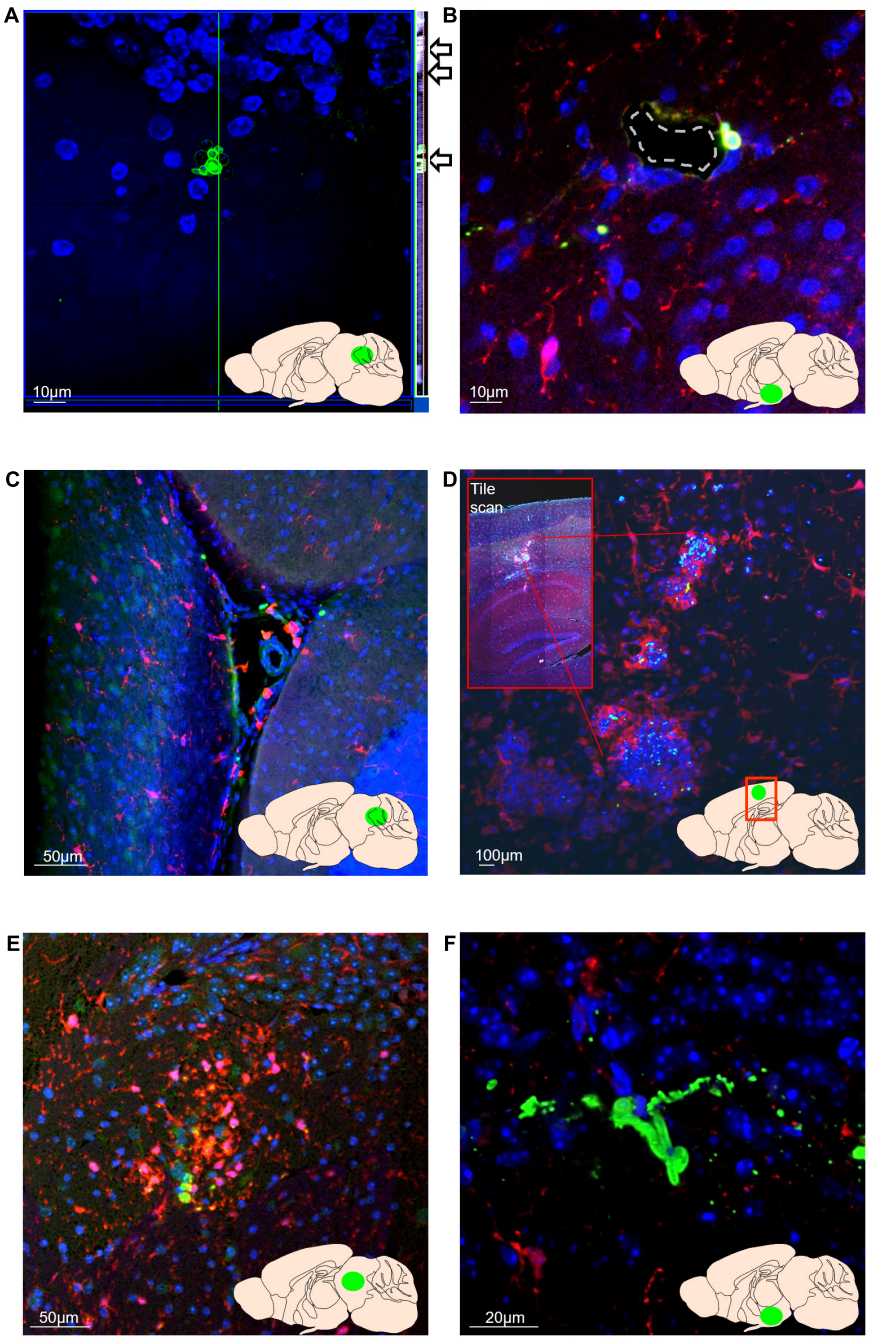

FIGURE 3 | *Candida albicans* can disseminate from the gut to the brain and can grow in the invasive hyphal form within brain tissue. In all images, blue = nuclei (Hoechst), green = *C. albicans*, red = Iba-1-positive microglia/macrophages. Green area in inset brain schematic indicates approximate position of image. **(A)** *C. albicans* cells are detectable within cerebellar brain tissue at 5 days post-colonisation. Z-stack orthogonal view (side bar and arrows) shows *C. albicans* cells are in the same plane as brain cell nuclei. Orthogonal side bar brightness and contrast has been enhanced here for visibility. **(B)** *C. albicans* cells in proximity to a hypothalamic blood vessel (dashed outline). **(C)** *C. albicans* cells and Iba-1 positive macrophages within the cerebral aqueduct (lobule II granule layer visible as dense Hoechst-stained area bottom right of image). **(D)** Foci of clustered Iba-1^{+} cells (red) around *C. albicans* cells (green) within the posterior parietal association area of the cortex. Inset box shows overview tile scan of the cortex and hippocampus. **(E)** Cluster of Iba-1 + cells around *C. albicans* cells within the midbrain **(F)** Entwined hyphal *C. albicans* hyphae within the hypothalamus.

mice, we found no evidence of fungal cells, either in yeast or hyphal form, within brain sections of either PBS control or antibiotic pre-treated colonised mice, either by staining specifically for *Candida*, or by using a non-specific fungal cell wall stain (example expected staining of positive control shown in **Supplementary Figure 1B**).

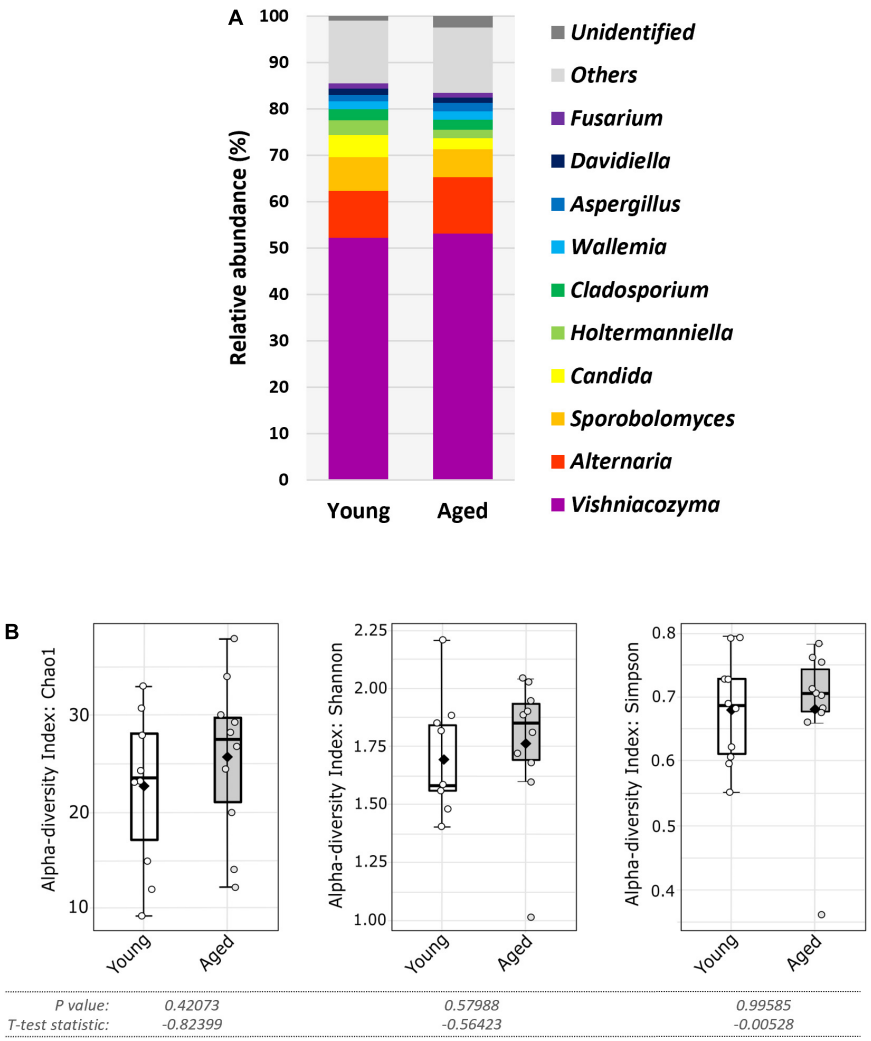

FIGURE 4 | Faecal fungal diversity and top ten genera in aged vs. young SPF mice. **(A)** Top ten most abundant genera (percentage mean relative abundance) in faecal samples of young vs. aged SPF mice (*n* = 10/group). **(B)** Alpha diversity (L-R: Chao1, Shannon, and Simpson indices) of faecal fungal composition of young vs. aged SPF mice (*n* = 10/group), whiskers show spread of data across all mice, solid black dot indicates the mean, horizontal line indicates the median.

Our data shows that disruption of the intestinal environment by antibiotic treatment permits increased fungal colonisation of the intestinal tract, but suggest that short term-antibiotic treatment is not sufficient to promote dissemination to the brain. On the other hand, recent data show that long-term chronic administration of antibiotics can promote systemic dissemination of both fungi and bacteria (Drummond et al., 2022). Advanced age is also associated with changing gut bacterial composition, as well as depleted barrier integrity promoting chronic systemic inflammageing (Fransen et al., 2017; Thevaranjan et al., 2017; Parker et al., 2022). Therefore, we next investigated whether the composition of the enteric mycobiota is altered in aged animals, and whether any fungal species detected are potential pathobionts/opportunistic pathogens with the capacity to cause serious infection.

Ageing Alone Is Not Sufficient to Select for or Drive Pathobiont Expansion in Specific Pathogen-Free Mice

High-throughput internal transcribed spacer 1 (ITS1) amplicon sequencing was used to characterise the faecal fungal communities in young (3-month) and aged (24-month) SPF mice. A total of 1,471,212 quality-trimmed ITS1 reads were obtained, ranging from 14,913 (A7, aged cohort) to 100,384 (Y2, young cohort), with a sample average of 73,560 reads (**Supplementary Figure 2A**). Over 2,000 amplicon sequence variants (ASVs) were used to determine the composition of the fungal microbiota in the young and aged mice at different taxonomic levels.

At the phylum level, most identified fungi in each age group belonged to either the Ascomycota or Basidiomycota

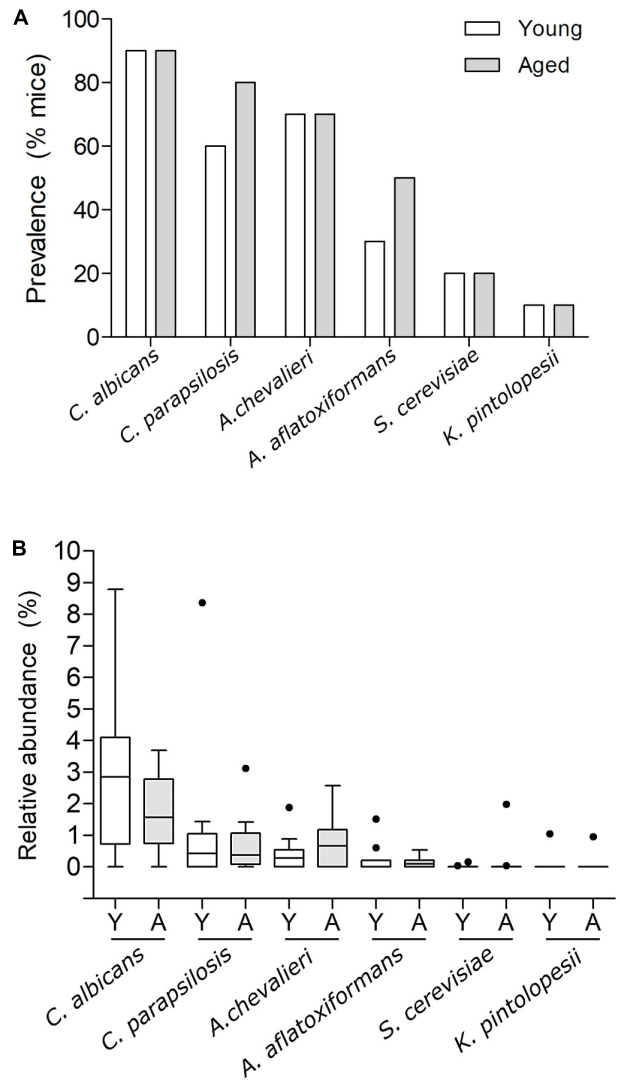

FIGURE 5 | Prevalence and relative abundance of putative gut commensal fungal species in faecal samples of young vs. aged SPF mice. **(A)** Prevalence of putative gut commensal fungal species *Candida albicans*, *Candida parapsilosis, Aspergillus chevalieri*, *Aspergillus aflatoxiformans*, *Saccharomyces cerevisiae*, and *Kazachstania pintolopesii* in faecal samples from groups of young and aged SPF mice (n = 10/group), percentage of mice harbouring each species displayed as % prevalence. **(B)** Percentage relative abundance of those same species in faecal samples from aged (A) vs. young (Y) mice (n = 10/group), Tukey whiskers, horizontal bars show the mean, outliers displayed as round points.

(**Supplementary Figure 2B**). A characteristic feature of the gut mycobiome of our C57BL/6 colony, irrespective of age, was the predominance of the Basidiomycota. At the genus level, when analyses were restricted to the most abundant genera (i.e., those with a relative abundance of 1% or more), which accounted for over 80% of all ITS1 reads, both age groups had broadly similar taxonomic profiles, with *Vishniacozyma* the predominant genus (**Figure 4A**). This basidiomycetous yeast genus had a mean relative abundancy of over 50% in each age group (young, 52.1%; aged, 53.1%). Other notable genera included *Alternaria*, *Sporobolomyces*, *Candida*, *Holtermanniella,* and *Cladosporium* (**Figure 4A**). Whilst most genera displayed comparable mean relative abundancies in both age groups (**Supplementary Figure 2B**), *Sporobolomyces*, *Candida* and *Holtermanniella* were all nominally less abundant, albeit not reaching statistical significance, in the aged mice (**Figure 4A** and **Supplementary Figure 2B**). At the genus level, there was no significant compositional change in alpha diversity between the two age groups ($p > 0.05$ in all three indices) (**Figure 4B**).

For taxa resolved to species level most were categorised as environmental fungi, typically found in soil and/or plant associated. This included *Vishniacozyma victoriae*, the most abundant taxon and a species present in every sample (**Supplementary Figure 3**). Six species were identified as candidate gut colonisers based on their ability to survive and proliferate at 37°C. These were *Aspergillus aflatoxiformans*, *Aspergillus chevalieri*, *Candida albicans*, *Candida parapsilosis*, *Kazachstania pintolopesii,* and *Saccharomyces cerevisiae*. Among these, *C. albicans* was the most prevalent in both age groups (**Figure 5A**), albeit at lower nominal relative abundance in the elderly mice compared to young mice (**Figure 5B** and **Supplementary Figure 3**), (young, 2.9%; aged 1.7%), although this was not statistically significant ($p > 0.05$) (**Figure 5B**, and **Supplementary Figure 3**). In contrast, *A. chevalieri,* which displayed similar prevalence in both cohorts (70%), was present at nominally higher relative abundance in the aged mice (young, 0.42%; aged, 0.77%), although this was also not statistically significant ($p > 0.05$) (**Figure 5B** and **Supplementary Figure 3**). *K. pintolopesii* a common rodent-associated yeast species (Kurtzman et al., 2005; Bendová et al., 2020) was found in only two of the mice (one from the 3-month-old group and one from the 24-month-old group), and at relatively low abundance (∼1%).

In summary, although the overall enteric mycobiota profiles of young and aged mice were broadly similar at the genus level, subtle differences in both the prevalence and abundance were evident at the species level. These differences were evident within a small group of putative commensal fungi, which included three opportunistic pathogens.

DISCUSSION

There is growing interest in the concept that intestinal microbial dysbiosis, as well as microbial infection, contributes to neuroinflammation and neurodegenerative disease, including dementias (Fung et al., 2017; Vogt et al., 2017). The majority of such studies have focused almost exclusively on the prokaryome, with only a small number investigating the mycobiome and implicating fungi in neurological disorders and AD (Alonso et al., 2014; Pisa et al., 2015a,b; Fung et al., 2017; Forbes et al., 2018; Ling et al., 2020). Gut-resident *C. albicans* populations represent the principal source of life-threatening disseminated candidiasis (Bougnoux et al., 2006; Gouba and Drancourt, 2015). In the gut, pathological invasion of *C. albicans* across the epithelial barrier and into the bloodstream occurs via hyphal penetration of cells,

hyphal production of a cytolytic peptide toxin (candidalysin), enterocyte necrosis and subsequent loss of epithelial barrier integrity (Dalle et al., 2010; Allert et al., 2018). Here, using oral delivery of *C. albicans* cells to the GIT of germ-free C57BL/6 mice, we demonstrate that *C. albicans* can traverse both the intestinal and blood-brain barriers and produce hyphae within the brain. We also observed clusters of Iba-1^+ activated immune cells surrounding *C. albicans* cells, in accordance with prior reports of gliosis in mouse models of candidiasis (Lionakis et al., 2011; Wu et al., 2019). Hyphae were not found within the brains of SPF C57BL/6 mice administered *C. albicans* via intravenous injection (Wu et al., 2019), which may reflect the use of different isolates of *C. albicans* between studies, or differences in SPF vs. germ-free mice.

We also found that while short-term antibiotic pre-treatment allowed for increased expansion of *C. albicans* in colonised SPF mice, compared to PBS pre-treated controls, no fungal cells, either in yeast or hyphal form, were detected in the brains of the colonised mice. Drummond and colleagues (Drummond et al., 2022) have recently shown that chronic exposure to antibiotics (>4 weeks in mice or >7 days in humans) can promote fungal and bacterial dissemination to other organs, however, brains were not assessed for fungal cell staining in the mouse studies so it is unclear whether a longer antibiotic regimen might allow for dissemination into the brain tissues. SPF mice may be resistant to brain infection by *C. albicans*, as intestinal mucins can inhibit hyphal formation by *C. albicans* (Kavanaugh et al., 2014) and the mucus layer differs in composition between SPF and germ-free mice (Johansson et al., 2014; Jakobsson et al., 2015). Furthermore, differences in immune responses between *C. albicans* cells and macrophages (Erwig and Gow, 2016) in germ-free versus SPF mice may also affect hyphal formation and persistence.

Considering that fungal processes can contribute to intestinal barrier damage and that age-related intestinal dysbiosis may increase the likelihood of gut-to-brain translocation of microbes in older hosts, we compared the fungal mycobiome of young and aged mice. Within the mycobiota of young mice three species, namely *C. albicans*, *C. parapsilosis* and *K. pintolopesii*, are recognised opportunistic pathogens of humans and mice, and are capable of causing life-threatening systemic infections (Kurtzman et al., 2005; Pfaller and Diekema, 2007). However, the relative abundances of these species were not significantly different in aged mice, nor was there any evidence of significant fungal dysbiosis in aged mice. This suggests that ageing alone is not a major driver of fungal composition in the mouse gut microbiota. In mice at least, it is more likely that other environmental factors including dietary changes, medications (antibiotics), infections and/or changes in host defence mechanisms and immune status might be required to permit fungal gut-brain translocation in aged, but otherwise healthy, hosts.

There is limited available data on the mycobiota profile of aged healthy human adults (Strati et al., 2016), although some studies have sequenced the mycobiota of patients with metabolic or neurodegenerative disease (Ahmad et al., 2020; Jayasudha et al., 2020; Ling et al., 2020; Nagpal et al., 2020). The gut microbiome in patients with mild cognitive impairment (MCI) and those with AD is reported to differ from healthy controls (Vogt et al., 2017; Zhuang et al., 2018; Saji et al., 2019). A study of patients from a United States cohort with MCI for example, found a higher proportion of the fungal genera *Botrytis, Kazachstania*, *Phaeoacremonium*, and *Cladosporium* but a reduced proportion of *Meyerozyma* compared to controls (Nagpal et al., 2020). A study of the faecal mycobiome of a Chinese cohort of AD patients reported increased abundance of the species *C. tropicalis*, *Trametes versicolor*, *Schizophyllum commune*, *Davidiella tassiana*, *Exophiala dermatitidis,* and *Erythrobasidium hasegawianum* compared to controls, but found no significant differences in the most prevalent C*andida* species, including *C. albicans* (Ling et al., 2020). In both the MCI and AD cohorts, no significant change in fungal alpha or beta diversity was seen compared to controls (Ling et al., 2020; Nagpal et al., 2020). With no evident overlap between studies of shared taxa with altered relative abundance, it is currently not possible to identify specific fungi (e.g., pathobionts) which may be associated with the development of these neurodegenerative disorders.

A major difficulty in attributing causality in MCI or AD development to an altered microbiome or mycobiome is identifying and measuring confounding factors, in particular the impact of age-associated changes in lifestyle, diet, behaviour, and co-morbidities. For example, many prescribed orally administered drugs including antibiotics, antidepressants and anti-inflammatory compounds, can significantly impact microbiota composition and function (Maier et al., 2018, 2021; Vich Vila et al., 2020), as can behavioural changes and shifts in diet or living conditions (Auchtung et al., 2018; Raimondi et al., 2019). These factors are of relevance to patients with MCI or AD. Such co-variables are minimised in animal models kept under environmentally controlled conditions and maintained on defined diets. However, when using transgenic mouse models of AD for example, it is often unclear what effects the genetic modifications may have on host immune, neural, or other responses that create an altered intestinal environment which is permissive for particular microbes and pathobionts. Whilst these considerations may help explain conflicting results between human studies, and when comparing results of animal and patient studies, it remains to be determined whether altered microbiota and mycobiota composition is a contributing factor in the development of dementias, or is merely a symptomatic or correlative phenomenon.

CONCLUSION

Here we show that in the absence of other enteric microbes, orally delivered *C. albicans* can translocate from the gut to the brain and induce cerebral inflammation. Furthermore, we also show that ageing alone did not alter the overall composition of the gut mycobiota in specific pathogen-free mice. This indicates that ageing alone is not sufficient to induce mycobiome dysbiosis and cerebral fungal infection, and that other disruptions to the gut microbiota and/or the intestinal barrier may be needed to permit gut fungal pathobiont escape and infection of the brain.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJEB49148.

ETHICS STATEMENT

The animal study was reviewed and approved by local (University of East Anglia) Animal Welfare and Ethical Review Body approval. All experiments involving animals were performed in accordance with EU and United Kingdom Home Office Legislation, revised Animals (Scientific Procedures) Act 1986 United Kingdom.

AUTHOR CONTRIBUTIONS

AP and SJ: conceptualization, methodology, investigation, formal analysis, data visualization, manuscript original draft, and review and editing. CP: investigation, formal analysis, data visualization, and manuscript content and review. AB, AG, and DB: methodology and investigation. AT: methodology, investigation, and formal analysis. SC: supervision, resources, funding, project administration, and manuscript review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.828429/full#supplementary-material

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Transplantation of fecal microbiota from APP/PS1 mice and Alzheimer's disease patients enhanced endoplasmic reticulum stress in the cerebral cortex of wild-type mice

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Background and purpose: The gut-brain axis is bidirectional and the imbalance of the gut microbiota usually coexists with brain diseases, including Alzheimer's disease (AD). Accumulating evidence indicates that endoplasmic reticulum (ER) stress is a core lesion in AD and persistent ER stress promotes AD pathology and impairs cognition. However, whether the imbalance of the gut microbiota is involved in triggering the ER stress in the brain remains unknown.

Materials and methods: In the present study, fecal microbiota transplantation (FMT) was performed with gut microbiota from AD patients and APP/PS1 mice, respectively, resulting in two mouse models with dysregulated gut microbiota. The ER stress marker protein levels in the cerebral cortex were assessed using western blotting. The composition of the gut microbiota was assessed using 16S rRNA sequencing.

Results: Excessive ER stress was induced in the cerebral cortex of mice after FMT. Elevated ER stress marker proteins (p-perk/perk, p-eIF2α/eIF2α) were observed, which were rescued by 3,3-dimethyl-1-butanol (DMB). Notably, DMB is a compound that significantly attenuates serum trimethylamine-N-oxide (TMAO), a metabolite of the gut microbiota widely reported to affect cognition.

Conclusion: The findings indicate that imbalance of the gut microbiota induces ER stress in the cerebral cortex, which may be mediated by TMAO.

KEYWORDS

FMT, Alzheimer's disease, ER stress, gut microbiota, TMAO

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that primarily involves the cerebral cortex and hippocampus (Masters et al., 2015). Pathological features of AD include extracellular senile plaques and intra-neuronal neurogenic fiber tangles (NFTs) (Hardy and Higgins, 1992; Niedowicz et al., 2011). Despite many efforts in this field, the pathogenesis of AD remains unclear. In recent years, the role of the gut microbiota in neurodegenerative illnesses has been extensively researched (Bedarf et al., 2017; Wu et al., 2017; Wright et al., 2018). The gut microbiota regulates the function and behavior of the host brain through the brain-gut axis. APP/PS1 mice treated with antibiotics and 3 × Tg AD mice administered probiotics exhibit reduced β-amyloid deposition in the brain (Minter et al., 2016; Bonfili et al., 2017). Furthermore, Aβ pathology can be propagated by transplantation of altered microbiota into sterile APP mice (Harach et al., 2017). Transplantation of gut microbiota from healthy mice attenuates brain pathology as well as memory impairment in ADLP^{APT} mice (Kim et al., 2020). These phenomena involve hypothesized mechanisms mediated primarily by vagal, neuroinflammatory, and gut microbiota metabolites (Ticinesi et al., 2018; Kowalski and Mulak, 2019). Trimethylamine-N-oxide (TMAO), a metabolite of the intestinal microbiota, has recently received increased attention (Brunt et al., 2020; Gatarek and Kaluzna-Czaplinska, 2021). In previous studies, elevated TMAO levels were shown to have a strong clinical correlation with AD and a biomarker for cognitive impairment (Vogt et al., 2018; Wang et al., 2020). Current research indicates that dysbiosis of the gut microbiota affects the onset and development of AD in different ways, rendering the brain-gut axis a promising area for future research.

Increasing evidence indicates that chronic ER stress is highly correlated with cognitive function and sustained stress is considered a pathological driver of AD (Cai et al., 2016). Endoplasmic reticulum (ER) stress markers were generally increased and appeared earlier in brain tissue from AD patients compared with brain tissue from non-demented patients (Hoozemans et al., 2005). ER stress results in the activation of a set of signaling pathways, called the unfolded protein response (UPR), which stimulates specific programs to restore ER function and ensure cell survival (Hetz et al., 2020). However, under prolonged or excessive ER stress, pathological changes in AD are promoted (Oakes and Papa, 2015). The PERK and eIF2α pathways are a branch of the UPR, and their sustained phosphorylation can inhibit synaptic protein synthesis, reduce synaptic plasticity, and exacerbate memory dysfunction (Brewer and Diehl, 2000; Ma et al., 2013; Hetz and Saxena, 2017).

In AD research, a variety of therapeutic approaches to reduce ER stress are under development and mostly include various small molecules that directly inhibit components of the UPR response, with significant efficacy in many preclinical models (Kraskiewicz and FitzGerald, 2012). However, safety aspects are of concern because the UPR has important roles in a variety of cell types and organ physiological states. Therefore, determining what causes ER stress is necessary for regulation at its source. Amyloid-β oligomers can disrupt ER calcium homeostasis, thereby causing ER stress (Gouras et al., 2000). Similarly, tau was shown to increase ubiquitinated protein levels in the brain and trigger activation of the UPR (Abisambra et al., 2013). However, ER stress occurs earlier in AD and may not be caused by amyloid or tau. The brain-gut axis links the brain to the gut microbiota, whether gut microbiota dysregulation causes ER stress in the brain has not been reported, and elucidating the relationship may help identify safe and effective targets for ER stress regulation. Therefore, in the current study, a fecal microbiota transplantation (FMT) approach was used to investigate whether gut microbial dysregulation causes cerebral cortical ER stress and identify the possible mediators involved.

Materials and methods

Reagents and antibodies

Ampicillin, metronidazole, neomycin, and vancomycin hydrochloride were purchased from MedChemExpress (Monmouth Junction, NJ, United States). Antibodies against PERK, p-PERK, and p-eIF2α were purchased from Cell Signaling (Danvers, MA, United States), and antibodies against eIF2α and β-tubulin were from Santa Cruz (Dallas, TX, United States). The enhanced chemiluminescence (ECL) kit was purchased from Thermo Fisher Scientific (Waltham, MA, United States). Mouse TMAO Elisa kit was purchased from Kisong Biotechnology (Beijing, China). Hs-CRP Elisa kit was purchased from Elabscience Biotechnology (Wuhan, China), mouse LPS Elisa kit from Cusabio (Wuhan, China), and 3,3-dimethyl-1-butanol (DMB) from Sigma-Aldrich (St. Louis, MO, United States).

Animals and experimental protocols

Male, 6-week-old C57BL/6J mice were purchased from Shanghai Slac Laboratory Animal Center (Shanghai, China). The mice were given adequate food and water and housed in a laboratory with 24°C temperature, 50–70% humidity, and 12-h light/12-h dark cycles. All animal experiments were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics and Welfare Committee of Shanghai Tenth People's Hospital.

Because an antibiotic cocktail was needed to empty the microbes from the mouse gut before FMT, the mice were divided into two groups to evaluate the potential effects of the antibiotic

cocktail, the ABX group (n = 6) and the control group (n = 6). Mice in the ABX group were administered a sterile PBS solution containing an antibiotic cocktail (ampicillin 1 g/L, neomycin 0.5 g/L, vancomycin 0.5 g/L, and metronidazole 1 g/L) by gavage once a day for 3 days in a total volume of 0.2 mL. The control group was not subjected to any intervention. On the 4th day, three mice in each group were sacrificed and the remaining mice were sacrificed on the 17th day after eating a normal diet.

To verify gut microbiota of AD patients and APP mice can cause ER stress in the cerebral cortex of wild-type (WT) mice, all animals were randomly divided into four groups: ABX group, ABX + PBS group, ABX + FMT-APP/PS1 group, and ABX + FMT-AD group. The ABX group was treated with an antibiotic cocktail for 3 days as described in the previous experiment (Bárcena et al., 2019). The ABX + PBS group (n = 7) was administered the antibiotic cocktail by gavage for 3 days followed by gavage with PBS containing 20% sterile glycerol three times a week for 2 weeks. The ABX + FMT-APP/PS1 group (n = 7) was first administered the antibiotic cocktail for 3 days followed by FMT treatment by gavage for 2 weeks (fecal microbiota of mice in APP/PS1 group). The ABX + FMT-AD group (n = 7) was administered the antibiotic cocktail for 3 days followed by FMT treatment (fecal microbiota of AD patients) for 2 weeks.

Furthermore, to verify whether ER stress in the cerebral cortex caused by gut microbial dysbiosis was associated with the gut microbial-associated metabolite TMAO, the mice were divided into six groups: ABX treatment, ABX + DMB treatment, ABX + FMT-APP/PS1, ABX + DMB + FMT-APP/PS1, ABX + FMT-AD, and ABX + DMB + FMT-AD. The duration and dose of the antibiotic cocktail and FMT treatment were the same as described above. DMB treatment was administered to mice from day 4 to day 17 of the experiment with drinking water containing 1% DMB and mice in the other groups were given normal drinking water.

Fecal microbiota transplantation treatment

Alzheimer's disease fecal donors were all from the cognitive impairment clinic of Shanghai Tenth People's Hospital. A total of six AD fecal donors were selected. The mean age of the donors was 72.2 (standard deviation [SD],4.8) years (range 67–80); 50% were women, and 33.3% were APOE ε4 carriers. The mean MMSE score was 18.7 (SD: 1.2). Fresh feces of AD patients and APP/PS1 mice were collected, diluted with sterile PBS solution, vortexed, centrifuged as previously described, and a bacterial suspension was obtained (Sun et al., 2018). Then, the bacterial suspension was mixed with 40% sterile glycerol in an equal volume and stored at −80°C until transplantation (Hamilton et al., 2012). Each recipient mouse was given 200 μL of bacterial suspension Once every 2 days for 2 weeks.

Elisa

The hs-CRP Elisa kit (Elabscience) was used to measure the serum hs-CRP concentration following the manufacturer's guidelines, The mouse LPS Elisa kit (Cusabio) and mouse TMAO kit (Kisong) were used to measure LPS and TMAO in serum, respectively, according to the manufacturer's protocol.

Western blot

Both cerebral hemispheres were removed from mice after cervical dislocation and stored at −80°C. The frozen cerebral cortex was homogenized in a pre-frozen RIPA buffer. The BCA kit was used to determine the protein concentration of the sample. Total protein (20 μg) was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Next, the membranes were blocked with 5% BSA solution to inhibit non-specific binding. The membranes were incubated with the following antibodies: rabbit anti-p-PERK (1:1,000), rabbit anti-PERK (1:1,000), rabbit anti-p-eIF2α (1:1,000), mouse anti-eIF2α (1:500), and mouse anti-β-tubulin (1:500). Next, the membranes were incubated for 1 h with the appropriate secondary antibodies (1:2,000). The β-tubulin level was used as the loading control. ImageJ was used to determine the intensity of various molecular bands.

16S rRNA sequencing and data analysis in gut microbes

The QIAamp DNA Stool Mini Kit was used to extract microbial genomic DNA from fecal samples following the manufacturer's instructions. Amplification and sequencing of the 16S rRNA gene were performed as previously described (Huang et al., 2019). The forward primer (338F 5′-ACTCCTACGGGGAGG CAGCA-3′) and the reverse primer (806R 5′-GGACTA CHVGGTWTCTAAT-3′) were used to amplify the V3-V4 region of the 16S rRNA gene (Huang et al., 2019). The sequencing data have been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) (Bioproject: PRJNA832124) to be released upon publication. QIIME (Quantitative Insights Into Microbial Ecology, v1.8.0)[1] and the R package 3.5.1[2] were used for 16S rRNA sequencing analysis. The UniFrac distance metric and primary coordinate analysis were used for diversity analysis to evaluate the structural changes of the microbial communities in the samples (PCoA). LEfSe was used to identify a wide range of

1 http://qiime.org/
2 https://www.r-project.org/

taxa. Heat-map was plotted using R package 3.5.1. The relative abundance of gut microbiota was analyzed by one-way ANOVA followed by *post-hoc* comparisons using LSD's test for multiple groups' comparisons.

Statistical analysis

In addition to the detailed description of the 16S rRNA data, one-way ANOVA followed by the least significant difference (LSD) pairwise comparison *post-hoc* test was used for data analysis with GraphPad Prism 9 software. Data were compared between the two groups using a two-tailed Student's *t*-test. The data are presented as mean ± standard error of the mean (SEM) with a statistical significance of $p < 0.05$.

Results

Antibiotic cocktail administration did not affect endoplasmic reticulum stress in the cerebral cortex of wild-type mice

Because the microbiota from the mouse gut had to be emptied with the antibiotic cocktail before conducting subsequent experiments, the ABX and control groups were established to examine the possible effects of the antibiotic cocktail on ER stress in the cerebral cortex of WT mice on days 4 and 17 (**Figure 1A**). Western blotting was used to assess the p-perk/perk and p-eIF2α/eIF2α levels in the cerebral cortex in response to ER stress. On day 4, statistical differences were not observed in the p-perk/perk (ABX group vs. control group, $p > 0.999$) and p-eIF2α/eIF2α (ABX group vs. control group, $p = 0.7$) levels in the cerebral cortex (**Figure 1B**). Similarly, on day 17, statistical differences were not observed in the p-perk/perk (ABX group vs. control group, $p = 0.655$) and p-eIF2α/eIF2α (ABX group vs. control group, $p = 0.1$) levels in the cerebral cortex (**Figure 1C**). Therefore, the effect of antibiotic cocktail administration on ER stress in WT mice's cerebral cortex was excluded.

Gut microbiome transplantation from Alzheimer's disease patients and APP/ps1 mice induced endoplasmic reticulum stress in the cerebral cortex of wild-type recipient mice

To determine the effect of gut microbiota on ER stress in the cerebral cortex, FMT was performed using fecal bacterial fluid from APP/PS1 mice and AD patients (**Figure 2A**). After 2 weeks of FMT, the ER stress in the cerebral cortex of mice in the different groups was compared. In the ABX + FMT-APP/PS1 group, p-perk/perk levels were increased (ABX vs. ABX + FMT-APP/PS1, $p = 0.041$) and p-eIF2α/eIF2α levels were increased (ABX vs. ABX + FMT-APP/PS1, $p = 0.0154$). Similarly, in the ABX + FMT-AD group, p-perk/perk levels were increased (ABX vs. ABX + FMT-AD, $p = 0.045$) and p-eIF2α/eIF2α levels were increased (ABX vs. ABX + FMT-AD, $p = 0.0111$). In contrast, p-perk/perk and p-eIF2α/eIF2α levels were not statistically different between the ABX and ABX + PBS groups (**Figure 2B**). In conclusion, these data indicate that recolonization of the gut microbiota in APP/PS1 mice and AD patients in WT recipient mice can induce ER stress in the cerebral cortex.

Mice in the ABX + FMT-APP/PS1 and ABX + FMT-Alzheimer's disease groups did not experience intestinal infection

To exclude FMT failure because mice were in the acute infection phase after FMT which could affect the experimental results, the serum of mice in each group was collected on the last day of the experiment (**Figure 2A**). Serum hs-CRP and LPS levels were measured using ELISA. The LPS (ABX vs. ABX + PBS vs. ABX + FMT-APP/PS1 vs. ABX + FMT-AD, $p = 0.595$) and hs-CRP (ABX vs. ABX + PBS vs. ABX + FMT-APP/PS1 vs. ABX + FMT-AD, $p = 0.768$) levels were not statistically different between the four groups (**Figures 2C,D**). Therefore, these results indicate the FMT did not cause acute infection in mice, excluding the possibility that cortical ER stress in mice is due to infection.

Profiles of intestinal microbiota alterations in the ABX + FMT-APP/PS1 and ABX + FMT-Alzheimer's disease groups

To explore the changes in intestinal microbiota structure in mice after FMT, the intestinal 16s rDNA sequencing results were evaluated and compared with the ABX-treated group, ABX + PBS-treated group, ABX + FMT-APP/PS1 group, and ABX + FMT-AD group. The microbial richness index (Chao1) and diversity index (Shannon) showed no significant difference between the groups (**Supplementary Figure 1**), indicating the FMT manipulation in this experiment did not affect the richness and diversity of the mouse gut microbiota. However, the UniFrac principal coordinates analysis (PCoA) results showed mice in the ABX + FMT-APP/PS1 and ABX + FMT-AD groups had a significantly different gut microbial composition than mice in the ABX and ABX + PBS groups (**Figure 3A**).

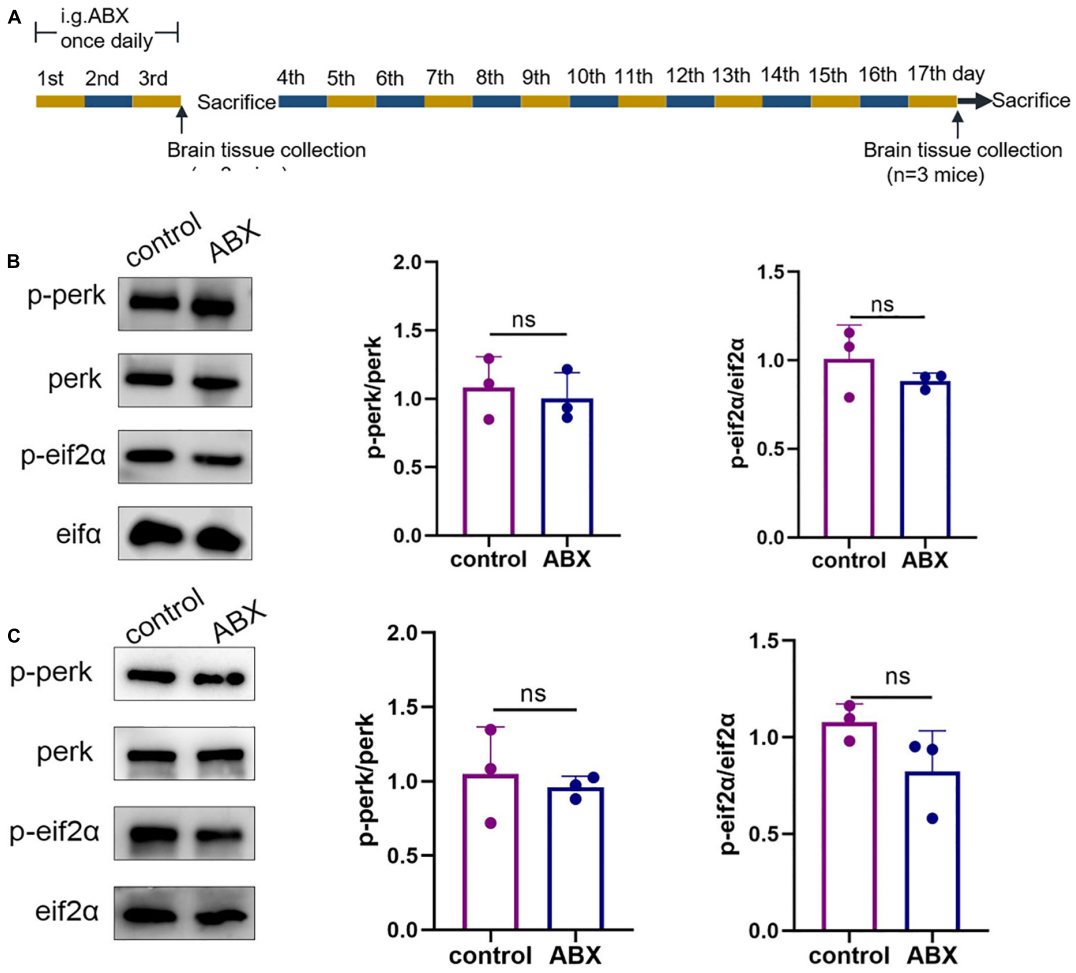

FIGURE 1
Antibiotic cocktail administration did not affect endoplasmic reticulum (ER) stress in the cerebral cortex of wild-type (WT) mice. **(A)** Experimental design of antibiotic cocktail-treated WT mice (mice were administered an antibiotic cocktail by gavage). **(B)** Representative immunoblots showing p-perk and p-eif2a levels in the cerebral cortex on day 3 of the experiment; $n = 3$ for each group. **(C)** Representative immunoblots on day 17 of the experiment showed p-perk and p-eif2a levels in the cerebral cortex. Data were compared between the two groups using a two-tailed Student's *t*-test.

Therefore, taxonomic changes were compared between taxa, mainly at the level of family or above, as shown in the heatmap (Figure 3B). Specifically, compared with the ABX and ABX + PBS groups, the ABX + FMT-APP/PS1 group showed a significant decrease in the relative abundance (RA) of *Bacteroidetes* (ABX vs. ABX + FMT-APP/PS1, $p = 0.0004$; ABX + FMT-APP/PS1 vs. ABX + PBS, $p = 0.0007$) at the phylum level and *S24-7* (ABX vs. ABX + FMT-APP/PS1, $p < 0.0001$; ABX + FMT-APP/PS1 vs. ABX + PBS, $p = 0.0002$) at the family level, and a significant increase in the RA of *Firmicutes* (ABX vs. ABX + FMT-APP/PS1, $p = 0.0036$; ABX + FMT-APP/PS1 vs. ABX + PBS $p = 0.0039$) at the phylum level and *Lachnospiraceae* (ABX vs. ABX + FMT-APP/PS1, $p = 0.0018$; ABX + FMT-APP/PS1 vs. ABX + PBS, $p = 0.0006$), *Desulfovibrionaceae* (ABX vs. ABX + FMT-APP/PS1, $p = 0.0009$; ABX + FMT-APP/PS1 vs. ABX + PBS, $p = 0.007$), and *Ruminococcaceae* (ABX vs. ABX + FMT-APP/PS1, $p = 0.0033$; ABX + FMT-APP/PS1 vs. ABX + PBS, $p = 0.0143$) at the family level. Furthermore, similar changes were observed in the ABX + FMT-AD group. At the phylum level, the RA of *Bacteroidetes* decreased (ABX vs. ABX + FMT-AD, $p = 0.0012$; ABX + FMT-AD vs. ABX + PBS, $p = 0.0024$) and the RA of *Firmicutes* increased (ABX vs. ABX + FMT-AD, $p = 0.0018$; ABX + FMT-AD vs. ABX + PBS, $p = 0.0021$). At the family level, the RA of *S24-7* decreased (ABX vs. ABX + FMT-AD, $p = 0.0007$; ABX + FMT-AD vs. ABX + PBS, $p = 0.0018$) and the RA of *Lachnospiraceae* (ABX vs. ABX + FMT-AD, $p = 0.0002$; ABX + FMT-AD vs. ABX + PBS, $p = 0.002$), and *Desulfovibrionaceae* (ABX vs. ABX + FMT-AD, $p = 0.0479$; ABX + FMT-AD vs. ABX + PBS, $p = 0.3996$) increased (Figure 3C). Taken together, the above

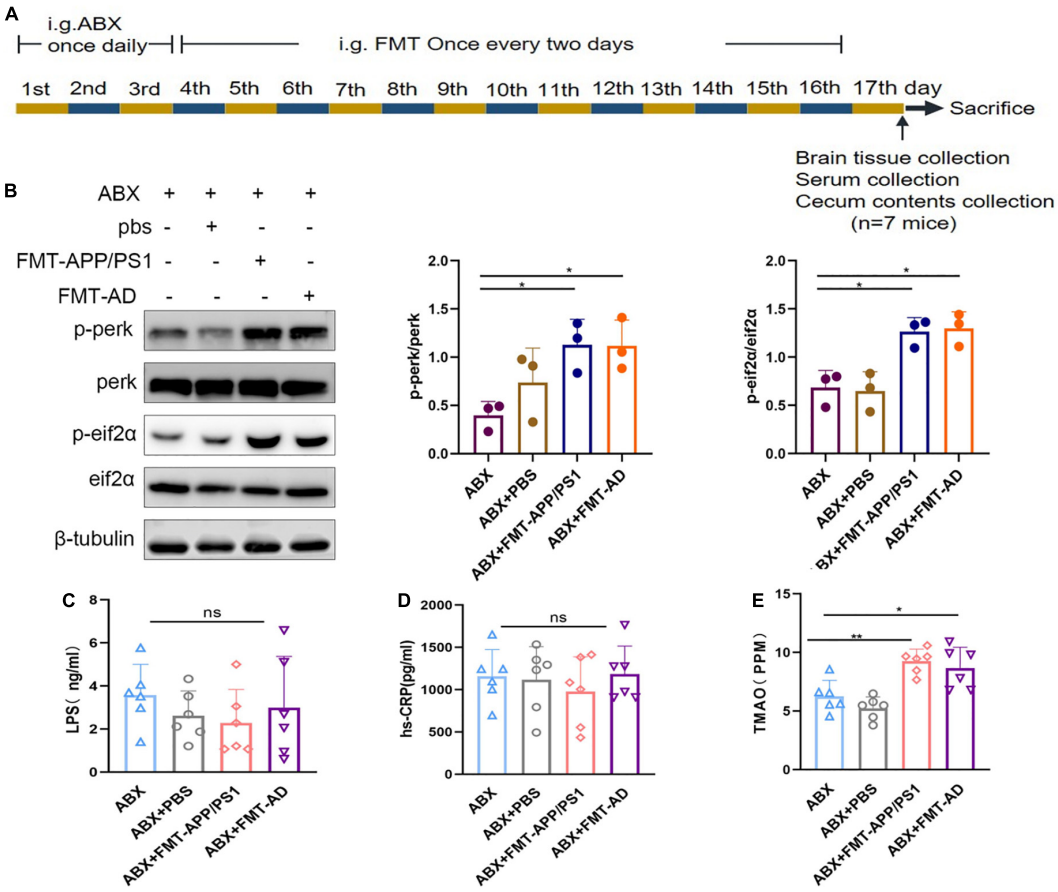

FIGURE 2
Cortical endoplasmic reticulum (ER) stress marker protein and serum hs-CRP, LPS, and TMAO levels in wild-type (WT) mice after fecal microbiota transplantation (FMT). **(A)** Experimental design of FMT-treated mice. **(B)** Representative immunoblots showing p-perk and p-eif2a levels in the cerebral cortex on day 17 of the experiment. **(C)** Serum hs-CRP levels after FMT. **(D)** Serum LPS levels after FMT. **(E)** Serum TMAO levels after FMT. $P < 0.05$ was set as the threshold for significance by one-way ANOVA followed by *post-hoc* comparisons using LSD's test for multiple groups' comparisons, $*p < 0.05$ and $**p < 0.01$.

data demonstrate the intestinal microbial characteristics of WT recipient mice after FMT.

Mice in the ABX + FMT-APP/PS1 and ABX + FMT-Alzheimer's disease groups showed elevated levels of trimethylamine-N-oxide in serum

Because ER stress occurs in the cerebral cortex after gut microbiota dysbiosis in WT recipient mice, the possible mediators between the gut microbiota and the brain were investigated. Increased production of TMAO (gut microbiota-associated metabolite) has been reported in AD patients and mouse models with gut microbiota dysbiosis. In addition, TMAO can enter the blood and cross the blood-brain barrier. In an *in vitro* TMAO study, TMAO was shown to cause ER stress in cells (Govindarajulu et al., 2020). Therefore, we measured TMAO levels in mouse serum using ELISA. We found that serum TMAO levels were significantly increased in both ABX + FMT-APP/PS1 and ABX + FMT-AD groups of mice compared to the ABX group (ABX vs. ABX + FMT-APP/PS1, $P = 0.0043$; ABX + FMT-AD vs. ABX, $P = 0.0232$) (Figure 2E). These results suggest that TMAO levels in the serum of mice were significantly increased after FMT, and therefore hypothesize that TMAO may mediate the communication between the gut microbiota and the brain.

Trimethylamine-N-oxide inhibition with 3,3-dimethyl-1-butanol reverses endoplasmic reticulum stress in the cerebral cortex

To further clarify whether TMAO is a mediator of gut microbiota dysbiosis and ER stress in the cerebral cortex, an

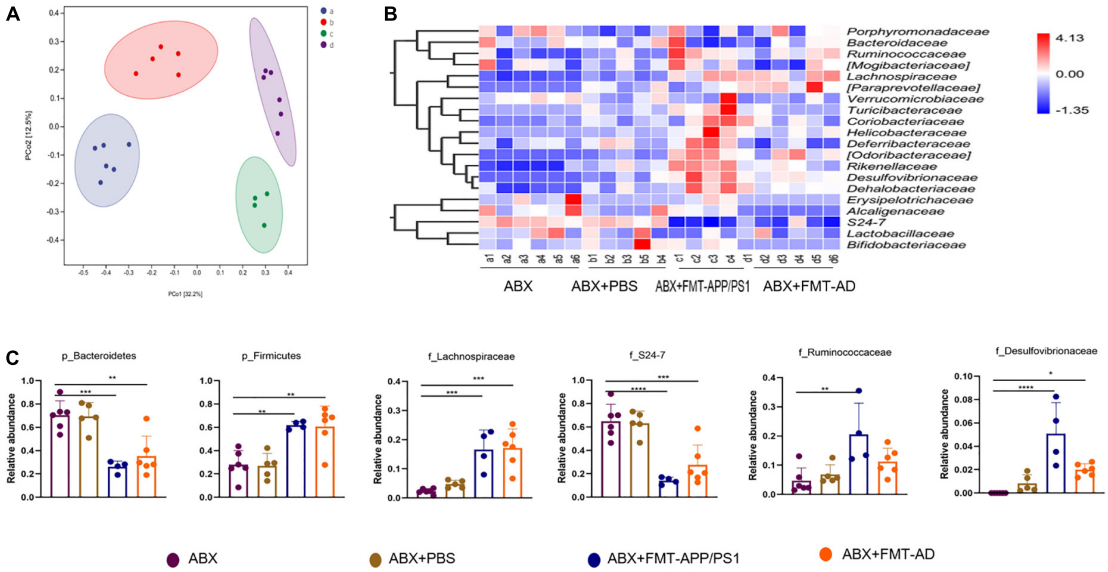

FIGURE 3
Alterations in the gut microbiota of wild-type (WT) recipient mice after fecal microbiota transplantation (FMT). **(A)** Principal coordinates analysis (PCoA) plots based on unweighted UniFrac distances. **(B)** Alterations in relative abundance (RA) of gut microbiota based on the heatmap. **(C)** RA bar plots of gut microbiota between groups at the phylum and family levels. $P < 0.05$ was set as the threshold for significance by one-way ANOVA followed by *post-hoc* comparisons using LSD's test for multiple groups' comparisons, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

additional FMT experiment (Figure 4A) was performed in which the drinking water was supplemented with DMB, which reduces TMAO production by inhibiting microbial TMA lytic enzymes and suppressing plasma TMAO concentrations. The results showed that after DMB supplementation, the TMAO levels in the serum of mice in the ABX + DMB + FMT-APP/PS1 and ABX + DMB + FMT-AD groups were significantly decreased compared to those in the ABX + FMT-APP/PS1 and ABX + FMT-AD groups, respectively (ABX + FMT-APP/PS1 vs. ABX + DMB + FMT-APP/PS1, $P < 0.0001$; ABX + FMT-AD vs. ABX + DMB + FMT-AD, $P = 0.0029$) (Figure 4B). And excessive ER stress occurred in the ABX + FMT-APP/PS1 and ABX + FMT-AD groups, consistent with the previous trend. In contrast, when DMB was administered, ER stress in the cerebral cortex due to gut microbiota dysbiosis was no longer significant in the ABX + DMB + FMT-APP/PS1 group. Similarly, the same effect was observed in the ABX + DMB + FMT-AD group (Figures 4C–E). Thus, gut microbiota dysbiosis-mediated ER stress in the brain may be associated with its effect on increased TMAO production.

Discussion

The present study findings indicate that FMT of WT recipient mice with fecal microbiota from AD patients and APP/PS1 mice may have increased plasma TMAO levels, which induced excessive ER stress in the cerebral cortex of the recipient mice. The Previous study results showed that FMT in AD model mice using microbiota from the gut of healthy mice improved the cognitive and pathological status of AD mice (Kim et al., 2020). In contrast, FMT of the gut microbiota of patients and model animals in WT recipient mice propagated their pathological features (Harach et al., 2017). Furthermore, transplantation of patient and model animal gut microbiota to WT recipient mice in the present study caused excessive ER stress in the brain cortex of recipient mice by disrupting the structure of their gut microbiota. In addition, the oral TMAO inhibitor DMB had a mitigating effect on ER stress, indicating that TMAO may be a mediator of ER stress in the cerebral cortex caused by dysregulated gut microbiota and TMAO production may be a potential target for AD intervention. The correlation between gut microbiome disorders and AD has been investigated in a variety of clinical and animal studies. Compared with healthy controls, AD patients had reduced gut microbiota diversity, different taxonomic composition, as well as reduced Firmicutes, and increased Bacteroidetes (Vogt et al., 2017). However, in another study, dementia was associated with increased biodiversity, reduced RA of Bacteroidetes, and an increased *Firmicutes*/*Bacteroidetes* ratio (Saji et al., 2019). Notably, in the present study, alterations in the gut microbiota of WT recipient mice caused by transplanting the gut microbiota of AD patients and APP/PS1 mice were similar to those observed in the latter study, with a decrease in RA of *Bacteroidetes* and an increase in RA

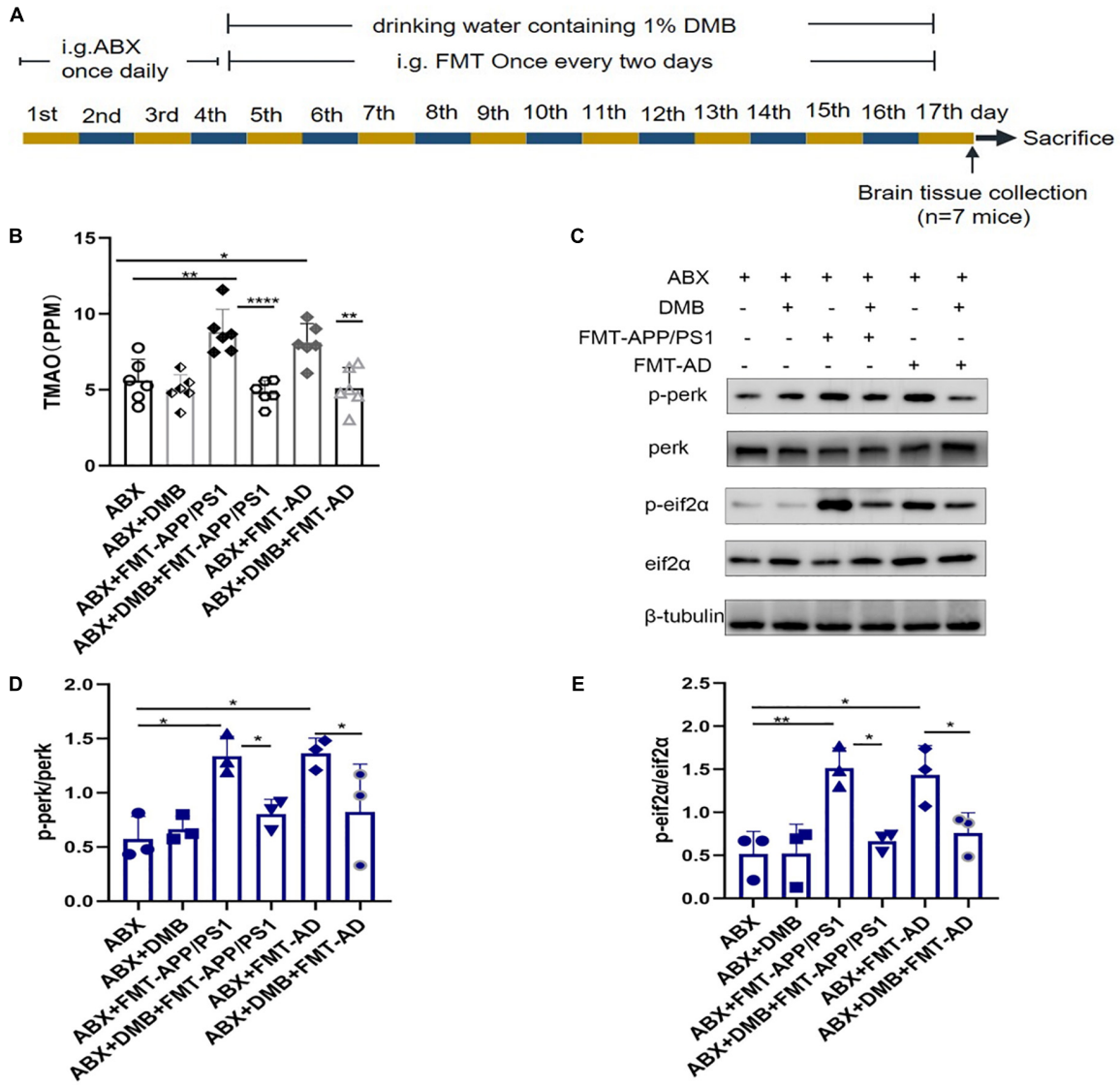

FIGURE 4
Trimethylamine-N-oxide (TMAO) inhibition with DMB reversed endoplasmic reticulum (ER) stress in the cerebral cortex. **(A)** Experimental design of DMB-treated mice. **(B)** Serum TMAO levels after DMB treatment. **(C–E)**. Representative immunoblots on day 17 of the experiment showed p-perk and p-eif2a levels in the cerebral cortex. $P < 0.05$ was set as the threshold for significance by one-way ANOVA followed by *post-hoc* comparisons using LSD's test for multiple groups' comparisons, $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$.

of *Firmicutes*, in contrast to the observations in the former study, which may be due to a different region and different interventions administered. As mentioned in many studies, excessive ER stress is present in the cerebral cortex of both AD patients and APP/PS1 mice, which can exacerbate amyloid deposition and tau phosphorylation, and in turn exacerbate ER stress (Ma et al., 2013). Notably, in the present study, WT recipient mice showed dysregulated gut microbiota and increased levels of cortical perk and eIF2α phosphorylation, exhibiting excessive ER stress. We hypothesize that dysbiosis of the gut microbiota leads to excessive ER stress in the cerebral cortex.

Furthermore, a 3-day ABX treatment was required before FMT to deplete the gut microbiota, and antibiotic administration significantly alters the gut microbiota composition (Suez et al., 2018) which may persist throughout life. Therefore, we needed to exclude the possible effect of altered gut microbiota caused by ABX treatment on ER stress in the cerebral cortex, thus, mice were sacrificed on days 3 and 14 after ABX treatment to assess ER stress markers (p-perk/perk, p-eIF2α/eIF2α) in the cerebral cortex. The results showed no ER stress in the cerebral cortex of mice sacrificed on days 1 and 14, indicating that antibiotic-induced gut flora depletion did not cause ER stress in the cerebral cortex and the cortical ER

stress observed was only associated with recolonization of the gut microbiota in AD patients and APP/PS1 mice.

In the present study, FMT was performed in mice, thus, the recipient mice were at risk of acute infection, and failure of FMT could result in unreliable experimental results. Simultaneously, LPS has been reported to cause ER stress (Sun et al., 2020). Therefore, the serum hs-CRP and LPS levels in the mice were examined after FMT and showed no significant difference compared with the control group, indicating the FMT did not cause acute infection, therefore, the possibility that infection could trigger ER stress in the cerebral cortex was excluded, and LPS was not a mediator of ER stress in the cerebral cortex caused by intestinal microorganisms although LPS is thought to cause ER stress.

The brain-gut axis has recently received increased research attention, in which TMAO (a gut microbiota-associated metabolite) may act as a key signaling mediator (Arrona Cardoza et al., 2021). In a recent clinical study, TMAO was significantly increased in the circulating and cerebrospinal fluid of patients with AD dementia compared with healthy controls (Vogt et al., 2018). In another study, TMAO promoted brain aging and cognitive impairment (Li et al., 2018). Notably, in cardiovascular disease and metabolic disorders, TMAO was shown to induce the ER stress signaling pathway by binding to the ER stress protein PERK (Chen et al., 2019). In human studies, TMAO can be detected in cerebrospinal fluid and elevated in AD (Vogt et al., 2018). Firmicutes and Proteobacteria appear the most active phyla in TMAO production, thus, the imbalance in their relative abundance is often accompanied by an increase in TMAO levels (Romano et al., 2015). In the present study, Firmicutes and Bacteroidetes were significantly increased in the ABX + FMT-APP/PS1 and ABX + FMT-AD groups, thus, TMAO levels may increase and be a key mediator between gut microbes and brain ER stress. Our experimental results did confirm higher TMAO concentrations in the serum of FMT mice, so we designed further experiments when performing FMT by adding the TMAO inhibitor DMB to the drinking water of the mice as previously described (Brunt et al., 2020). ER stress in the cerebral cortex was significantly reduced compared with the control group, indicating that TMAO may play a dominant role in mediating gut microbiota disruption leading to cortical ER stress.

There are some limitations to our study. First, our study used only male mice, so it can only reflect the relationship between gut microbiota dysregulation and ER stress in the male mouse population. Second, the cognitive function of the recipient mice and the pathology of AD in the brain, such as Aβ and p-tau, were not explored further. Therefore, further exploration of these issues is also the next step of our study. Third, extracellular bacterial DNA has been shown to play an important role in protein misfolding (Tetz et al., 2020; Tetz and Tetz, 2021), yet the current study did not analyze whether fecal transplantation increases extracellular bacterial DNA in plasma, so this will be a direction for our future research. Finally, the specific communication pathways between brain and gut microbiota are not fully understood, and how TMAO causes ER stress in the cerebral cortex is unclear, so more in-depth studies are needed to test and verify our findings.

Conclusion

In summary, the present study results indicate for the first time that host microbiota imbalance may lead to ER stress in the cerebral cortex and ER stress can be rescued by inhibiting TMAO production. In addition, an association was found between gut microbiota and the brain. These results can be used as a basis for reducing ER stress in the brain through modulation of the gut microbiota or its metabolites, contributing to the understanding and treatment of neurological disorders associated with ER dysfunction.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI–PRJNA832124.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai Tenth People's Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Ethics and Welfare Committee of Shanghai Tenth People's Hospital.

Author contributions

XL and YZ designed the experiments and revised the manuscript. FW, CX, and YG performed fecal microbial transplantation and drafted the manuscript. FW performed the western blot experiments. KD and CZ performed the analysis of 16srDNA sequencing results. All authors agreed to be accountable for the content of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.858130/full#supplementary-material

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Neurodegenerative diseases: from gut-brain axis to brain microbiome

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KEYWORDS

dementia, amyloid, Alzheimer's, microbiota, Aducanumab

Introduction

The proportions of global populations that are over 65 years of age are growing rapidly. In Japan there are now 2.5 times more diapers sold for adults than for children. Because the neurodegenerative disorders Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS) are all linked to aging the number of cases with these conditions are expanding greatly. It is estimated that there are 55 million people currently with dementia in the world and it is projected that there will be 139 million globally with dementia by 2050. Despite intense efforts by scientists and drug companies worldwide there is still no disease modifying therapy for Alzheimer's disease (AD), the most common cause of dementia.

On June 16, 2022, it was announced that an anti-amyloid antibody, Crenezumab, failed in a large clinical trial. This result followed the complex story of another anti-amyloid antibody, Aducanumab. In June 2021, the US Food and Drug Administration (FDA) granted accelerated approval to Aducanumab, developed by Biogen, for the treatment of AD. Randomized placebo-controlled studies had shown that the antibody removed amyloid deposits in the brain. These plaques of aggregated molecules have been an important finding in AD for over 120 years. Despite this encouraging development, the drug did not provide clinically meaningful cognitive benefit. And disturbingly, over 40% of subjects experienced potentially hazardous side effects involving brain swelling. Because of these findings an FDA scientific advisory panel had recommended in November 2021, by a vote of 10 to 0 (with one voting "uncertain"), that the antibody should not be approved.

Responses to the FDA's approval of the Aducanumab have been overwhelmingly negative. Three members of the FDA's advisory panel which recommended against approval resigned following the FDA's decision to approve the antibody. Biogen was criticized for the cost of $56,000 for 1 year of treatment, and they have lowered the cost to $28,000. In response to the high cost and lack of efficacy the European Medicines Agency has refused marketing authorization for Aducanumab and the US Centers for Medicare and Medicaid Services will pay for the antibody only for patients enrolled in qualifying clinical trials (Center for Medicare and Medicaid Services, 2022). In addition, the US Veterans Administration will not include the drug in its formulary because of its lack of effectiveness.

There have now been over 20 studies that show that reducing amyloid in the brain is not helpful in significantly improving the clinical outcome in persons with AD (see Imbimbo et al., 2020, for review). The failure of Crenezumab and Aducanumab to provide clinical benefit is the latest of a series of failures in AD treatment trials going back several decades. Lecanemab, a humanized immunoglobulin gamma 1 (IgG1) monoclonal antibody directed against aggregated soluble and insoluble forms of amyloid beta, has also been shown to have limited cognitive benefit. Of concern also is the risk of brain edema, hemorrhage

TABLE 1 Side effects of anti-amyloid monoclonal antibody therapy.

- ARIA-E cerebral edema
- ARIA-H small and large brain hemorrhages
- Superficial siderosis
- Cortical atrophy

ARIA, Alzheimer related imaging abnormality.

TABLE 2 Potential microbial based therapies.

- Prebiotics, probiotics, synbiotics, postbiotics
- Curds
- Fecal microbiota transplants (FMT)
- Phages
- Diet
- Dental care
- Antibiotics
- Antibodies
- Vaccines
- Medical foods
- Amyloid inhibitors (e.g., polyphenols)

and death associated with these agents (Table 1). Their use will require careful monitoring, genetic testing and exclusion of patients suspected of having cerebral amyloid angiopathy or taking anticoagulants.

We are concerned that these disappointing therapeutic developments have obscured recent advances in our understanding of the role of the microbiota in the molecular mechanisms of Alzheimer's disease.

The microbiota and neurodegeneration

Research in the last 10 years has shown that we are all home to a complex community of microorganisms, referred to as the microbiota. Considerable evidence demonstrates that they are highly involved in the initiation and maintenance of disease mechanisms in AD and related disorders.

The microbiota are composed of bacteria, fungi, viruses and other microbes which live on our body surfaces, as well as inside us (Parker et al., this issue, hyperlink). The number of our own cells is similar to the number of these partner organisms and they contain over a hundred times more genetic information than does our own DNA. They are with us at every moment of our lives, beginning shortly after birth. All of our ancestors going back 1 billion years also had microbial companions. *This means that we evolved with them and they evolved with us.*

This co-evolution is key to understanding their importance in our lives. The microbiota evolved the capacity to enhance the tolerance of our immune system to their presence. This acceptance allows them to thrive without causing an aggressive immune response from the host which would be damaging. At the same time, our immune system evolved the capacity to monitor the presence of our microbial communities and control our immune responses, so that a symbiotic relationship, which benefits us both, can be maintained. It has been shown that the microbiota are key to the healthy development of the immune system. They also play important roles in metabolism nutrition learning memory and protection from disease causing organisms (Bostick et al., 2022).

A critical role of the microbiota in AD and the related neurodegenerations such as Parkinson's diseases (PD), and ALS has been recently demonstrated (Klann et al., Li et al., Shen et al., this issue, hyperlinks, Kurlawala et al., 2023). Key features of all these conditions include deposits of aggregated proteins (such as the amyloid β protein in AD and alpha synuclein in PD), activation of the immune system in the brain and the presence of reactive oxygen molecules, which are unstable, highly reactive and destructive. The microbiota in the gut influence all of these processes through diverse molecular mechanisms, some of which are being determined in studies conducted in labs around the world.

Microbial amyloid proteins

Microbes in the gut, including the nose, mouth, and intestines make functional bacterial amyloid proteins which have been shown to cause templated cross-seeding of neuronal proteins in the brain, accelerating cerebral amyloid deposition in neurodegenerative disease models (Sampson et al., 2020). Otzen et al. in Denmark have shown that the microbiota make functional amyloid proteins that may be involved in neurodegeneration (Christensen et al., 2021). Landau et al. in Haifa have demonstrated the cross-seeding of the amyloid beta protein by curli *in vitro* (Perov et al., 2019). Importantly, passage of amyloid proteins from the gut to the brain via the vagus nerve has been documented in rats by Holmqvist et al. (2014), as discussed in this special issue by Geng et al. (hyperlink). A genome wide screen of Wang et al. (2021) from Hong Kong showed that the functional bacterial amyloid protein curli made by *Escherichia coli* and other bacteria colocalized with neuronal amyloid inside neurons, promoted aggregation though cross-seeding and promoted disease in worm models of AD, ALS and Huntington's.

Furthermore, functional bacterial amyloids proteins in the gut are recognized as pathogen associated molecular patterns by the innate immune system, which has been shown to increase the immune response to neuronal amyloids in the brain and worsen functional deficit (Chen et al., 2016; Friedland and Chapman, 2017). It has been shown that functional bacterial amyloids activate Toll-Like receptors 1 and 2, NFkB and iNOS leading to enhanced inflammation and oxidative toxicity. This is a similar pathway by which neuronal amyloids are recognized by the brains immune system (Friedland and Chapman, 2017). Heneka and associates in Bonn, Germany, have shown that activation of the innate immune system in the brain contributes to aggregation of the amyloid beta protein in AD model mice (Ravichandran and Heneka, 2021).

Therapeutic potential of microbiota in AD

An exciting feature of this work is that our microbial partners are relatively easy to adjust. They are our captives and must eat what we give them. Studies show that a change in diet in humans in as little as 2 weeks can significantly change the nature of bacterial populations in the intestine with beneficial effects on health (O'Keefe et al., 2015). This effort to improve our health through changes in diet can be referred to as "gene therapy in the kitchen," because a change in diet changes our internal bacterial

communities and their DNA. it is critical that we be aware of the influence of nutrition on our microbiota (Friedland, 2022).

Discussion

Furthermore, considerable effort is being extended globally to develop agents which will affect the gut microbiota and their metabolic products with benefits for our health and fitness (Table 2). It is clear that there will be drugs which influence gut bacteria to provide benefit for the brain, without the need for the drug itself to enter the blood or cross the blood-brain barrier. There are many potential avenues for exploration of this therapeutic approach: probiotics provide live bacteria; prebiotics provide food for desirable bacteria, and postbiotics are beneficial products produced by the microbes (Kim et al., this issue, hyperlink). Antibiotics also have strong influences on gut bacteria, as shown by Liu et al. in this issue (hyperlink). Bacterial transplants are being explored and agents which alter bacterial metabolism with benefits for health are being developed (Zhang et al., 2021; Wang et al. both from this issue, hyperlinks). For example, Sampson et al. (2020) has shown that oral intake of polyphenols with anti-amyloid effects have beneficial influences in Parkinson model mice. In this issue Chung et al. (hyperlink) reports the influence of the polyphenol resveratrol on the gut-brain axis.

Ninety to ninety-nine percent of cases of neurodegenerative diseases are not caused by genes. The microbiota may be a major environmental factor influencing the course of their development. The amyloid deposits in the brain, which anti-amyloid treatments remove, are a biomarker of AD, not the disease itself. The papers in a recent special issue of *Frontier of Aging Neuroscience* provide good reasons to believe that new approaches based on microbial mechanisms will powerfully influence the critical primary pathways of disease development with powerful effect (Tetz, 2022).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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