INTERACTIONS OF THE NERVOUS SYSTEM WITH BACTERIA

EDITED BY: Elisa L. Hill-Yardin, Mastura Monif, Andreas Martin Grabrucker, Ruth Ann Luna and Ashley Edwin Franks

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INTERACTIONS OF THE NERVOUS SYSTEM WITH BACTERIA

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EDITORIAL
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Editorial: Interactions of the Nervous System With Bacteria

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Editorial on the Research Topic

Interactions of the Nervous System With Bacteria

Recent evidence that microbes influence mood and behavior via the gut-brain axis has opened up new avenues for research into neurological disorders. Hence, many studies now employ multidisciplinary approaches assessing for changes in microbial diversity, neuroinflammation as well as alterations in neuronal circuitry that impact brain function in health and disease. Such collaborative research was virtually unheard of in previous decades but holds remarkable promise for identifying novel pathways and therapeutic targets within the gastrointestinal tract to treat brain disorders. This editorial highlights these exciting developments in neuroscience, microbiology, and immunological research by examining 13 articles focused on how the nervous system interacts with bacteria in preclinical and clinical settings. A common theme is the dissection of complex interactions between the nervous system and bacteria as well as the resulting influences on inflammatory pathways, symptoms, or behavior in patient studies and mouse models. Specifically, neuronal-microbial interactions in the context of nervous system disorders ranging from autism, Attention Deficit Hyperactivity Disorder, Alzheimer's Disease and Major Depressive Disorder to migraine and epilepsy are investigated. Overall, we propose that via leveraging our understanding of the gut-brain axis, the modulation of gut microbes leading to significant benefits for brain health can become a reality.

The recent years have seen substantial progress in the understanding of gut-brain interactions. Today, evidence is mounting that the microbiota-gut-brain axis is a key contributor to healthy brain development and function. Accordingly, gastrointestinal (GI) problems and microbial dysbiosis have been linked to several neurological and neuroinflammatory disorders. Consequently, targeting gut microbiota composition to regulate peripheral and central inflammation could serve as means of developing novel treatments or disease modifying strategies for several key neuroinflammatory conditions. This is a rapidly emerging field of neuroscience research and is highlighted in the current issue.

This Research Topic is dedicated to understanding the influence of microbes on brain health. Alterations in the gut microbiota may affect gut-brain signaling via neuronal, endocrine and immunological mechanisms, thereby influencing a range of neuronal network activities and ultimately host behaviors. Zhao et al. showed that emotional behavior is among those behaviors

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affected. Antibiotic-treated mice with a lower richness and diversity of microbiota display increased anxiety-like, depression-like, and aggressive behaviors (Zhao et al.). These effects on behavior may be caused by a dysregulation of the immune and endocrine system. In line with this, a link between altered microbiota composition and immune system abnormalities was also reported by Sauer and Grabrucker. In their study, a deficiency in the essential trace metal, zinc, changed the composition of the microbiota. Of interest, zinc deficiency has been linked to depression-like behavior in rodents. In this study, the microbial changes were accompanied by increased inflammatory cytokine levels. Furthermore, this study reported abnormal GI morphology and increased GI permeability in these mice, hinting that an increase in intestinal barrier permeability may mediate pro-inflammatory processes (Sauer and Grabrucker).

Relevant to the findings of Sauer and Grabrucker, the role of microbes in modifying the permeability of the mucosal epithelium lining the GI tract was discussed in a review of microbiome-derived neurotoxins by Lukiw. How microbiota might impact the properties of the mucus membrane in the GI tract could also be important in brain disorders. Lukiw discussed the role of the zinc metalloproteinase (also known as *B. fragilis Toxin; BFT* or *fragilysin*), which is released by *Bacteroides fragilis* microbes. *Fragilysin* cleaves tight junction components such as cadherins which can alter the permeability of mucosal and blood-brain barriers. This action would also alter neuro-immune interactions and is age-related and progressive. An important part of the epithelial barrier of the GI tract is the mucus biofilm, which provides a supportive environment for specific microbial populations. Because patients with many neurological diseases have GI issues and microbial dysbiosis, Herath et al., reviewed the evidence for mucus as a potential treatment target in the context of neurological disease (Herath et al.). This review outlined a range of pathways by which gene mutations associated with brain disorders could influence the GI tract's mucus and its microbial populations.

Sharna and colleagues investigated neuro-immune interactions in a mouse model of autism expressing a gene mutation in the nervous system (Sharna et al.). Surprisingly, the weight of the caecum (the equivalent of the human appendix) was reduced in these mice. When analyzing the enteric nervous system in the caecum, mutant mice had more neurons overall and a higher proportion of neurons synthesizing the major inhibitory neurotransmitter (nitric oxide) of the gut. In the same study, Sharna and coauthors found changes in the gut immune system. In these mice, macrophages were labeled with Iba-1 (a marker of microglia in the brain) in the gut-associated lymphoid tissue of the caecal patch. Mutant mice had more Iba-1 labeled macrophages, and these macrophages were smaller and rounder in shape. Interestingly, this could indicate increased immune activity in the autism model. More broadly, these findings suggest that communication between the nervous system and immune cells could be altered in neurological disorders such as autism, and that the gut microbiota plays a key role in regulating such interactions.

Further evidence of the nervous system's interactions and inflammatory pathways in brain disorders include a role for lipopolysaccharide (LPS), lifestyle factors, and both the oral and GI microbiome in Alzheimer's Disease. The accumulation of gut bacteria-derived LPS in neurons is associated with Alzheimer's disease. Lukiw et al. report that LPS entry into human neurons is facilitated by amyloid beta-42 (Aβ42) and discuss the associated pathogenic role of LPS in neurons of Alzheimer's disease brains (Lukiw et al.). The encapsulation of neurons by LPS reduces the release of mRNA from the neuronal nuclei, likely via the interaction of Aβ42 with nuclear pore complexes. Therefore, manipulating the gut microbiome by diet and pre/probiotics to reduce neurotoxic effects within the CNS is an exciting avenue for future research. In line with these findings, Askarova and colleagues brought together a wide range of research to link the brain-gut-microbiota axis and Alzheimer's Disease (Askarova et al.). These authors noted that several lifestyle factors linked to Alzheimer's disease also drive microbiome changes, such as dietary habits, sedentary behavior, and disturbances in circadian rhythms. Changes in the oral microbiome in addition to chronic periodontitis have also been associated with Alzheimer's Disease (Paganini-Hill et al., 2012; Harding et al., 2017; Liu et al., 2019; Panza et al., 2019; Olsen and Singhrao, 2020). Given the potential for factors within the gastrointestinal tract to contribute to the development of neuroinflammation and neurodegeneration, further studies are warranted.

Gut microbiota profiles may contribute to behavioral symptoms associated with a range of neurological disorders, including attention-deficit/hyperactivity disorder (ADHD) and major depressive disorder (MDD). Wan et al. investigated the gut microbiota in children with ADHD. Their results of a case-control study reveal that specific bacteria are significantly increased or decreased in children with ADHD and align with alterations in neurotransmitter metabolic pathways (Wan et al.). However, as Isaiah et al. point out, in addition to the gut signaling to the brain, the reverse (i.e., brain-to-gut signaling) may occur as well (Isaiah et al.), a phenomenon that is currently understudied in the context of ADHD. Yong et al. discussed the role of the microbiota-gut-brain axis, stress, and lifestyle factors for major depressive disorder (MDD) (Yong et al.). The antidepressive effects of probiotics and potential biological mechanisms (including the production of metabolites) to benefit gut health were compared in clinical and animal studies. In addition, Westfall and Pasinetti discussed the role of dietary polyphenols as a possible diseases-modifying treatment for depression (Westfall and Pasinetti). Their review highlighted that synbiotics that combine probiotics with dietary polyphenols such as those found in fruits, tea, herbs, cereal, or wine might be a novel therapy for MDD. This may occur via modulation of multiple metabolic pathways, including the breakdown of kynurenic acid and tryptophan (with influences on glutamatergic activity and microglial function in the brain), serotonergic mechanisms, and immune pathways, including via interferon-gamma and inflammasome activation. Importantly, these authors highlight that the heterogeneity of MDD must be taken into consideration when evaluating the potential therapeutic effects of probiotics.

Migraine is a common, recurrent, and disabling neurological disorder that is associated with alterations in the neurovascular and immunological system. The changes in the nervous system activity that occur in the setting of migraine are commonly associated with gut disorders such as irritable bowel syndrome and inflammatory bowel disease. Chen and others examined the potential for interactions between the nervous system and bacteria in their study of fecal samples from 108 elderly women with and without migraine, which revealed a significant decrease in species diversity and metabolic functions in the gut microbiota of migraine sufferers. These findings suggest that monitoring harmful bacteria such as Clostridium could help with alteration of migraine frequency and potentially even prevention of disease (Chen et al.). Future randomized control trials that assess the efficacy of controlled delivery of "safer" and "less inflammatory" microbiota as a means of controlling migraine frequency are warranted.

Epilepsy is another neurological disorder for which a role for the microbiome is understudied. The ketogenic diet is commonly used to treat medically refractive seizures and epilepsy. Importantly the anti-seizure effects of the ketogenic diet are thought to be mediated by the gut microbiome (Olson et al., 2018). Altering the gut bacteria is thought to regulate seizure frequency and modify disease severity. Rubin and Glazer outlined a potential role for subclinical infection with Bordetella pertussis (BP) in epilepsy (Rubin and Glazer). This work highlighted that cases of subclinical infection are vastly more prevalent than reported pertussis cases and describe incidences of epilepsy occurring after BP infection. Further research into the relationship between epilepsy and BP infection, including BP screening, medical history, and pertussis vaccination is required to assess for an association between BP and seizures.

In summary, this Research Topic brings together evidence for microbial influences in a range of neurological, neuropsychiatric and neurodegenerative disorders including autism, Alzheimer's Disease, Major Depressive Disorder, migraine, and epilepsy. Gut microbiota are thought to influence not only local immunological processes, but also exert effects on the CNS through the disruption of the gastrointestinal mucosal barrier and BBB. The future of gut-brain research holds promise for identifying novel therapeutic approaches to treat many disabling CNS conditions traditionally considered to be associated with dysfunction of the CNS itself. Further research into how modifying gut microbes influence processes in the brain has enormous potential.

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

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The Potential Role of Subclinical *Bordetella pertussis* Infection in Epilepsy

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Keywords: *Bordetella pertussis*, epilepsy, seizure, *Bordetella pertussis* toxin, subclinical infection

The etiology of epilepsy remains unknown in 14–39% of cases across multiple continents (Banerjee et al., 2009). Given the increased risk for seizures and epilepsy in children after symptomatic *Bordetella pertussis* (BP) infection (Olsen et al., 2015), an association recognized for nearly a century (Eley, 1930), we briefly review the evidence and propose a role for subclinical BP colonizing infections in epilepsy.

Subclinical BP infections are vastly more prevalent than reported pertussis (Ward et al., 2005). In multiple countries with high BP vaccination rates, evidence of subclinical BP infection is demonstrated in 4.8–7.1% of asymptomatic individuals by nasal swab PCR (Klement et al., 2003; Zhang et al., 2014; Naeini et al., 2015), and in 6.6–14.1% by serology indicative of infection during the past year (de Melker et al., 2006; De Greeff et al., 2010; Palazzo et al., 2016). Based on serology, investigators in the United States (US) acellular BP vaccine trial estimated the number of undocumented BP infections at 1 to 10 million cases in the US annually from 1997 to 1999 (Ward et al., 2005), years when the CDC reported approximately 7,000 cases per year (http://www.cdc.gov/pertussis/surv-reporting/cases-by-year.html), a ratio of up to 1,400 subclinical BP infections for every reported pertussis case.

Multiple lines of evidence support the hypothesis that subclinical nasopharyngeal BP colonizing infections have unrecognized clinical consequences including epilepsy. *B. pertussis* secretes pertussis toxin, which compromises the blood-brain barrier in human brain endothelium models (Kugler et al., 2007), as seen in epilepsy (Oby and Janigro, 2006). Murine respiratory BP infection induces inflammatory cytokines in the brain (Loscher et al., 2000), and intracerebroventricular pertussis toxin lowers drug-induced seizure thresholds (Durcan and Morgan, 1991), though findings documented in mice should be interpreted with caution. At the neuronal level, mechanistic plausibility is supported in that pertussis toxin increases excitatory neuronal glutamate release (Cullen et al., 1994) and decreases Gi/o receptor-mediated neuroinhibitory GABA activity (Padgett and Slesinger, 2010), as well as GABA receptor binding (Moss and Vaughan, 1988). In summary, mechanisms by which pertussis infection may play a causal role in epilepsy include immunologic and inflammatory responses to pertussis infection, direct action of pertussis toxin on neurons, and a combination of these factors.

Clinical observation also supports the association between BP and epilepsy. In children < 2 years of age admitted to the hospital with pertussis, new seizures were reported in 2.3%, and encephalopathy in 0.5% of patients (Halperin et al., 1999). In BP-associated encephalopathy, elevated antibody titers to BP toxins have been demonstrated with 10-fold higher concentrations in CSF compared with serum, indicating entry of BP antigens to the CNS (Grant et al., 1998). In Denmark between 1978 and 2011, the incidence of epilepsy at 10 years of age was 1.7% for patients with a history of hospital-diagnosed pertussis, and 0.9% in a matched cohort [HR 1.7 (95% CI, 1.3–2.1)] (Olsen et al., 2015). Almost all of the increased epilepsy risk occurred in the first 1.5 years after clinical pertussis, and did not vary with age at pertussis diagnosis.

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Investigating the hypothesis that subclinical BP colonizing infections are a cause of epilepsy could begin by screening patients presenting with an initial idiopathic seizure. Subjects and controls could be tested for serum BP antibody titers and nasopharyngeal BP by swab PCR. In future BP-seizure risk analyses, BP vaccination status should be accounted for to avoid the confounding effects of vaccine-induced BP immunoglobulins on estimates of BP exposure history. The particular form of BP vaccination is also important since the diphtheria, tetanus toxoid and whole-cell pertussis vaccine (DTP) has been associated with febrile seizures (but not epilepsy) (Barlow et al., 2001), while the combination acellular pertussis vaccine (DTaP) has not (Huang et al., 2010). Of note, some historically reported associations between pertussis vaccination and neurologic disorders may be due to early unmasking of genetically determined disease such as Dravet syndrome in those with sodium channel gene SCN1A mutations (McIntosh et al., 2010). Since these mutations may occur without a prior family history, referral for specialty testing should be considered to help identify all potential causes of new onset seizures.

As subclinical BP colonizing infections are prevalent in highly BP-vaccinated populations, and non-human primate studies demonstrate the failure of DTP and DTaP to prevent nasopharyngeal BP colonization (Warfel et al., 2014), evidence suggests that current pertussis vaccines do not prevent nasopharyngeal BP colonization. Since the number of subclinical BP infections may be more than 1,000 times greater than clinically reported cases as noted above, it would not be surprising to observe a minimal or even lack of epilepsy risk reduction following DTP and DTaP vaccination.

In light of the available evidence, we suggest that a causal role for subclinical BP colonizing infection in epilepsy is plausible and worthy of further investigation. Regression analysis of epilepsy risk, incorporating BP screening assays, medical history, and pertussis vaccination status would be a compelling first step in assessing the potential relationship between epilepsy and subclinical BP infection.

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REVIEW
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The Gut Microbiota Links Dietary Polyphenols With Management of Psychiatric Mood Disorders

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The pathophysiology of depression is multifactorial yet generally aggravated by stress and its associated physiological consequences. To effectively treat these diverse risk factors, a broad acting strategy is required and is has been suggested that gut-brain-axis signaling may play a pinnacle role in promoting resilience to several of these stress-induced changes including pathogenic load, inflammation, HPA-axis activation, oxidative stress and neurotransmitter imbalances. The gut microbiota also manages the bioaccessibility of phenolic metabolites from dietary polyphenols whose multiple beneficial properties have known therapeutic efficacy against depression. Although several potential therapeutic mechanisms of dietary polyphenols toward establishing cognitive resilience to neuropsychiatric disorders have been established, only a handful of studies have systematically identified how the interaction of the gut microbiota with dietary polyphenols can synergistically alleviate the biological signatures of depression. The current review investigates several of these potential mechanisms and how synbiotics, that combine probiotics with dietary polyphenols, may provide a novel therapeutic strategy for depression. In particular, synbiotics have the potential to alleviate neuroinflammation by modulating microglial and inflammasome activation, reduce oxidative stress and balance serotonin metabolism therefore simultaneously targeting several of the major pathological risk factors of depression. Overall, synbiotics may act as a novel therapeutic paradigm for neuropsychiatric disorders and further understanding the fundamental mechanisms of gut-brain-axis signaling will allow full utilization of the gut microbiota's as a therapeutic tool.

Keywords: gut-brain-axis, neuroinflammation, polyphenols, resilience, probiotics, synbiotics

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INTRODUCTION

Major depressive disorder (MDD) is a recurrent psychological disorder with numerous pathophysiological characteristics that result in prolonged periods of sadness and emptiness coupled with anhedonia, elevated anxiety and eventual cognitive dysfunction (Pellegrino et al., 2013). Depression is a significant global affliction present in more than 350 million people (World Health Organization [WHO], 2018), 16 million whom reside in the United States accounting for 6.7% of the total population (National Institute of Mental Health, 2017). Based on clinical observations, depression is defined as a multifactorial disorder with several heterogeneous neuropathological indications including reduction in the size and density of γ-amino butyric acid

(GABA) neurons in the prefrontal cortex and limbic regions (Savitz et al., 2014), according to the "gliocentric theory," abnormalities in glial density and functioning (Czeh and Nagy, 2018), imbalances in monoamine neurotransmitters in synaptic clefts (Meyer et al., 2006) and loss of hippocampal volume and neuronal loss in the hypothalamus (Manaye et al., 2005). The classical cause of depression is defined as a deficiency in noradrenaline and serotonin in the hippocampus and frontal cortex, and this remains to be a prominent hypothesis due to the efficacy of pharmacological monoaminergic reuptake blockers toward improving mood status (Taylor et al., 2005). Despite this understanding, the fundamental neurobiological changes in depression remain elusive and at present are loosely attributed to the interaction of genetic predispositions and environmental factors (Bleys et al., 2018). It is known that chronic psychological or physical stress induce a battery of depressive phenotypes through mechanisms related to abnormalities in hypothalamic-pituitary-adrenal (HPA) axis signaling including hypersecretion of C-reactive protein from the hypothalamus, impaired negative feedback of the HPA axis and hypercortisolemia (Pruessner et al., 2003). However, without a causal pathological etiology to define the manifestation of depression, therapeutic development has become rooted in the alleviation of depressive-like symptoms and such therapies remain inconsistent between patients, invoke significant side effects and result in a large proportion of patients being unresponsive to them (Kupfer, 2005).

An emerging trend in the pathology of chronic neurological diseases including depression- and anxiety-like disorders is the concept of a multifactorial causation due to multisystem abnormalities. The concept of cognitive resilience has recently gained traction as a viable therapeutic strategy for these multifactorial chronic diseases and is defined as the ability to physiologically adapt to external stresses in order to maintain normal psychological and physical functioning and avoid pathological states that can drive disease (Aburn et al., 2016). Considering that the epidemiological cause of anxiety and depression include the interaction of environmental stresses and genetic disposition in a variety of physiological systems, a treatment regime incorporating probiotics and natural polyphenols may prove to be superior compared to classical pharmacological treatments as probiotics promote the production of a diverse host of bioactive metabolites from the dietary polyphenols capable of simultaneously ameliorating multiple risk factors of depression and anxiety.

POLYPHENOLS IN NEUROPSYCHIATRIC DISORDERS

Dietary polyphenols expand the definition of prebiotics, which were previously believed to contain various fibrous foods that predominantly generate the short-chain fatty acids (SCFAs) propionate, acetate and butyrate following fermentation by the gut microbiota. Diets rich in SCFAs or that include prebiotics that promote SCFA production such as fructooligosaccharides (FOS), galactooligosaccharides (GOS) or inulin, have been shown to have multiple beneficial immune- and metabolic- effects that can ultimately improve cognition (Bourassa et al., 2016; Stilling et al., 2016; Dinan and Cryan, 2017). Although extremely effective at promoting neurological health, the range of SCFA activity is limited compared to the variety of polyphenols and their gut-derived metabolites making dietary polyphenols an emerging therapeutic option in neurological conditions.

Polyphenols are a broad category of heterogeneous botanicals composed of hydroxylated phenyl moieties found abundantly in fruits, tea, herbs, cereals and wine (Vinson et al., 2001). Many polyphenol-rich botanicals are considered to be adaptogenic: stress-modifying phytochemicals that increase organisms' non-specific resistance to stress by increasing their ability to adapt and survive to external stressors and stimuli (Panossian, 2017). Due to their heterogeneous nature, individual polyphenols have distinct biological activities; however, as they are found in combination in nature, they inherently have synergistic activity that must be considered when designing polyphenolic pharmaceutical agents. Indeed, many natural occurring polyphenolic mixtures have been shown to have extensive beneficial effects on cognition and mood in both healthy and diseased subjects (**Table 1**).

Much of the interindividual variability of the aforementioned clinical studies can be explained by the polyphenols' bioavailability, which is dependent on their fermentation by the gut microbiota and consequent secondary xenobiotic biotransformation in the liver. There are several comprehensive reviews exploring the prebiotic activity of polyphenols and how ingestion of polyphenols can beneficially alter the composition of the gut microbiota (Cardona et al., 2013; Westfall et al., 2018a) so this topic will not be discussed in further detail here. However, fewer studies have indicated how the specific metabolites produced by the gut microbiota from dietary polyphenol sources can impact the potential biological signature of depression.

MICROBIOTA AND THE GUT-BRAIN-AXIS

The gut microbiota is a synergistic community of microorganisms residing in the gastrointestinal tract (GIT) composed of trillions of bacterial cells classified into thousands of species, each with a distinct metabolic profile (Qin et al., 2010). The two predominant phyla constituting approximately 98% of the gut microbiota are the Firmicutes and Bacteroidetes, with the remainder belonging to the phyla Proteobacteria, Verrucomicrobia, Fusobacteria, Cyanobacteria, Actinobacteria, and others (Backhed et al., 2004). The composition of the gut microbiota is highly amenable to diet, antibiotic usage, hygiene, pharmaceuticals and stress and changes in the composition of the gut microbiota result in dysbiosis, or imbalances in the composition of the gut microbiota and/or its metabolism (Aziz et al., 2013). Dysbiosis has been shown to influence the onset and/or progression of a battery of chronic diseases including metabolic syndrome, inflammatory bowel disease, depression, cardiovascular disease and neurodegeneration (Quigley, 2017).

The gut-brain-axis (GBA) is a bidirectional neuroendocrine system linking the GIT, including the microbiota, and the brain. The GBA consists of the enteric (ENS), peripheral (PNS) and

TABLE 1 | Effect of complex polyphenolic substances on markers of depression.

Dietary Polyphenol	Contents	Biological Effect	References
Concord Grape Juice (CGJ)	Flavanols Flavones Quercetin Phenolic Acids Proanthocyanidins Anthocyanins	In aging rats, grape juice fed *ad libitum* at concentrations of 10% enhanced cognitive performance and dopamine release while at 50%, improved motor function	Shukitt-Hale et al., 2006
		In older adults with memory decline, 6–9 ml/kg of concord grape juice for 12 weeks significantly improved cognitive function, but not depressive symptoms	Krikorian et al., 2010a
		In healthy middle-aged working women, 355 ml of CGJ consumption daily for 12 weeks significantly improved spatial memory and driving performance	Lamport et al., 2016
		In 20 healthy young adults, 230 ml of purple grape juice improved reactive time, increased calm ratings, elicited a positive effect on memory reaction time	Haskell-Ramsay et al., 2017
		A biosynthetic epicatechin metabolite derived from grapes, 3′-*O*-methyl-epicatechin-5-*O*-β-glucuronide, promotes basal synaptic transmission and long-term potentiation in hippocampal slices through mechanisms associated with CREB signaling	Wang J. et al., 2012
Cocoa	Catechins Anthocyanins Proanthocyanins Flavanols Epicatechin	Dark chocolate fed to rats exposed to air pollution of Mexico city prevented the associated neuroinflammation, COX-2 expression, IL-1β and CD14 mRNA expression in the dorsal vagal complex	Villarreal-Calderon et al., 2010
		Administration of a cocoa polyphenolic extract (22.9 mg/kg/day) to rats after heat exposure protected animals against the associated cognitive impairments as measured in the Morris Water Maze, associated with reduced free radical production by leukocytes	Rozan et al., 2007
		In healthy subjects, consumption of a dark chocolate drink mix containing 500, 250, or 0 mg of polyphenols over 30 days improved measured of mood	Pase et al., 2013
		In the Cocoa, Cognition and Aging (CoCoA) study, consumption of an enriched cocoa flavanol drink containing high (990 mg), medium (520 mg) or low (45 mg) levels of cocoa flavanols per day over 8 weeks improved cognitive function in 90 elderly adults with mild cognitive impairment in a dose-dependent manner	Desideri et al., 2012; Mastroiacovo et al., 2015
Blueberries	Anthocyanins	After 8 weeks of feeding a 2% blueberry supplements diet to aged rats, anthocyanins were found to cross the BBB and improve memory performance	Andres-Lacueva et al., 2005
		In 9 older adults consuming a wild blueberry juice for 12 weeks, improved paired associate learning and word recall was observed with a trend suggesting reduced depressive symptoms	Krikorian et al., 2010b
		In healthy older adults supplemented for 12 weeks with 30 ml of blueberry concentrate providing 387 g of anthocyanins, significant increases in brain activity were observed associated with improved working memory	Bowtell et al., 2017
		Another study demonstrated that acute administration of a flavonoid-rich blueberry extract in both young adults and children improved positive effect on mood	Khalid et al., 2017
Coffee	Flavanols Caffeic Acid Chlorogenic Acid	In aged rats, coffee at an equivalent dose of 5 cups per day, but not caffeine, improved the aged animals' psychomotor control and working memory	Shukitt-Hale et al., 2013
		In a pilot clinical trial, decaffeinated coffee enriched with chlorogenic acids had a greater impact on cognitive performance than regular decaffeinated coffee	Cropley et al., 2012
Green Tea	Catechins (-)-epigallocatechin gallate (EGCG)	In a cross-sectional study involving 1003 elderly Japanese subjects, green tea consumption was associated with attenuated cognitive impairment	Kuriyama et al., 2006; Wang Y. et al., 2012
		In an elderly population with clinical mild cognitive impairment, 16 weeks of treatment with a combination of green tea extract with L-theanine, a protein found in green tea, improved memory and selective attention associated with elevated brain theta waves, which is an indicator of cognitive alertness	Park et al., 2011
		One study involving 27 elderly subjects showed that 2 g/day of green tea powder containing 220.2 mg of catechins did not impact cognitive impairment, despite having a positive effect on oxidative stress	Ide et al., 2016

central (CNS) nervous systems, neuroendocrine connections, humoral pathways, cytokines, neuropeptides and other signaling molecules derived from the gut microbiota itself or produced by the enterochromaffin cells in the gut epithelium in response to the gut microbiota (Mayer et al., 2014). There are several independent and distinct pathways that contribute to the GBA's bidirectional signaling including inflammatory mediators, metabolic signaling, oxidative stress markers, stress modulators, neurohormone factors and direct neuronal communication through the vagus nerve (Kohler et al., 2016; Westfall et al., 2017).

One of the major mechanisms of GBA signaling influencing neurological health is inflammation. The gut microbiota influences inflammation in several ways beginning with management of the epithelial barrier's integrity, constituting

the host's first line of physical defense against invading pathogens. The gut microbiota maintains the thick layer of highly glycosylated mucus on the gut epithelium that promotes the production of tissue repair factors and antimicrobial proteins (Rakoff-Nahoum et al., 2004). In addition, toll-like receptor (TLR)2 signaling, activated by various gram-positive bacterial ligands such as lipoteichoic acid found on *Lactobacillus plantarum* strains, is required for the microbiota-mediated protection of the epithelial barrier and formation of tight junctions (Podolsky et al., 2009). A healthy gut microbiota also regulates the expansion of invading pathogens, some of which harbor immune-activating ligands. Pattern recognition receptors (PRRs), specifically the TLRs and Nod-like receptors (NLRs), on host immune effector cells recognize a variety of antigens known as pathogen-associated molecular patterns (PAMPs) on bacteria, fungi, etc. that normally maintain the GIT's basic immune tone; however, if activated in excess, initiate an innate immune response. The gut microbiota can also influence the cytokine profile of dendritic cells, which is critical to determine the fate of naïve CD4+ T helper (Th)0 cells into Th1, Th2, Th17 or regulatory T cells (Treg) in secondary lymphoid tissues (Barberi et al., 2015), which determines the inflammatory tone in the periphery and brain. It was previously shown that the ratio of Firmicutes to Bacteroidetes determines the balance of Th17 and Treg cells while *Bifidobacterium breve, B. infantis,* and *L. salivarius* were each shown to dose-dependently inhibit the differentiation and activity of early precursor dendritic cells (Round and Mazmanian, 2010). In addition, fecal transplant from inflammatory bowel disease patients into gnotobiotic mice was shown to alter the balance of gut Th17 and RAR-related orphan receptor (ROR)γT^{+} cells favoring elevated numbers of proinflammatory Th17 cells, ultimately exasperating the colitis phenotype in mice (Britton et al., 2019). Although there is little information about the specific gut bacteria that regulate the immune system, one study showed that *Bacteroides fragilis*, which produces a specific bacterial polysaccharide, directly impacts the cellular and physical maturation of the immune system including correcting T cell deficiencies and Th1/Th2 imbalances observed in germ-free mice (Mazmanian et al., 2005).

Apart from inflammatory signaling, the gut microbiota can communicate with the brain through direct nervous afferents. The ENS is the brain of the GIT governing its activity and homeostasis. From the ENS, afferent sensory pathways innervate the nucleus of the solitary tract (NTS) in the brainstem, which integrates the GIT-derived sensory information with autonomic and homeostasis-related functions in the GIT (Browning and Mendelowitz, 2003). Vagal efferents release acetylcholine to excite enteric neurons and inhibit gastric functions, which is a major contributor to symptomatic GIT dysfunctions in response to stress (Travagli et al., 2006). In addition, the vagus nerve originating in the NTS/dorsal vagal complex innervates several key visceral organs including the heart, lungs and GIT through the cholinergic system (Pavlov et al., 2003). Of particular importance, vagal efferents are known to have counter-inflammatory roles through the activation of nicotinic receptors on macrophages, downregulation of T cells and downregulation tumor necrosis factor (TNF)α production via α7-nAChR-agonistic signaling (Ghia et al., 2006). This demonstrates the complexity of the vagal connections and how dysbiosis may have a broad influence on the general inflammatory state in the body.

Finally, the gut microbiota is fundamental in managing the availability of neurotransmitters both through their synthesis in the epithelial lining and the metabolism of their precursors in the GIT lumen. An important study conducted by Asano et al. (2012) demonstrated that the gut microbiota is critical for the production of catecholamines in the luminal space. In addition, *Clostridium* ssp. are required for the biotransformation of catecholamines into their bioactive form owing to their β-glucuronidase enzymes (Asano et al., 2012). It was later determined that several microbiota species produce dopamine including *Bacillus cereus*, *B. subtilis* and *Staphylococcus aureus* (Wall et al., 2014). The gut microbiota is also critical in managing the bioavailable levels of tryptophan and consequently the synthesis of serotonin. Indeed, 95 % of all serotonin synthesis occurs in the GIT, which influences its availability in the brain (O'Mahony et al., 2015). There is also evidence suggesting that *L. plantarum* can produce acetylcholine (Stanaszek et al., 1977), *Bifidobacteria* and *Lactobacillus* spp. can produce micromolar concentrations of GABA (Barrett et al., 2012) and histamine may be produced by several gram-negative species (Devalia et al., 1989). In most of these instances, there also indications that these neurohormone producing species could invoke behavioral effects in animals. For example, the GABA-producing *L. brevis* FPA 3709 can significantly reduce depressive-like behavior in rats as effectively as the known antidepressant fluoxetine (Ko et al., 2013).

The GBA is an integrated and complex bidirectional communication system (Westfall et al., 2017) that is heavily impacted by the composition of the gut microbiota and its metabolites. Modulation of the basal GBA signaling by dietary polyphenols can have important consequences for the prevention and management of neuropsychiatric disorders by simultaneously attenuating multiple risk factors including inflammation, neuronal innervation through ENS-CNS communication and bioavailability of neurotransmitters and their precursors.

THE GUT MICROBIOTA IN NEUROPSYCHIATRIC DISORDERS

The first indication that the gut microbiota can influence a psychiatric disorder was found with irritable bowel syndrome (IBS) (Mulak and Bonaz, 2004); however, this understanding has since been expanded to several other conditions including depression and anxiety (reviewed in Inserra et al., 2018). For example, germ-free mice have reduced anxiety compared to specific pathogen free mice, which is correlated to reduced brain-derived neurotrophic factor (BDNF) expression in the amygdala (Arentsen et al., 2015). This corroborates an earlier study that showed how antibiotic treatment could promote exploratory behavior and hippocampal expression of BDNF linking the composition of the gut microbiota

directly with neurochemical and neurobehavioral effects (Bercik et al., 2011). These early animal studies have since been extended to demonstrate that the gut microbiota of depressed patients is significantly altered, potentially causatively driving symptoms of depression. In a cohort of 10 individuals with severe depression, comparative metaproteomic analyses revealed that there are significant variations in the phyla Bacteroidetes, Proteobacteria, Firmicutes, and Actinobacteria extending to altered abundances in 16 families including reduced *Bifidobacteriaceae* and *Prevotellaceae* with elevated *Enterobacteriaceae* and *Ruminococcaceae* (Chen Z. et al., 2018). A clinical study of 35 patients with major depression showed that depressive symptoms were associated with reduced gut microbiota richness and diversity while a fecal transplant from depressed patients into germ-free mice could reconstitute the depressive phenotype indicating that the gut microbiota causatively promotes depression (Kelly et al., 2016). In a similar cohort, significant variations in the phyla Firmicutes, Actinobacteria, and Bacteroidetes were observed in clinically depressed patients and again, fecal transplantation into mice could transfer the phenotype (Zheng et al., 2016). Interestingly, there are reported gender differences in the gut microbiota composition of depressed patients where female patients have a characteristic increase in the phyla Actinobacteria, male patients have reduced Bacteroidetes and both genders display disrupted Firmicutes homeostasis and elevated *Collinsella* spp. abundance (Chen J.J. et al., 2018).

There have also been intervention studies to understand how supplementation with psychobiotics, probiotics that can impact neuropsychiatric conditions, may influence depressive and anxiety phenotypes. In wild-type mice fed a western-style diet incorporating 33% fat and 49% refined carbohydrates, a phenotype resembling elevated anxiety and memory deficits was observed though prevented by treatment with *L. helveticus R0052* (Ohland et al., 2013). In a study of 124 healthy volunteers (mean age 62 years), those who consumed a mix of psychobiotics (*L. helveticus* and *B. longum*) exhibited less anxiety and depression than controls (Dinan and Cryan, 2013). In a more recent study with cohort of 79 participants with self-reported mood measures, a probiotic preparation also containing *L. helveticus* and *B. longum* did not significantly alter the mood or depression scores compared to the placebo group, however this could be from the heterogeneity, severity or chronicity of the treatment cohort (Romijn et al., 2017). In a large cohort of pregnant women, supplementation with *L. rhamnosus* HN001 lead to significantly less postpartum depression and anxiety compared to placebo controls (Slykerman et al., 2017). In another cohort of patients diagnosed with both IBS and major depression, a twice daily dose of *Bacillus coagulans* MTCC 5856 was administered and treated patients demonstrated reduced depressive phenotypes on multiple scales (Majeed et al., 2018). This accumulation of fecal transplant and psychobiotic intervention studies demonstrate that through the GBA, variations in the composition and consequently metabolism of the gut microbiota has potential therapeutic efficacy for the treatment of neuropsychiatric conditions. To amplify this effect, psychobiotics can be administered in conjunction with dietary polyphenols, as a synbiotic, increasing the production of bioactive metabolites acting on the aforementioned GBA mechanisms.

THE GUT MICROBIOTA INCREASES BIOAVAILABILITY OF DIETARY POLYPHENOLS

Interaction of the Gut Microbiota With Dietary Polyphenols

The interaction of gut bacteria with dietary polyphenols has a two-fold impact on health. First, dietary polyphenols act as prebiotics enhancing the growth of specific beneficial bacterial species that elicit health benefits (Cardona et al., 2013; Duenas et al., 2015; Ozdal et al., 2016). Second, autochthonous gut microbiota can increase the production of bioactive phenolic acids derived from dietary polyphenols increasing their beneficial biological activity (Espin et al., 2017). The former prebiotic effect of botanicals has been extensively described in several reviews (Cardona et al., 2013; Duenas et al., 2015; Ozdal et al., 2016) so will not be further elaborated here; however, the biotransformation of dietary polyphenols by the gut microbiota creating a diverse host of bioactive phenolic acids is a developing understanding, especially toward the promotion of cognitive resilience to depression and anxiety. The gut microbiota contains approximately 10^{14} bacterial cells, 10 times more than mammalian cells present in the human body, which contributes to its huge metabolic potential (Kardum and Glibetic, 2018). In this sense, the gut microbiota can be considered as both a metabolic and an endocrine organ that is critically important for numerous biological activities.

Only 5–10% of dietary polyphenols are absorbed in the small intestine where they subsequently undergo phase I biotransformation (i.e., oxidation) in the endothelial cells and phase II biotransformation (i.e., conjugation) in hepatocytes liberating water-soluble conjugate metabolites (Manach et al., 2005) (**Figure 1**). The remaining polyphenols transit through the small intestine into the colon where the gut microbiota with their specific enzymatic makeup facilitate the bioconversion of various polyphenols and their intermediate metabolites (Braune and Blaut, 2016; Espin et al., 2017). In their natural form, dietary polyphenols are present as conjugates with sugars or organic acids that need to first be liberated before absorption. In the colon, microbial enzymes de-conjugate polyphenols producing the less-polar aglycone forms that can be either absorbed or processed though subsequent microbial reactions in the colon (Murota et al., 2018). There are three major catabolic mechanisms elicited by the gut microbiota to produce bioactive phenolic acids: hydrolysis (*O*-deglycosylations and ester hydrolysis), cleavage (C-ring cleavage, delactonization, demethylation) and reduction (dehydroxylation and double bond reduction) (reviewed in Espin et al., 2017). Indeed, several studies have identified specific enzymes in various gut microbiota that conduct these reactions; however, it must be recognized that ultimately, it is a combination

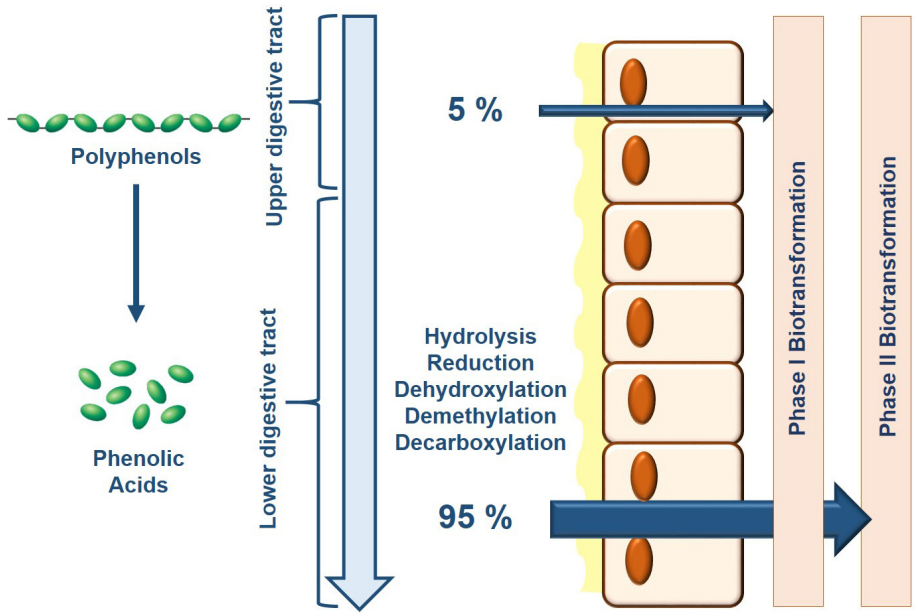

FIGURE 1 | Bioavailability of dietary polyphenols is primarily determined by the composition of the gut microbiota. As consumed, only 5% of dietary polyphenols are absorbed where consequently, Phase I and Phase II biotransformation takes place in the epithelial cells and liver which generates a limited battery of bioavailable metabolites. Upon reaching the lower colon, the gut microbiota breaks down the remaining dietary polyphenols through their endogenous enzymatic activity increasing their relative bioavailability for downstream processing by Phase I and Phase II biotransformation.

of reactions conducted by several microbiota species that produce the final bioactive phenolic acids, a process known as cross-feeding (Duda-Chodak et al., 2015).

One study reported that in rats, 85% of blueberry anthocyanins reached the colon, though 69% disappear from the GIT after 4 h indicating that dietary anthocyanins are heavily metabolized by the gut microbiota (Kahle et al., 2006). Further, an anthocyanin-rich extract from black currants only demonstrated metabolic benefits in the presence of an intact gut microbiota (Esposito et al., 2015). Another study showed that mulberry anthocyanins were specifically transformed by *S. thermophilus* (46.2%) and *L. plantarum* (43.6%) into chlorogenic acid, cypto-chlorogenic acid, caffeic acid and ferulic acid: all phenolic acids with potent anti-inflammatory benefits (Cheng J.R. et al., 2016). Anthocyanins can also be broken down into protocatechuic acid, further into the bioactive phenolic acid cyanidin-3-glucoside (Vitaglione et al., 2007) and finally into 3-hydroxycinnamic acid, which has potent anti-depressive effects possibly through mechanisms implicating inflammation (Hanske et al., 2013).

Since there is high interpersonal variability in the composition of the gut microbiota, production of bioactive metabolites is also highly variable making the physiological health benefits of dietary polyphenols unpredictable in diseased populations whose display dysbiosis. As such, to standardize the physiological benefits of dietary polyphenols, they can be delivered together with probiotics (i.e., synbiotics) normalizing the production of bioactive metabolites, which can then be optimized toward having a beneficial effect. Some authors have identified that these interactions between specific gut microbial species and dietary polyphenols may have negative impacts on the host (Galati and O'Brien, 2004; Nunes et al., 2008), however the vast majority of the interactions are positive, and increase the bioavailability of the ingested polyphenols to elicit beneficial health effects.

Synbiotics Increase the Bioavailability of Polyphenolic Metabolites Enhancing Their Biological Effect

There are only a handful of studies investigating the impact of synbiotics on cognition, and even fewer that utilize a polyphenolic prebiotic. In general, clinical studies with synbiotics are inconclusive as there are a broad cohort diversities and inadequate regulation of treatment regimens as was demonstrated with meta-analyses on IBS patients (Ford et al., 2018), ulcerative colitis (Asto et al., 2019), and diabetes (Zheng et al., 2019). Nevertheless, a few notable studies have been conducted to date demonstrating the potential of synbiotics to act as powerful therapeutic agents in the management of neurological disorders. In a study of a healthy elderly population, patients were separated into either placebo or synbiotic groups where the latter were exposed to two daily doses of a FOS and a probiotic formulation containing *L. paracasei*, *L. rhamnosus, L. acidophilus,* and *B. lactis.* Although there were no significant differences in the depression scores, the synbiotic did improve inflammatory markers of the healthy elderly individuals, notably with an increase in the anti-inflammatory IL-10 cytokine, associated to improvements in cognition (Louzada and Ribeiro, 2018). In a separate study, a cohort of 75 hemodialysis patients were administered a synbiotic

containing *L. acidophilus, B. bifidum, B. lactis,* and *B. longum* with the prebiotics FOS, GOS and inulin. In a subset of patients with depressive symptoms, the synbiotic significantly reduced the depressive score compared to both the probiotic-alone and placebo groups, which correlated to an increase in BDNF serum levels (Haghighat et al., 2019). In one comprehensive animal study, the effects of probiotic (*L. paracasei*), prebiotic (xylooligosaccharide) and synbiotic treatment on chronic high-fat diet (HFD) induced obesity and insulin resistance was evaluated including measures of the associated HFD-induced cognitive decline. Interestingly, all treatment groups reduced HFD-associated inflammation, hippocampal oxidative stress, apoptosis and microglial activation. Although there were no statistical differences between the prebiotic or probiotic groups with the synbiotic, the synbiotic did have a trending beneficial effect on multiple measures of hippocampal activity including dendritic spine density, soma area and apoptosis measures, which could be potentially amplified with the use of a more complex prebiotic formula such as a polyphenol (Chunchai et al., 2018). Although interesting, these synbiotic studies only utilize the traditional fiber-based prebiotics, and to the author's knowledge, only one study to date has tested how a polyphenol-rich synbiotic can affect multiple markers of cognition, using an Alzheimer's Disease (AD) model in *Drosophila*. In this study, a synbiotic that was previously shown to enhance production of polyphenolic metabolites (Westfall et al., 2018a) and promote longevity in *Drosophila melanogaster* (Westfall et al., 2018b) was shown to rescue the AD phenotype in humanized transgenic *Drosophila* (Westfall et al., 2019). In particular, the synbiotic-derived metabolites provided potent anti-inflammatory and antioxidant activity while reestablishing metabolic homeostasis. Of particular interest, when considering all of the AD risk factors as a whole, the synbiotic consistently rescued all of the risk factors to a greater or same extent as its components establishing its combinatorial activity. Despite the lack of studies truly investigating the combinatorial action of synbiotics containing polyphenolic prebiotics, below is a description of potential polyphenolic precursors that are known to require the gut microbiota to produce its full extent of metabolites and elicit potential beneficial effects on cognition and mechanisms associated with depression.

Roasted green coffee beans contain a high level of hydroxycinnamates, which are partially bioavailable yet extensively metabolized, mainly by the colonic microbiota. In subjects who drank a roasted coffee blend containing 269.5 mg of chlorogenic acids, the majority of metabolites in the urine (75.7%), composed of dihydrohydroxycinnamic acids and feruloylglycine, were of colonic origin (Gomez-Juaristi et al., 2018b). The same group identified that the polyphenolic-rich yerba mate was mainly metabolized by the colonic microbiota with up to 81 % of the metabolites composed of dihydroferulic acid, dihydrocaffeic acid and dihydrocoumaric acids (Gomez-Juaristi et al., 2018a). Another example is ester hydrolysis of chlorogenic acid to release caffeic acid, which was determined to be carried out by *B. animalis* by a specific enzyme identified as Balat_0669 (Raimondi et al., 2015).

The urolithins are an important class of bioactive microbial metabolites derived from ellagitannin and ellagic acid precursors. Several bacterial species have been identified that produce their intermediate metabolites including *Gordonibacter urolithinfaciens* and *G. pamelaeae* (Selma et al., 2014); however it has recently been shown that a new class of microbiota species, the *Eggerthellaceae* family, is essential to produce the final urolithin metabolite isourolithin A (Selma et al., 2017; Beltran et al., 2018). It is well known that the microbial-derived urolithins have potent anti-inflammatory effects. In human colonic fibroblasts, urolithins, but not their ellagitannin precursor, inhibited nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation into the nucleus and consequent activation of downstream inflammatory events (Gonzalez-Sarrias et al., 2010). These effects were extended to indicate the microbial-derived urolithins are neuroprotective and pomegranate's anti- AD's neuroprotective ability was attributed to the production of microbial-derived urolithins (Yuan et al., 2016).

Catechins and epicatechins are major constituents of grape seed extracts and the production of their bioactive metabolites is dependent on the presence of the microbiota (Ou et al., 2014). In two grape-seed extracts containing either 70% monomers and 28% procyanidins or 21% monomers and 78% procyanidins, the growth of *Lactobacillus* and *Enterococcus* spp. were elevated while the *Clostridium histolyticum* group was inhibited indicating that specific gut bacteria are responsible for the metabolism of grape seed flavan-3-ols. These changes in the gut microbiota were associated with increases in 4-hydroxyphenylacetic acid, phenylpropionic acid, phenylacetic acid and 4-hydroxybenzoic acid (Cueva et al., 2013). *Eggerthella lenta* JCM 9979 was shown to facilitate the C-ring cleavage of epicatechins and catechins and the subsequent 4'-dehydroxylation to produce different intermediate metabolites (Takagaki and Nanjo, 2015). *L. plantarum* IFPL935 was found to be important in the first step of catechin and procyanidin catabolism involving ring fission, however did not impact the production of phenolic metabolites unless in the context of a complete microbiota indicating that there is another microbe using the metabolic intermediate of *L. plantarum* to produce the bioactive metabolites (Barroso et al., 2013).

The natural flavonoid, quercetin, is also heavily processed by the gut microbiota. In an *in vitro* anaerobic fermentation model, the fecal microbiota was shown to deconjugate rutin, isoquercetin and a mixture of quercetin glucuronides through deglycosylation, ring fission and dehydroxylation reactions to produce the metabolites 3,4-dihydrophenylacetic acid and 3-hydroxyphenylacetic acid (Aura et al., 2002). In an elderly Japanese population, interindividual variations in quercetin concentrations with respect to fecal microbiota compositions were observed and the level of quercetin consumption was negatively correlated with the abundance of *Sutterellaceae* and *Oscillospiraceae* spp. and positively correlated with the families *Fusobacteriaceae* and *Enterobacteriaceae* (Tamura et al., 2017).

When considering neurological diseases, it is important not only to understand the bioavailability of the phenolic acids in the colon and plasma, but also in the brain where their activity is

warranted. Grape seed polyphenol extract (GSPE) is a rich source of flavan-3-ols including catechin, epicatechin, and anthocyanins which were shown to produce a variety of bioavailable phenolic acids both in the plasma and brain (Ho et al., 2013; Wang et al., 2015). Importantly, the production of bioactive phenolic acids derived from the anthocyanin-rich GSPE is dependent on the microbiota, and two of the microbiota-derived metabolites 3-hydroxybenzoic acid and 3-(3′-hydroxyphenyl)propionic acid accumulate in micromolar concentrations in the brain where they can interfere with the assembly of amyloid-beta peptides (Wang et al., 2015). We also found that after moderate wine consumption in rats, there is an accumulation of the polyphenol metabolite quercetin-3-*O*-glucuronide in the brain, which specifically reduced the generation of amyloid-beta from primary neuron cultures generated from the Tg2576 AD mouse model (Ho et al., 2013). At the same time, this metabolite significantly improved AD-type deficits in hippocampal formation basal synaptic transmission. Going further, a Bioactive Dietary Polyphenol Preparation (BDPP) containing GSPE, concord grape juice and resveratrol was shown to attenuate sleep deprivation-induced contextual memory deficits. Supplementation of BDPP lead to the accumulation of malvidin-3-*O*-glucoside and quercetin-3-*O*-glucuronide in the brain where the former activated target of rapamycin (mTOR) signaling and the latter cAMP response element-binding protein (CREB) signaling (Zhao et al., 2015). Combining malvidin-3′-*O*-glucoside with another BDPP metabolite, dihydrocaffeic acid (DHCA) significantly promoted cognitive resilience to stress-induced depression by modulating neuronal plasticity and peripheral inflammation in stressed rats. Mechanistically, DHCA was shown to inhibit DNA methylation on CpG-rich interleukin (IL)-6 sequences while malvidin-glucoside increased histone acetylation of the regulatory sequences that modulate synaptic plasticity (Wang J. et al., 2018).

Despite the small number of studies on the specific activity of synbiotics including a polyphenol-rich prebiotic on the specific mechanisms of depression, it can be concluded that the gut microbiota is essential for producing the full battery of plasma- and brain-bioactive polyphenolic metabolites that have potential neuroprotective activity. Below is a description of how some of the individual microbial-derived polyphenolic metabolites can impact different mechanisms that lead depression, strengthening the argument for the use of synbiotics as depressive therapies.

GUT-BRAIN-AXIS MECHANISMS LINKING POLYPHENOLIC MICROBIAL METABOLISM TO DEPRESSION

Gut Microbiota-Anti-inflammatory Effects Modulate Depression

Neuroinflammation Implicates Neurological Changes in MDD

Chronic neuroinflammation is a major risk factor for neurological diseases as it leads to changes in brain structure and synaptic plasticity resulting in neural deficits (Maes, 1999). Neuroinflammation also modulates neurotransmitter concentrations by upregulating monoamine transporters, reducing synaptic reuptake of monoamines and reducing monoamine synthesis by decreasing tetrahydrobiopterin availability, a cofactor necessary for both tyrosine and tryptophan hydroxylases (Miller and Raison, 2016). The elevated release of cytokines observed with neuroinflammation also drives glutamatergic neurotransmission inducing excitotoxicity and consequently neuronal death (Haroon et al., 2014). Although neuroinflammation is not specific to depression, it does account for a large part of its pathophysiology and anti-inflammatory medication has been successful in alleviating some depressive symptoms (Kohler et al., 2014; Ebada, 2017). As previously described, stress is a major risk factor for depression and neuroinflammation could explain, in part, how stress induces the psychological impairment characteristic of depression. Neuroinflammation, especially elevation in interferon(IFN)-α and IL-6 cytokines, inhibits negative-feedback regulation of the HPA axis, therefore maintaining hypercortisolemia in the context of chronic stress. This elevated glucocorticoid release exasperates the stress response and reduces sensitivity of peripheral immune cells to anti-inflammatory feedback (Frank et al., 2012). Hence, neuroinflammation, when coupled with a reduction in neuroprotection and neuronal repair due to elevated glucocorticoid levels, may be among the initial pathological markers of depression and controlling neuroinflammation with a dietary regime incorporating synbiotics could be a viable prophylactic approach for preventing the onset and/or progression of depression.

Several associations between inflammatory conditions and susceptibility to depression have been made. Patients with rheumatoid arthritis, cancer, autoimmune diseases or other chronic inflammatory conditions are predisposed to depression (Pollak and Yirmiya, 2002). Also, several inflammatory markers have also been used as diagnostic indicators of MDD (Musselman et al., 2001; Vogelzangs et al., 2012). Based on post-mortem studies, depressed patients were found to have area-dependent elevation in proinflammatory cytokine mRNA and protein expression, which is linked to the prominent downregulation in both number and density of oligodendrocytes in areas associated with the depressive phenotype (Mechawar and Savitz, 2016). Depressed suicide completers in particular have elevated mRNA and protein levels of TNF-α, IL-1β and IL-6 in the prefrontal cortex (Pandey et al., 2012), consistent with elevated TLR expression in macrophages and microglia in the corresponding areas (Pandey, 2017).

In depressed patients, there is also evidence of increased blood-brain-barrier (BBB) permeability (Bechter et al., 2010) and through a compromised BBB, cytokines and chemokines infiltrate the CNS, stimulating microglia and astrocyte activation (Wohleb et al., 2016). In a mouse preclinical repeated social stress model, BBB impairment in the nucleus accumbens region was observed in stress-susceptible mice and confirmed in postmortem depressed patients as observed with decreased expression of a key tight-junction protein, claudin 5. This loss of barrier integrity was associated with elevated infiltration of cytokines and subsequent expression of depression-like behaviors

(Menard et al., 2017). The circumventricular organs (CVOs) border the brain's ventricular spaces and lack the typical tight junction integrity of the BBB (Petrov et al., 1994). TLRs on macrophage-like cells in the CVOs are uniquely activated by systemic inflammation and can increase production of proinflammatory mediators. One CVO in particular, the area postrema, is highly interconnected with the nucleus of the solitary tract and dorsal motor nucleus of the vagus nerve (Maolood and Meister, 2009), directly linking inflammatory signals from the GIT with compromised BBB integrity through the CVOs. A compromised BBB also allows the infiltration of peripheral immune cells, including monocytes, dendritic cells and T lymphocytes, into the brain (Najjar et al., 2013). Stress can bias myeloid lineage cells to increase their trafficking ability promoting their increased infiltration into the brain parenchyma (Wohleb et al., 2013). This elevated trafficking could be due to elevated expression of key immune adhesion molecules in specific brain regions after stress. As such both intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 were observed to be increased on endothelial cells in the prefrontal cortex and hypothalamus following a repeat social defeat paradigm in mice, which parallels the patterns of macrophage trafficking and microglial activation in the brain. Once in the brain, these infiltrated monocytes alter behavior and promote microglial reactivity exasperating the neuroinflammatory response (Ataka et al., 2013).

Compromised Gut-Brain-Axis Signaling Instigates Neuroinflammation

A stable and healthy commensal microbiota plays a cardinal role in maintaining the host's immune status (Bercik et al., 2011). On one side, the gut microbiota is immunomodulatory (Round and Mazmanian, 2009; El Aidy et al., 2015), while on the other side, the immune system works to shape the composition and diversity of the intestinal microbiota (Hooper et al., 2012). Incorporation of dietary polyphenols dually impacts the gut microbiota-mediated immunomodulatory effects as they alter both the composition of the gut microbiota with their prebiotic activity while providing precursors for the production of many microbial-derived metabolites that have protective influences on the gut barrier, the first line of immune modulation.

As a first line of defense, the gut microbiota maintains the integrity of the intestinal epithelium, which prevents infiltration of bacteria and other immune-triggering substances into the host's circulation. The microbiota accomplishes this through a variety of mechanisms including maintaining tight junction proteins, production of the mucus layer and secretion of antibactericial proteins and factors, such as IgA, to fend off invading pathogens (Daulatzai, 2014). A major gut-derived mediator of inflammation is lipopolysaccharide (LPS), which through TLR4 signaling activates NF-κB-mediated transcription of proinflammatory cytokines from monocytes and macrophages (Tanti et al., 2012). GIT-mediated inflammation stimulates barrier breakdown and consequently, elevated infiltration of LPS and other bacterial components. One study specifically showed that elevated GIT permeability with an increased translocation of LPS from gram-negative bacteria plays a significant role in the pathophysiology of MDD (Maes et al., 2008). LPS dose-dependently increases IL-1β levels in the dorsal vagal complex, as well as in the hypothalamus, hippocampus, cerebellum, neocortex and pituitary gland (Hansen et al., 2000). Indeed, a single injection of LPS is associated with elevated systemic and central inflammatory mediators and significant cognitive deficits (Kahn et al., 2012), while chronic LPS injections over 5 days in female mice at 1 month intervals induces chronic anhedonia (Kubera et al., 2013).

Interestingly, the gut microbiota has also been associated with deficits in BBB integrity. Germ-free mice have increased BBB permeability and lower expression of occludin and claudin-5 in different brain regions implicated in depression including the frontal cortex, hippocampus and the striatum (Braniste et al., 2014). This group also confirmed that mono-colonization of germ-free mice with the butyrate producing *Clostridium tyrobutyricum* elevated occludin expression in the frontal cortex and hippocampus while reducing BBB permeability (Braniste et al., 2014).

The vagus nerve is also an important player in the bidirectional communication between the gut microbiota and the brain as it monitors the physiological homeostasis of the GIT and connects it to the cognitive and emotional centers in the CNS (Carabotti et al., 2015). One of the major functions of vagal afferents is activation of the HPA axis, which coordinates the adaptive responses of the organism with external stressors, and directly links the health of the microbiota and GIT to depressive phenotypes (Howland, 2014). The vagus nerve also implements an inflammatory reflex where pathogenic species that induce proinflammatory cytokines can activate afferent sensory vagal fibers synapsing in the nucleus tractus solitarius. Efferent vagal signals communicate with the periphery and HPA axis to reduce inflammatory tone by inhibiting the release of TNF-α by splenic nerves (Breit et al., 2018). Notably, there are also receptors on the vagal afferents for various cytokines and TLRs, which can initiate the synthesis and release of inflammatory cytokines from cells within the CNS (Dantzer et al., 2008).

Gut-Derived Polyphenols Reduce Neuroinflammation

There is ample information supporting the anti-inflammatory activity of polyphenols and their metabolites in the context of neurological disorders. However, most studies neglect to integrate the importance of the gut microbiota in producing the polyphenolic bioactives thereby underestimating their full anti-inflammatory potential. Ferulic acid is a hydroxycinnaminic acid produced as a microbial metabolite from several *Lactobacillus* species (Tomaro-Duchesneau et al., 2012) and the production of many of its bioactive metabolic products (dihydroferulic acid or vanillic acid) is dependent on the gut microbiota. Ferulic acid has been shown to have potent implications in depression. *Ligusticum officinale* is an anti-inflammatory plant used in oriental medicine which is rich in ferulic acid and potently can attenuate NF-κB activation in BV2 microglial cells following LPS stimulation (Zeni et al., 2017). In another study utilizing a defeat stress paradigm, ferulic acid at 1 mg/kg reduced oxidative stress and neuroinflammatory markers in the blood, hippocampus and cerebral cortex of mice (Lenzi et al., 2015). In

an outbred ICR mouse model, ferulic acid, in combination with the bioavailability enhancer piperine, reduced immobility in the tail suspension and forced swim test by 60% possibly by inhibiting monoamine oxidase activity in the frontal cortex, hippocampus and hypothalamus (Li et al., 2015). Going further, in a model of chronic unpredictable mild stress, ferulic acid ameliorated depressive-like behaviors possibly through the upregulation of BDNF, postsynaptic protein PSD95 levels, and synapsin I in the prefrontal cortex and hippocampus (Liu Y.M. et al., 2017).

Epicatechin, catechin and the proanthocyanidins are the main flavan-3-ols metabolized by the gut microbiota that also elicit beneficial anti-inflammatory effects. Catechin pretreatment to the chemotherapeutic agent Doxorubicin in rats dose-dependently prevented neurodegeneration while at 100 mg/kg, reduced memory deficits by decreasing oxidative stress, acetylcholinesterase activity and neuroinflammation in the hippocampus (Cheruku et al., 2018). In a rat model of traumatic brain injury, catechin treatment was shown to be neuroprotective by dually protecting both BBB integrity and excessive neuroinflammation (Jiang et al., 2017). A major green tea catechin, (-)-epigallocatechin gallate (EGCG) has many neuroprotective abilities, neuroinflammation being just one of them. Pretreatment of outbred ICR mice with EGCG for 3 weeks (1.5 and 3 mg/kg/day) prior to LPS injection for 7 days prevented the LPS-induced memory impairment and apoptotic neuronal cell death. This included preventing astrogliosis associated with the LPS injections and the consequent production of inflammatory mediators (Lee et al., 2013). In a similar model, EGCG rescued LPS-induced inhibition of adult neurogenesis by restoring proliferation and differentiation of neural stem cells in the dentate gyrus and modulating neuroinflammation through the TLR4-NFκB pathway (Seong et al., 2016).

There is also ample evidence that quercetin can reduce neuroinflammation and as previously indicated, the urolithin metabolic products derived from quercetin have more bioactivity than quercetin itself. In a rat cardiopulmonary resuscitation model of depression, quercetin inhibited ROS generation, neuroinflammation and metalloproteinase-2 protein expression corresponding to recovering left ventricular ejection fraction reduced by the depression paradigm (Wang D. et al., 2018). Mice undergoing chronic unpredictable stress for 21 days were simultaneously treated with 30 mg/kg of quercetin, which alleviated both anxiety and depression behavioral dysfunctions. Simultaneously, quercetin treatment reduced the stress-induced elevation in oxidative stress markers and proinflammatory markers (Mehta et al., 2017). Adriamycin is a chemotherapeutic agent that induces depression- and anxiety-like behaviors in rats. Quercetin (60 mg/kg), alleviated the anxiety and depressive behaviors while attenuating brain oxidative stress and suppressing the excessive corticosterone induction in rats treated with Adriamycin (Merzoug et al., 2014). Quercetin was also shown to reduce depressive behavioral deficits in olfactory bulbectomized rats by simultaneously reducing the oxidative, inflammatory and stress-induced changes in the cerebral cortex and hippocampus. This group suggested that quercetin may elicit its neuroprotective effects through a microglial inhibitory pathway as subclinical amounts of quercetin potentiated the activity of minocycline, a known microglial inhibitor (Rinwa and Kumar, 2013).

The known anti-inflammatory action of resveratrol has been translated to be beneficial in various neuroinflammatory models of depression. Resveratrol inhibits several proinflammatory mediators, modifies eicosanoid synthesis and inhibits enzymes including cyclooxygenase (COX)2, NF-κB, AP-1, TNFα, IL-6 and vascular endothelial growth factor (VEGF) (Namasivayam, 2011). In a social defeat paradigm, resveratrol at 30 mg/kg body weight per day blocked neuroinflammation in the locus coeruleus, but not neurotransmitter release, associated with reduced anhedonia to the sucrose preference test (Finnell et al., 2017).

Caffeic acid is among the main constituents in coffee that have been shown *in vitro* and *in vivo* to have beneficial effects in modulating neuroinflammation (Hall et al., 2015). The derivative of caffeic acid, caffeic acid phenethyl ester, also has a battery of neuroprotective activities including anti-inflammatory and immunomodulatory properties (Noelker et al., 2005) whose production is dependent on the gut microbiota (Peppercorn and Goldman, 1971). Indeed, a negative correlation between the level of coffee consumption and depression has been recorded (Pham et al., 2014). In mice, caffeic acid (4 mg/kg) was shown to have antidepressant-like activity independent of monoamine transduction suggesting that caffeic acid works through a non-monoameringeric system (Takeda et al., 2002). Caffeic acid, at a dose of 30 mg/kg body weight in mice, prophylactically inhibited LPS-induced sickness behavior specifically by reducing cytokine production in serum and brain thus eliciting a protective effect against neuropathologies associated with depression (Basu Mallik et al., 2016).

The anti-inflammatory activity of polyphenols is well known, albeit interesting as a potential therapeutic application in depression. The activity of polyphenols, however, may only be considered in the context of the gut microbiota. As demonstrated previously, the diversity and richness of the gut microbiota is critical to determine the bioavailability of dietary polyphenols, which in their parent form, remain relatively inactive. Likewise, combining dietary polyphenols with optimized probiotic formulation as a synbiotic, would increase the production of specific metabolites whose anti-inflammatory mechanisms could be elucidate and optimized for the treatment of depression.

Activated Microglia Impact Depressive Neuropathology

Microglia are the principle immune mediators in the CNS and their improper activation is associated with neuroinflammation and clinical psychiatric phenotypes (Steiner et al., 2008). In a resting state, microglia conduct immunosurveillance, mediating brain homeostasis and innate immune responses against a range of pathogenic insults primarily through phagocytosis (Hanisch and Kettenmann, 2007). However, under conditions of stress or elevated peripheral inflammation, the microglia transform into an activated state where there is an upregulation of the major histocompatibility complexes

(MHCs) and complement receptors stimulating the production of large amounts of inflammatory cytokines and chemokines (Hanisch and Kettenmann, 2007).

Recently, depression has been described as a microglia-associated disorder with many depressed patients suffering from excessive microglial activation (Yirmiya et al., 2015). In preclinical depression models following a stress paradigm, elevated numbers of activated microglia in the hippocampus, prefrontal cortex, nucleus accumbens and amygdala have been reported (Biesmans et al., 2013; Hinwood et al., 2013). In rats subjected to a mild stress paradigm for 12 weeks as a model of depression, significant microglial activation in the hippocampus was observed (Wang J. et al., 2018). In suicide completers, there is evidence of elevated microgliosis (Steiner et al., 2008, 2011), which has been confirmed with postmortem enrichment of the microglial ionized calcium binding adaptor molecular (IBA)1 marker in the dorsal anterior cingulate cortex of depressed suicide victims (Torres-Platas et al., 2014). In addition, an association between reactive microglia and major depression was shown in a positron emission tomography study demonstrating that the density of translocator protein-18, a mitochondrial protein expressed almost exclusively in activated microglia, was significantly elevated by 30% in the prefrontal cortex, anterior cingulate cortex and insula of patients with major depression (Setiawan et al., 2015). This was confirmed in a recent study in mild to major depression patients where translocator protein 18 was more highly expressed in the anterior cingulate cortex and insula of major depression patients with suicidal thoughts (Holmes et al., 2018).

Gut-Brain-Axis Signaling Abrogates Microglial Activation

The first study showing that the gut microbiota can influence microglial dynamics was conducted by Erny et al. (2015), which showed distinct variations in the microglial transcriptomes of germ-free versus specific pathogen free mice. In particular, many genes involved in cellular activation were down-regulated in the microglia of germ-free animals while flow cytometry analyses indicated that these microglia were immature. Further, this group showed that chronic treatment with SCFAs could reverse the microglial immaturity and malformation observed in germ-free mice indicating the importance of microbial-derived metabolites in shaping the microglial responses (Erny et al., 2015). One study showed that repeated treatment of sodium butyrate attenuated LPS-induced depressive behaviors while simultaneously attenuating microglial activation in the hippocampus, possibly through epigenetic regulation of various promoter elements (Yamawaki et al., 2018). Another study also compared microglial activation between conventional and germ-free mice subjected to a LPS stressor. Using a cytometric bead array analysis from hippocampal and prefrontal cortex samples, germ-free mice demonstrated attenuated production of cytokines in both these areas, which correlated to the observed increase in microglial activation in conventional, but not germ-free mice. Further, the microglia in germ-free mice lacked MHCII markers, CD44 and CD62L, confirming their inability to be stimulated (Campos et al., 2016).

Microbial-Derived Polyphenolic Metabolites Inhibit Microglial Activation

Phenolic acids produced by the gut microbiota also modulate microglial activation. In the AD APP/PS1 mouse model, a pomegranate extract was shown to reduce microgliosis and amyloid-beta plaque deposition in association with reduced anxiety-like behavior and increased memory performance. This effect was attributed to two polyphenolic compounds, punicalagin and ellagic acid, and likely its bioactive microbial-derived metabolite EGCG (Rojanathammanee et al., 2013). Similar to EGCG, resveratrol was shown in neuron-glial primary cultures to inhibit LPS-induced microglial activation and subsequent production of TNFα, nitric oxide and IL-1β likely through modulation of inflammasome signaling (Zhang et al., 2013). In a follow up study, resveratrol reduced hypoxia-induced microglial activation in BV-2 cells, consequently reducing proinflammatory factor release by inhibiting hypoxia-induced NF-κB inhibitor (IkB)-α degradation (Zhang et al., 2015). Chronic constriction injury causes significant glial activation and neuroinflammation in the spinal trigeminal nucleus. Resveratrol treatment after the constriction injury showed an inhibitory effect on the associated microglia and astrocyte activation while reducing the production of inflammatory cytokines through a mechanism implicating MAPK activation (Yang et al., 2016). Quercetin invokes a dose-dependent decrease in nitric oxide production in BV2 microglial cells 1 h prior to LPS treatment. Mechanistically, the authors observed that quercetin suppressed cPLA2 phosphorylation, an activity that was shown to prevent microglia-induced neurotoxicity in differentiated SH-SY5Y neuroblast cells (Chuang et al., 2016). Quercetin was also shown to inhibit obesity-induced hypothalamic inflammation by inhibiting microglia-mediated inflammatory responses, likely through mechanisms involving heme oxygenase induction. These results were verified *in vivo* where microglial activation markers in the hypothalamus of high fat diet fed obese mice were reduced in quercetin-supplemented animals (Yang et al., 2017). Various anthocyanin-rich extracts, particularly from the purple basal, were also shown to attenuate nitrite release from microglial cells stimulated by LPS (Strathearn et al., 2014). Anthocyanins inhibit LPS-induced microglial activation in BV2 microglial cells by inhibiting NF-κB translocation into the nucleus and consequently cytokine release including nitric oxide and prostaglandin E2 release (Jeong et al., 2013).

Similar to the investigation on the anti-inflammatory activity of polyphenolic metabolites, many of the dietary polyphenols discussed for the management of microglial activation require the activity of the gut microbiota to produce the appropriate bioactive metabolites. With an appropriately designed synbiotic formulation, multiple bioactive polyphenolic metabolites may be produced with multiple actions promoting

neuroprotection against exasperated microglial activation, consequently protecting against depressive-like phenotypes.

Inflammasome Activity Drives Neuroinflammation in Depression

The importance of the inflammasome and sterile inflammation in translating psychological stressful stimuli into neuroinflammatory responses has become recently recognized (Herman and Pasinetti, 2018). Pharmacological inhibition (Zhang et al., 2015) or genetic depletion (Iwata et al., 2016) of the inflammasome's assembly abolishes the depressive phenotype in response to various stress models. There are several inflammasome complexes in the body; however, the nod-like receptor pyrin containing 3 inflammasome (NLRP3), implicated specifically in caspase-1 activation, is found predominantly in the microglia under conditions of mild chronic stress (Pan et al., 2014), but can also be induced in neurons under conditions of severe stress (Zendedel et al., 2016). Indeed, NLRP3 gene expression was found elevated in PBMCs of patients with major depression corresponding with elevated serum levels of IL-1β and IL-18, supporting the clinical applicability of inflammasome activation in depression.

The inflammasome can be activated through sterile inflammation making the inflammasome an intracellular sensor to cellular stress and damage instead of direct pathogenic load. Canonical inflammasome activation requires two activating signals. The first signal stimulates the transcription of *Nlrp3*, *IL-1*β and *IL-18* proinflammatory cytokines and is under the control of the PRRs, TLR or NLR, and the subsequent activation of the NF-κB transcriptional program. As such, damaged neurons or psychological stressors release danger associated molecular patterns (DAMPs) including high mobility group box 1 (HMGB1), mtDNA, ATP and the S100 proteins which trigger TLR-associated pathways and present a major risk factor for depression (Fleshner et al., 2017). Each DAMP has a different affinity for either TLRs, or other PRRs such as RAGE (receptor of advanced glycation end products) or P2X7 (reviewed in Franklin et al., 2018) that leads to the same downstream inflammatory cascades including assembly and activity of the inflammasome. The second signal, such as ATP release, instigates assembly of the NLRP3 multimeric complex including recruitment of the apoptosis speck-like (ASC) protein and pro-caspase-1 (Lechtenberg et al., 2014). The assembled proteasome is responsible for the catalytic cleavage of pro-IL-1β and pro-IL-18 by activated caspase-1 leading to inflammatory-driven cellular damage, autophagy and pyroptosis (Gurung et al., 2014).

The stress-induced production of IL-1β is critical for the development of depressive-like behaviors. In a chronic unpredictable stress model in rats, IL-1β mRNA and protein levels produced from inflammasome activation were found elevated in the prefrontal cortex, but not in the serum or CSF (Pan et al., 2014). Interestingly, *Nlrp3*-null mice are resilient to restraint stress-induced depressive-like behaviors including the associated microglial activation or reduced hippocampal neurogenesis (Alcocer-Gomez et al., 2016). Following a foot-shock paradigm, HMGB1 was found to be specifically upregulated in the hippocampus and associated with elevated chemokine and cytokine production (Cheng Y. et al., 2016). Similarly, S100b is elevated in the plasma of major depression patients, and overexpression of S100 is associated with depressive-like behaviors observed with the forced swim test in mice (Stroth and Svenningsson, 2015).

The Gut Microbiota and Related Polyphenolic Metabolites Regulate Inflammasome Activation

Recently, the microbiota-inflammasome hypothesis of major depression was proposed (**Figure 2**) (Inserra et al., 2018). This theory suggests that there is a feedback loop where the gut microbiota-induced production of peripheral inflammation reduces the integrity of the BBB leading to inflammasome activation and consequently, imparts depressive symptoms while simultaneously disrupting the composition of the gut microbiota. A variety of microbial pathogens that can activate the NLRP3 inflammasome have been identified including *Salmonella typhimurium*, *Escherichia coli,* and *Shigella flexneri* (Brodsky and Monack, 2009); yet the mechanisms that drive this activation remain to be fully characterized. The gut bacteria can either directly activate the inflammasome, or indirectly. Through direct activation, inflammasome receptors will recognize bacterial antigens instigating the canonical inflammatory cascades while indirect activation involves sensing changes in the host's response to infection known as "patterns of pathogenicity." The latter includes changes in oxidative stress, potassium efflux or lysosomal destabilization (Storek and Monack, 2015) which can be sensed by the sterile inflammatory response. In one study, specific gut microbiota species were shown to stimulate IL-1β release through inflammasome signaling following spinal cord injury including the *Enterobacteriaceae* family and in particular, the pathobiont *Proteus mirabilis*. This study suggested that these selective members of the gut microbiota could stimulate newly recruited monocytes to induce NLRP3-dependent IL-1β release, promoting inflammation in the intestine and further studies may demonstrate their importance in the depressive clinical phenotypes (Seo et al., 2015).

There are several indications that polyphenol supplementation can reduce inflammasome activation. In line with its aforementioned anti-inflammatory activities, EGCG has been shown to impact inflammasome signaling in multiple models. In a contrast-induced model of renal failure, EGCG downregulated *Nlrp3* gene expression through a pathway involving a known inflammatory regulator heme oxygenase-1 (Gao et al., 2016). In another study, prophylactic EGCG treatment attenuated lupus nephritis symptoms and several inflammatory pathological targets leading to tangible preclinical benefits (Tsai et al., 2011). In endothelial cells, palmitate-induced oxidative stress lead to the strong upregulation of the NLRP3 inflammasome associated IL-1β release and apoptosis, an effect that was ameliorated by EGCG supplementation through mechanisms involving AMPK signaling (Wu et al., 2014).

Quercetin also shows promising ability to attenuate inflammasome-induced inflammation. In a spinal cord injury model in rats, quercetin significantly decreased ROS production, inhibited NLRP3 inflammasome activation and reduced

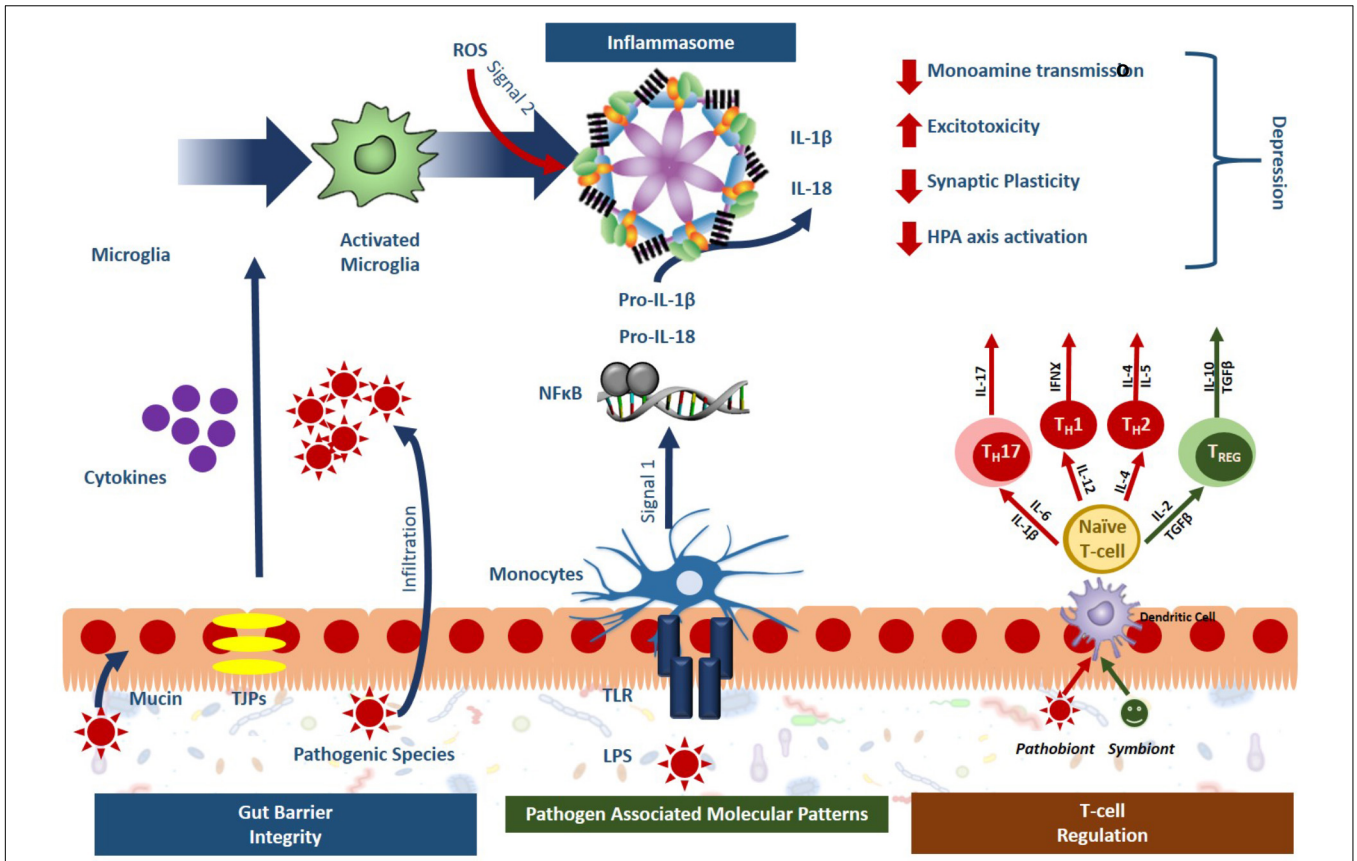

FIGURE 2 | Gut microbiota activated inflammasome signaling leads to phenotypes specific to depression. Sterile inflammatory responses involving inflammasome signaling is a key mechanism of stress-induced depression and the gut microbiota has been shown to impact inflammasome signaling at several levels. Symbionts, or beneficial bacteria, have been shown to promote gut barrier integrity, and guide T-cell regulation toward an anti-inflammatory phenotype (green). Pathobionts, or bacteria with a negative effect, have the opposite effect, compromising the gut barrier integrity, activating immune response through the release of pathogen associated molecular patterns (PAMPs) and pushing T cell development toward a proinflammatory state (red). Overall, these effects lead to inflammasome activation in the periphery and the microglia that ultimately promote the development of depression.

inflammatory cytokine levels (Jiang et al., 2016). *In vitro*, the LPS-producing *E. coli* O157:H7 induced significant upregulation of NLRP3 assembly along with caspase-1 activation and oxidative stress. Quercetin protected NLRP3 activation upon *E. coli* infection in Caco-2 epithelial cells demonstrating its potential to protect the GIT epithelial barrier against pathogenic insults (Xue et al., 2017). Another study suggested that quercetin specifically inhibited NLRP3, and not NLRP1, inflammasome activation by interfering with ASC oligomerization in a dose-dependent manner resulting in lower IL-1β release (Domiciano et al., 2017). Finally, in a streptozotocin-induced diabetes nephropathy model, quercetin suppressed NLRP3 inflammasome activation via, in part, its anti-hyperuricemic effects (Wang C. et al., 2012) demonstrating quercetin's inflammasome-inhibition action *in vivo*. Apigenin, a natural flavone, normalized the expression levels of NLRP3 and IL-1β following microglial activation caused by chronic unpredictable stress in the prefrontal cortex of rats via upregulation of peroxisome proliferator-activated receptor (PPAR)γ receptors (Li et al., 2016). Grape seed-derived procyanidins, rich in apigenin, significantly attenuated gout pain in CD-1 mice caused by macrophage-mediated inflammation and inflammasome activation (Liu H.J. et al., 2017). Finally, in macrophages, apigenin was shown to inhibit LPS-induced production of cytokines primarily through the inhibition of caspase-1 activity and disruption of the NLRP3 inflammasome assembly as well as inhibiting ERK1/2 activation (Zhang et al., 2014).

Several polyphenols have been shown to attenuate the onset of sterile inflammatory cascades. For example, resveratrol can normalize P2X7R expression in a model of chronic pain in rats (Wu et al., 2017). Resveratrol was also shown to exhibit a hepatoprotective effect in diabetic rats mostly through the modulation of RAGE receptor expression (Khazaei et al., 2016). *In vitro*, GSPE attenuated the advanced glycation end products (AGE)-modified bovine serum albumin insult in HUVEC cells by attenuation of surface RAGE expression (Zhang et al., 2013). A GSPE extract was also shown to reduce encephalopathy associated with chronic diabetes through modulation of the AGE/RAGE/NF-κB pathway in the hippocampus (Lu et al., 2010). In a long-term high-fructose fed

model of neurodegeneration in rats, therapeutic supplementation with 6% polyphenol rich grape powder for 12 weeks reduced RAGE expression and tau hyperphosphorylation (Liao et al., 2017). Apigenin and diosmetin, both grape-derived polyphenols, potently and dose-dependently inhibited AGE-induced nitric oxide and TNFα release (Chandler et al., 2010). Finally, there has been some evidence suggesting that quercetin can modulate the RAGE/NF-κB cascade as quercetin attenuated atopic dermatitis symptoms, including downregulation of cytoplasmic HMGB1, RAGE and nuclear NF-kB translocation (Karuppagounder et al., 2015).

Tryptophan Metabolism

Serotonin depletion is one of the most robust blood markers of severe depression (Anderson et al., 1990) and the classical "serotonin hypothesis" describes how diminished serotonin levels play a causative role in depressive phenotypes. However, this hypothesis has been routinely challenged in recent years as the serotonin hypothesis has failed to be substantiated. Diets depleted in tryptophan, the precursor to serotonin synthesis, fail to show any alterations in mood in healthy participants (Smith et al., 1997) indicating that reduced serotonin is neither necessary nor sufficient to cause depression. If this is true, then why are reduced levels of plasma serotonin such a strong biochemical marker of depression? The answer may be the reallocation of tryptophan toward its pro-inflammatory kynurenine degradative pathway, a transition that is dependent on the gut microbiota.

There are two competing pathways for tryptophan metabolism, the methoxyindole and kynurenine pathways. Along the methoxyindole pathway, only 1–5% of dietary tryptophan is synthesized into serotonin, which occurs namely in the enterochromaffin cells in the GIT tract, producing 95% of the body's serotonin (Gershon and Tack, 2007). In the GIT, serotonin is responsible for controlling motility, secretion and absorption of nutrients, intestinal transit time and colonic tone. Approximately 10–20% of the tryptophan allocated toward serotonin development will directly pass through the BBB initiating serotonin synthesis in the brain (Gal and Sherman, 1980). The remaining tryptophan is metabolized along the kynurenine pathway, which forms several metabolites important for the pathophysiology of depression. The balance of tryptophan metabolism is determined by the activation of the rate-limiting enzymes of kynurenine production, which under normal physiological conditions is controlled by the availability of tryptophan itself and the kynurenine pathway remains stabilized (Cervenka et al., 2017). However, under pathophysiological conditions, elevated inflammation and stress can disrupt the balance of kynurenine production.

The rate-limiting enzymes of tryptophan metabolism are indoleamine- 2,3-dioxygenase (IDO) found in all extrahepatic tissues including the brain and tryptophan-2,3-dioxygenase (TDO) found in the liver. Of particular importance, IDO, inducible by IFNγ, is found in the astrocytes, microglia, endothelial cells and macrophages (Gal and Sherman, 1980). TDO, however, is more heavily influenced by corticosteroids produced by the stress response (O'Mahony et al., 2015) linking HPA activation with tryptophan metabolism. There are two competing pathways that further metabolize kynurenine and the resultant metabolites, namely kynurenic acid (KA) and quinolinic acid (QA), are potent neuro- and immuno-modulatory factors. KA is regarded as neuroprotective as its primary function is to antagonize the glycine co-agonist site on NMDA receptors to prevent excitotoxicity (Kessler et al., 1989). On the other hand, QA is neurotoxic, agonizing the same site on the NMDA receptors promoting excitotoxicity (Guillemin, 2012). In the brain, KA is mostly produced in the astrocytes while QA by the microglia and macrophages (Guillemin et al., 2005). Under a state of chronic inflammation, the elevation in corticosterones and inflammatory cytokines increase the peripheral and central production of kynurenine, consequently reducing serotonin production in the brain (Li et al., 2017). In addition, proinflammatory cytokines activate the enzyme kynurenine-3-monooxygenase, which shifts the metabolism of kynurenine from KA to QA increasing the production kynurenine's more neurotoxic downstream metabolites.

Elevated brain QA has been recorded in brains of patients with inflammatory neurological diseases (Stone, 2001) and in depressed patients that attempted suicide for up to 2 years after their attempt (Bay-Richter et al., 2015). In the serum of patients with major depression, there is a reduced ratio of KA to QA (Savitz et al., 2015) associated with an inverse correlation to hippocampal volume, a canonical marker of MDD. Additionally, one of the intermediates between kynurenine and QA, 3-hydroxykynurenine, a potent free-radical generator, directly causes neuronal apoptosis, in addition to activating inflammasome activity (Okuda et al., 1998).

Unfortunately, the development of pharmaceutical interventions to modulate the kynurenine pathway have been unsuccessful. Blocking the activity of IDO or TDO enzymes will leave too much circulating tryptophan to potentially toxic levels, while blocking kynurenine-3-monooxygenase to prevent QA production will skew the KA/QA balance too in favor of KA, which can reduce overall NMDA receptor activity. Further, modulation of IFNγ, or other activators of IDO or TDO, is not specific, and will have widespread side effects (reviewed in Jeon and Kim, 2017). Based on the limitations of pharmacological intervention for kynurenine pathway activity, strategies utilizing the gut microbiota and its ability to produce microbial polyphenolic metabolites may prove successful.

Tryptophan and the Gut Microbiota

The availability of tryptophan is dependent both on diet and importantly, the composition of the gut microbiota as some species utilize tryptophan for the local synthesis of serotonin while others break it down with their endogenous tryptophanase enzyme into the microbial metabolite indole (O'Mahony et al., 2015). Indeed, germ-free animals have elevated circulating tryptophan levels (El Aidy et al., 2012) and elevated circulating tryptophan is associated with increased serotonin levels in the hippocampus (Clarke et al., 2013). Interestingly, the tryptophanase activity of *B. fragilis* was linked to the pathology of autism spectrum disorders (Hsiao et al., 2013). In another study, administration of *B. infantis* resulted in reduced serotonin metabolite (5-HIAA) concentrations in the frontal

cortex (Desbonnet et al., 2008). Further, *L. johnsonii* reduced serum kynurenine concentrations by 17% while correspondingly elevating serotonin levels by 1.4-fold, a result associated with the ability of *L. johnsonii* to suppress IDO activity (Valladares et al., 2013).

Serotonin production in the GIT tract directly connects the gut to neurological signaling as approximately 90% of the dietary tryptophan is metabolized along the kynurenine pathway (O'Mahony et al., 2015), which has a dramatic impact on central serotonin availability. As such, studies have shown that peripherally produced serotonin has neuroactivity, which is critical in many neuropsychiatric conditions including depression (O'Mahony et al., 2015). Interestingly, a fecal microbiota transplant from patients with major depression into germ-free rats induced alterations in tryptophan metabolism, anhedonia and anxiety-like behavior (Kelly et al., 2016) directly linking the gut microbiota composition to depressive-like symptoms.

Polyphenols Impacting Tryptophan Metabolism

There are several instances where polyphenols or their metabolites were shown to modulate signaling through the kynurenine pathway. A bolus dose of resveratrol (5 g) in humans significantly reduced tryptophan levels 2.5 and 5 h after treatment in healthy volunteers resulting in a 1.33- and 1.30-fold increased the in kynurenine to tryptophan ratio, respectively (Gualdoni et al., 2016). However, in a preclinical study, neither IDO activity nor serotonin levels were correlated with resveratrol-mediated protective effects on social-stress-induced cytokine release or depressive-like behavior (Finnell et al., 2017). Polyphenols present in black tea, notably catechins and epicatechins, increased kynurenine levels in healthy volunteers resulting in a higher kynurenine to tryptophan ratio (Gostner et al., 2015). Similarly, EGCG dose-dependently inhibited IDO mRNA and protein expression in human colorectal cells, in correlation with reduced IFNγ levels, possibly through modulating the phosphorylation status and hence activity of STAT1 (Ogawa et al., 2012). In contrast, a group of flavone polyphenols where shown to inhibit IDO activity, but not mRNA expression, in human neuronal stem cells with apigenin having the greatest inhibitory activity and genistein and quercetin the lowest (Chen et al., 2012). It is clear that polyphenols impact tryptophan metabolism; however some of the effects seem inconsistent, likely due to the variable bioavailability of the polyphenols dependent on the composition of the microbiota. Nevertheless, development of synbiotic strategies to optimize the production of polyphenolic metabolites may successfully modulate the activity of the kynurenine pathway to regulate serotonin levels in the brain of depression patients.

Neurogenesis and Synaptic Plasticity in Depression

Most animal models of depression are focused on stress-induced inflammatory models that result in neurodegeneration of specific brain areas and consequently, a depressive phenotype. However, under natural conditions, although neuroinflammation does play a major role in depression, reduced neurogenesis is another major pathological concern (Mahar et al., 2014). Many groups believe that suppressed neurogenesis leads to depression (Kim, 2016) and that this fact is underestimated based on the use of animal models as adult neurogenesis in humans is higher compared to rodents (Spalding et al., 2013). Chronic stress impairs hippocampal neurogenesis, which consequently impacts HPA axis regulation (Dranovsky and Hen, 2006). This feedforward mechanism exacerbates affective behavioral responses, while predisposing an individual to subsequent depressive episodes (Mahar et al., 2014). Indeed, elevated microglial activity is associated with reduced hippocampal neurogenesis, which could account for the canonical loss of hippocampal volume associated with depression (Kempermann, 2002).

BDNF is a neurotrophic factor that is a key modulator of hippocampal neurogenesis. BDNF binds to the tropomyosin receptor kinase B (TrkB) whose downstream signaling pathways play an important role in the structural plasticity induced by depression. Several studies have implicated BDNF levels in multiple brain areas with the pathophysiology of depression with decreased levels in the dentate gyrus and the CA3 of the hippocampus and prefrontal cortex or elevated levels in the nucleus accumbens promoting depressive phenotypes (reviewed in Zhang et al., 2016). As such, TrkB receptor agonists such as 7,8-dihydroxyflaone and receptor antagonists such as ANA-12 have antidepressant effects (Jang et al., 2010), which indicates the sensitivity of physiology to variable levels of BDNF. In a chronic unpredictable stress model of depression, depressive symptoms were correlated to reduced BDNF levels in the hippocampus resulting in the mounting decrease in hippocampal CA1 pyramidal neurons (Qiao et al., 2017). Studies have shown that peripheral levels of BDNF can stimulate overall hippocampal neurogenesis (Schmidt and Duman, 2010) indicating that peripheral physiological effects, such as that mitigated by the gut microbiota, could potentially have antidepressant effects through modulating neurogenesis.

Indeed, there is evidence suggesting that the gut microbiota can alter the expression of neurotrophins such as BDNF in the hippocampus and proteins involved in their synaptic transmission such as synaptophysin and PSD-95 in the striatum (Bercik et al., 2011). Treatment of post-weaned mice with antibiotics was shown to reduce anxiety-like behaviors while promoting cognitive deficits and significantly reducing BDNF levels in the adult brain (Desbonnet et al., 2015). Similarly, depleted microbiota in adult mice also lead to significant depletion of BDNF in the brain, associated with greater susceptibility to depressive-like phenotypes (Hoban et al., 2016). All of these studies indicate that the gut microbiota plays a significant role in managing the levels of BDNF in the brain; however only a handful of studies have investigated how supplementation with probiotics can alter these levels. In one study, supplementation with *L. helveticus* NS8 reduced restraint-stress induced behavioral and pathophysiological markers of depression, specifically including elevated levels of hippocampal BDNF (Liang et al., 2015). In an aged model of Fisher rats, *L. pentosus* var. *plantarum* C29 restored age-reduced loss of motor activity and reduced BDNF levels, while simultaneously ameliorating variations of Akt, mTOR and NF-κB in the

hippocampus (Jeong et al., 2015). Similarly, in aged mice, *L. brevis* OW38 reduced the associated inflammaging and increased the spontaneous alternation behavioral phenotype through the restoration of BDNF expression (Jeong et al., 2016). Recently, C57BL/6J mice subjected to a 5-week chronic unpredictable stress paradigm were supplemented with *Bifidobacterium longum* subsp. *infantis* E41 and *Bifidobacterium breve* M2CF22M7, which together reduced the depressive phenotype partially through rescuing BDNF levels in the brain (Tian et al., 2019). A similar study also showed that supplementation with *Clostridium butyricum* could reduce the depression phenotype and reduced BDNF levels in male C57BL/6J mice undergoing the same stress paradigm (Sun et al., 2018). In contrast, in a randomized controlled clinical trial, 79 patients with moderate scores of self-report mood measures were allocated to take a mixture of *L. helveticus* and *B. longum* for 8 weeks. Although there was significant improvement in the depression score (60%), there was no variation in several plasma biomarkers including BDNF (Romijn et al., 2017) indicating that there is a complex relationship between the composition of the gut microbiota and its effect on neurogenesis and neuroplasticity metabolites.

Likewise, a positive relationship between the consumption of polyphenols with markers of neurogenesis including BDNF has been observed. Several polyphenol-rich natural extracts have been shown to be key modulators of neuroplasticity (Sangiovanni et al., 2017) while many isolated polyphenols have been shown to promote neurite outgrowth *in vitro* including resveratrol, EGCG, ferulic acid, caffeic acid and quercetin derivatives, through mechanisms involving BDNF activity (reviewed in Moosavi et al., 2016). A low-dose unfractionated green tea polyphenol

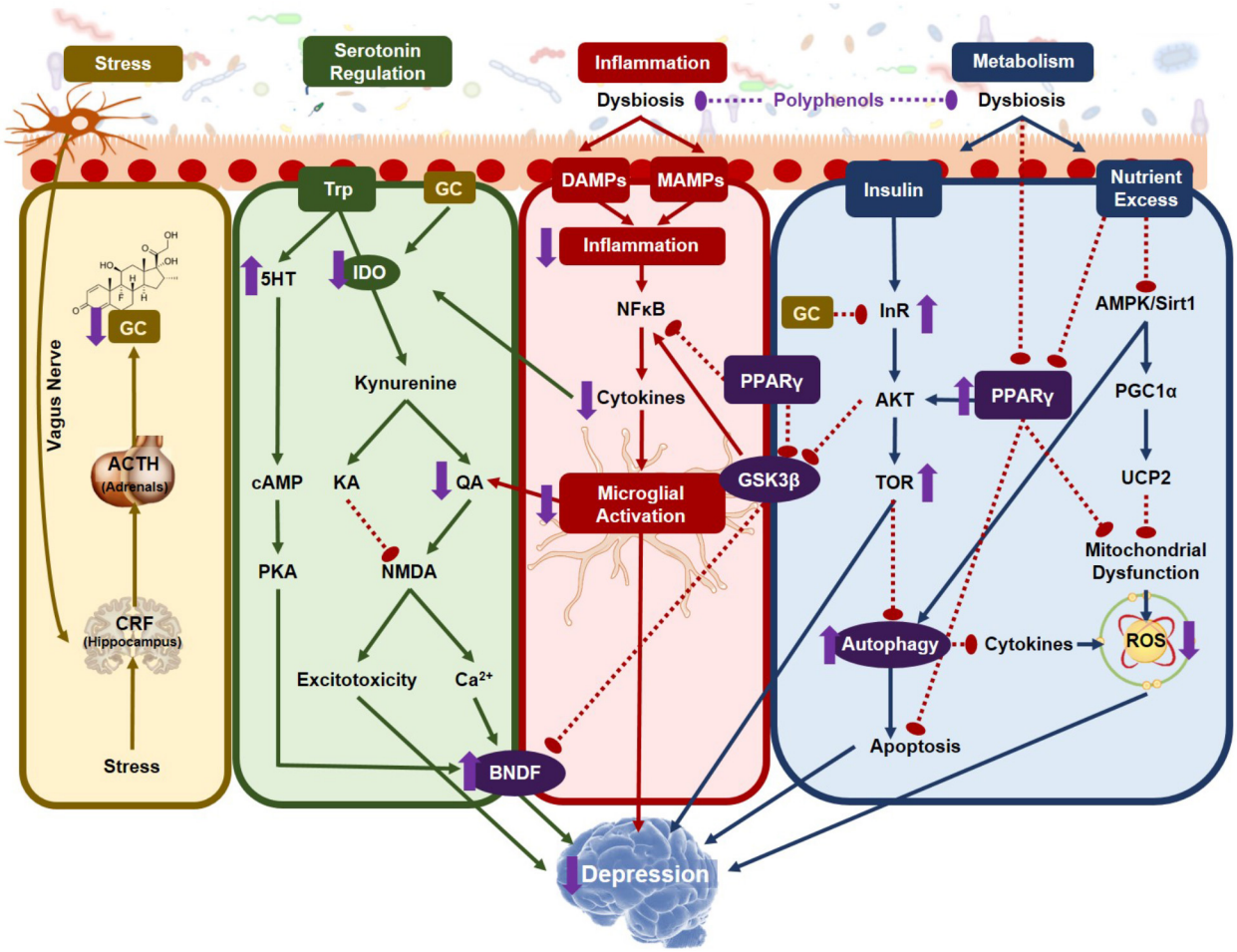

FIGURE 3 | Mechanisms connecting gastrointestinal dysbiosis with biological signature of depression. Depression is a multifaceted disorder with several coordinating pathologies, most which can be modulated by gut microbiota modifying agents including dietary polyphenols. The main gut-brain-axis mechanisms through which polyphenols and their microbial-derived metabolites can elicit a positive effect on depression are stress (yellow), serotonin regulation (green), inflammation (red) and metabolism (blue), effects which are shown as purple arrows. GC, glucocorticoids; ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; 5HT, serotonin; Trp, tryptophan; IDO, indolamine-2,3-dioxygenase; KA, kynuric acid; QA, quinolinic acid; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; BDNF, brain derived neurotrophic factor; DAMPs, danger associated molecular patterns; MAMPs, microbe associated molecular patterns; PPARγ, peroxisome proliferator activated receptor gamma; GSKβ, glycogen synthase kinase 3 beta; InR, insulin receptor; AKT, Protein kinase B; AMPK, 5′ AMP-activated protein kinase; PGC1, proliferator-activated receptor gamma coactivator; UCP2, uncoupling protein 2; ROS, reactive oxygen species.

preparation (<0.1 μg/ml) or a low-dose of one of its active ingredients EGCG (<0.5 μM) potentiated the neuritogenic ability of a low concentration of BDNF in PC12 cells (Gundimeda et al., 2014). In an oxidative stress model of anxiety in rats, GSPE (15 g/L/day) treatment over 3 weeks significantly reduced anxiety-like behavior while restoring, among other markers, BDNF levels indicating that oxidative-stress induced changes in behavior can be rescued by grape seed polyphenol treatment (Allam et al., 2013). In a rat model of posttraumatic stress with a single-prolonged stress through foot shock, grape powder administered at 15 g/L for 3 weeks following the stress protocol reduced anxiety-like behavior while preventing the loss of BDNF levels in the amygdala of affected animals (Solanki et al., 2015). Finally, in a human intervention study, subjects were given a single dose of whole coffee fruit concentrate powder, green coffee caffeine powder, grape seed extract powder or green coffee bean extract powder. It was found that the grape seed extract powder and the green coffee caffeine powder increased the levels of BDNF in the serum by 31% while the whole coffee fruit concentrate powder increased BDNF levels by 143% (Reyes-Izquierdo et al., 2013). As indicated earlier in this review, all of these extracts are modulated by the gut microbiota. It can therefore be predicted that there is a synergistic impact of the dietary polyphenols with the gut microbiota in modulating the plasma and presumably peripheral and central levels of BDNF.

CONCLUSION

Depression is a multifactorial disorder reflecting an accumulation of several pathophysiological conditions including neuroinflammation, elevated microglia activation, an imbalance of tryptophan metabolites and altered BDNF levels. Due to its complexity, no single pharmacological agent targeting one specific aspect of depression's etiology would be sufficient to ameliorate such a diverse set of risk factors. Recently the gut microbiota's interaction with dietary polyphenols has been shown to produce a large battery of bioactive metabolites with the ability to simultaneously modulate the multiple risk factors of depression. As each of the microbial-derived bioactive metabolites produced by a single polyphenol-rich botanical have the potential to overlap or complement the bioactivity of other metabolites produced by the same botanical, the possibility of synergistic and multiplexed activity against multiple depression risk factors is enhanced (**Figure 3**). As demonstrated in this review with the support of mechanistic studies, this synbiotic approach may instigate a paradigm shift in the treatment regime of depression as probiotic and polyphenol-rich botanical supplementation is a cost-effective, long-term treatment option with limited side effects that may be more robust that traditional pharmacological paradigms that target specific depression risk factors.

AUTHOR CONTRIBUTIONS

SW conceived, outlined, and prepared the manuscript. GP directed in all aspects of the manuscript's preparation.

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Zinc Deficiency During Pregnancy Leads to Altered Microbiome and Elevated Inflammatory Markers in Mice

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Zinc is an essential trace metal for bacteria of the intestinal flora. Approximately 20% of dietary zinc – intake is used by intestinal bacteria. The microbiome has recently been described as an important factor for healthy brain function via so-called gut-brain interactions. Similarly, zinc deficiency has been associated with neurological problems such as depression, mental lethargy and cognitive impairments in humans and animal models. However, the underlying pathomechanisms are currently not well understood and a link between zinc deficiency and altered microbiota composition has not been studied. Especially during pregnancy, women may be prone to low zinc status. Thus, here, we investigate whether zinc deficiency alters gut-brain interaction in pregnant mice by triggering changes in the microbiome. To that end, pregnant mice were fed different diets being zinc-adequate, deficient in zinc, or adequate in zinc but high in zinc uptake antagonists for 8 weeks. Our results show that acute zinc-deficient pregnant mice and pregnant mice on a diet high in zinc uptake antagonists have an altered composition of gastro-intestinal (GI) microbiota. These changes were accompanied by alterations in markers for GI permeability. Within the brain, we found signs of neuroinflammation. Interestingly, microbiota composition, gut pathology, and inflammatory cytokine levels were partially rescued upon supplementation of mice with zinc amino-acid conjugates (ZnAA). We conclude that zinc deficiency may contribute to abnormal gut-brain signaling by altering gut physiology, microbiota composition and triggering an increase of inflammatory markers.

Keywords: Zn, microbiota, gastrointestinal, gut-brain, postpartum depression, mood disorder, immune disease, trace metal

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INTRODUCTION

Zinc is one of the most prevalent trace metal ions in the body and plays a major role in the functions of the brain, immune system and endocrine system (Sauer et al., 2016). In humans, acute zinc deficiency is associated with the occurrence of skin lesions, anorexia, diarrhea, growth retardation, depressed wound healing, altered immune function, sensory impairments, and behavioral changes such as lethargy and depression (Aggett and Harries, 1979; Chasapis et al., 2012). Especially the ability to precipitate

depression hints toward an interesting gut-brain interaction, given that zinc required for cognitive processes is ultimately taken up via the gastrointestinal (GI) system (Vela et al., 2015; Rafalo et al., 2016).

Low zinc status has been associated with several mood disorders including major depressive disorders and bipolar depression (Cope and Levenson, 2010). Depression is a mental disorder caused by changes in brain chemistry affecting thought processes, emotions, behaviors and overall physical health (Bondy, 2002). Interestingly, zinc status may be a biomarker of mood disorder (Styczeń et al., 2017). Several studies show that zinc deficiency induces depression while supplementing zinc improves mood as well as cognitive function in humans with depression and animal models (Piao et al., 2017).

Clinical studies have reported a reduction of blood zinc concentration in depressed patients (McLoughlin and Hodge, 1990; Maes et al., 1997; Siwek et al., 2013; Swardfager et al., 2013; Pfaender and Grabrucker, 2014) A recent meta-analysis found that depression in subjects was associated with significantly lower peripheral blood zinc concentration (Swardfager et al., 2013). Interestingly, lower zinc blood concentrations were also reported in women with postpartum depression (Etebary et al., 2010).

In animal models, acute zinc deficiency often was reported to result in depression-like behavior (Hagmeyer et al., 2014). The administration of low zinc diets leads to the development of depressive-like behavior in mice and rats (Tassabehji et al., 2008; Watanabe et al., 2010; Młyniec and Nowak, 2012; Młyniec et al., 2012, 2013a,b), assessed through measuring immobility time in the forced swim test (Corniola et al., 2008; Tassabehji et al., 2008; Whittle et al., 2009; Watanabe et al., 2010; Młyniec et al., 2012, 2013b) or tail suspension test (Młyniec and Nowak, 2012), indicating that zinc deficiency contributes to the development of this behavior. However, the underlying molecular pathomechanisms are still not well known. On a molecular level, zinc has been associated with the GPR39 receptor modulating monoaminergic and glutamatergic neurotransmission, NMDA receptor signaling (Paoletti et al., 2009), glucocorticoids (Nowak, 2001), and BDNF levels, which are all affected in depression (Młyniec et al., 2015). Further, zinc deficiency can disrupt energy metabolism and contributes to chronic inflammation (Bao et al., 2010). There is a tight interplay between zinc levels and inflammation (Prakash et al., 2015), with decreased zinc associated with a pro-inflammatory state. In particular, increased levels of circulating pro-inflammatory cytokines, which may lead to the activation of brain-resident microglia may contribute to neurobiological changes seen in depression (Wohleb et al., 2016) such as dysfunction of the monoamine system, impaired neurogenesis, and alterations in synaptic function (Horowitz and Zunszain, 2015) that result in abnormalities in regional brain activity.

While zinc deficiency negatively affects mood possibly triggering the development of depressive-like symptoms, evidence is mounting that zinc supplementation can be used to improve depressive symptoms in humans and animal models (Nowak et al., 2003; Siwek et al., 2010; Petrilli et al., 2017). Zinc supplementation exhibits antidepressant-like effects in both preclinical and clinical studies (Nowak et al., 2003; Siwek et al., 2009). In addition, the results of several randomized controlled trials show effectiveness of zinc as adjunctive therapy in depressed individuals (Nowak et al., 2003; Siwek et al., 2009; Sawada and Yokoi, 2010; Lai et al., 2012; Ranjbar et al., 2014; Solati et al., 2015). Similarly, the treatment of animal models for depression with zinc showed antidepressant effects, and zinc supplementation enhanced the effectiveness of antidepressants (Rafalo et al., 2016).

Zinc is also an essential trace metal for bacteria of the intestinal flora. A comparison between germ-free rats and rats with pathogen-free intestinal flora revealed that approx. 20% of the dietary intake of zinc was used by intestinal bacteria (Smith et al., 1972). Intriguingly, changes in gut microbiota have been implicated in a variety of conditions including depression (Dinan and Cryan, 2017). In a recent study, depression was reported associated with decreased gut microbiota richness and diversity (Kelly et al., 2016). A feedback loop between depressive states and dysregulation of the microbiome was suggested. An open question is, whether zinc deficiency alone may correspondingly alter the gut microbiome as demonstrated in mice in which chronic depression- and anxiety-like behaviors were induced by olfactory bulbectomy (Park et al., 2013).

In mice, the offspring of mice with zinc deficiency during pregnancy show autism-like behavior (Grabrucker et al., 2014, 2016). Intriguingly, in humans, maternal postpartum depression is associated with the presence of autistic traits in the offspring at 18 months of age (Salvanos et al., 2010). Pregnant women are at increased risk of developing zinc deficiency as the required daily intake almost doubles in this period (Grabrucker, 2016). Further, supplementation of folic acid, high levels of calcium and iron, as well as a diet high in phytates may lower the bioavailability of zinc (Sauer et al., 2017) during pregnancy.

Thus, here, we investigated whether dietary-induced acute zinc deficiency during pregnancy can elicit alterations in the gut microbiota composition in pregnant mice, which may translate into increased inflammatory signaling and ultimately induce changes in brain function in mice. We included a diet adequate in zinc but enriched in zinc uptake inhibitors (folic acid, phytic acid, high Ca/Fe) for comparison with a diet deficient in zinc in our study. We further investigated whether rescuing zinc levels using zinc amino-acid conjugates (ZnAA) ameliorates the observed alterations and thus may be a potential future prevention strategy. ZnAAs have advantages in overcoming especially low bioavailability of zinc (Sauer et al., 2017) and are currently available on the market as a mineral supplement for animals, where they are safe and effective and may be used in human studies in future.

MATERIALS AND METHODS

Materials

PBS with Ca^{2+}/Mg^{2+} was purchased from PAA. Paraformaldehyde was purchased from Merck, and D-Saccharose from Carl Roth. Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich. Primary antibodies were purchased from the following companies: Thermo Fisher

Scientific (ZO-1 polyclonal antibody, 61-7300, mouse specific; FABP2 polyclonal antibody, PA5-18700, mouse specific), Abcam (Anti-Claudin 3 polyclonal antibody, ab15102, mouse specific; Anti-GFAP monoclonal antibody [GF5], ab10062, mouse specific), Origene (monoclonal antibody to *Escherichia coli* LPS (J5 LPS)[Clone ID: 2D7/1], BM1091, besides *E. coli* J5 LPS, the antibody has also been found to react with *K. pneumoniae, S. sonnei*, and *S. typhimurium* LPS, the antibody detects LPS and LPS with modifications), Cell signaling Technologies (IL-6 (D5W4V) XP monoclonal antibody, #12912, mouse specific), Sigma Aldrich (Anti-Iba1/AIF1 monoclonal antibody, MABN92, mouse specific) and Merck Millipore (Anti-NR2B polyclonal antibody, 06-600, mouse specific). Alexa Fluor conjugated secondary antibodies were obtained from Invitrogen/Life Technologies Europe. Secondary HRP conjugated antibodies were purchased from Dako. Zinc amino acid complexes (ZnAAs) were obtained from Zinpro Corporation (Eden Prairie, MN, United States). Special diets for mice were purchased from Ssniff diets, Germany.

Animals

8-week old C57BL/6JRj mice were purchased from Janvier Labs and housed upon arrival in the animal facility in plastic cages under standard laboratory conditions and provided with food and water available *ad libitum*. The housing room was maintained at 22°C, with lights automatically turned on/off in a 12 h rhythm (lights on at 7 am). After 2 weeks of acclimation, mice were divided into 4 groups: the control group (3 females) was fed with standard laboratory food (41 mg/kg zinc) (diet 1), the second group (3 females) was fed a zinc-deficient diet (19 mg/kg zinc) (diet 2). The third group received the standard laboratory food (41 mg/kg zinc), with increased levels of phytates (9.5 mg/kg), folic acid (1.9 mg/kg), Ca (1.13 mg/kg) and Fe (503 mg/kg) (diet 3). The fourth group was given diet 3 with 41 mg/kg ZnAA supplement (Sauer et al., 2017). Mice were given access to distilled, demineralized drinking water *ad libitum*. After 5 weeks, animals became pregnant and were maintained for 3 weeks of pregnancy on the respective diet. All animal experiments were performed in accordance with the guidelines and regulations for the welfare of experimental animals issued by the Federal Government of Germany and by the local ethics committee (Ulm University). The protocol used was approved by the Regierungspräsidium Tübingen, state of Baden-Württemberg, and the Ethics Committee of Ulm University (ID Number: 1257).

Measurement of Trace Metal Concentrations

The Zn-concentration of solutions was measured by flame atomic absorption spectrometry (AAS) at the Department of Clinical Chemistry (ZE klinische Chemie) of the University Hospital Ulm using a PinAAcle 900T from Perkin Elmer.

Immunohistochemistry

Frozen brain sections were cut at 14 μm thickness with a cryostat (Leica CM3050 S) and stored at −80°C until further use. Prior to fluorescent staining, sections were thawed for 20 min in a hydrated staining chamber, fixed in 4% paraformaldehyde (PFA)/4% sucrose/PBS for 20 min and washed three times in PBS for 5 min each. Then, sections were treated with 1x PBS with 0.2% Triton X-100 for 20 min at RT and 1 × PBS with 0.05% Triton X-100 for 10 min at RT. To prevent unspecific antibody binding, blocking was performed with blocking solution (BS) (10% FBS in 1x PBS) for 1 h at RT. Primary antibodies were diluted in BS and incubated overnight at 4°C in a humid chamber. Subsequently, sections were washed 1 × PBS with 0.05% Triton X-100 for 10 min and then incubated in a humidity chamber with secondary antibody (alexa488 or alexa568) in BS for 2 h at 37°C in darkness. After washing of tissue with 1 × PBS plus 0.05% Triton X-100 for 5 min each for three times, and additionally 5 min in 1× PBS, brain sections were counterstained with DAPI (4′,6-Diamidin-2-phenylindol) for 5 min at RT. After washing with aqua bidest, sections were mounted with Vecta Mount. Fluorescence images were obtained using an inverted confocal microscope (Zeiss LSM710) and an ImageXpress Micro Spinning Disc Confocal High-Content Imaging System (Molecular Devices), and analyses of signal intensities were performed with ImageJ 1.48r. For quantitative analysis, signals were thresholded and signal intensities of GFAP/IL-6 immunoreactivity in the vicinity of DAPI labeled cell nuclei measured using the selection tool to determine cytoplasmic protein levels. Exposure times and threshold values were equal for all groups. Brain sections from three animals per group were used and signal intensities from 10 cells from three sections per animal measured in the hippocampal CA1 + CA2 area.

Protein Biochemistry

Liver tissue was immersed in Hepes Sucrose buffer (10 mM Hepes, 0.32 M Sucrose) and disrupted using a sonicator (Fisherbrand sonic dismembranator 120). To obtain homogenate from GI tissue, gut mucus was removed by gently squeezing it out of the intestine with the blunt point of tweezers, and tissue was submerged in PBS. Afterward the cleaned tissue was lysed in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate) plus added protease inhibitor cocktail (complete EDTA-free Protease Inhibitor Cocktail tablets, Roche). Tissue samples in lysis buffer were disrupted with a sonicator (Fisherbrand sonic dismembrator 120). Afterward the obtained homogenate was incubated for 2 h at 4°C on a rotator. Besides semi-quantitative measurement of protein levels, the resulting lysate allows for the analysis of LPS in tissue. All materials used for lysate preparation were sterile and endotoxin-free.

LPS analysis was performed according to Sturm et al. (1984). The optional step of "baking" the nitrocellulose membrane after transfer was not performed due to simultaneous detection of ACTIN. Therefore, more diffuse LPS banding patterns are observed representing higher molecular weight LPS molecules. Using this protocol, the detection of LPS is limited to molecules having side chain lengths of approx. 30 repeat units and greater.

Western Blotting

Protein concentrations were determine by Bradford protein assay and Pierce BCA Protein assay, and equal concentrations loaded per lane. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (GE Healthcare). Immunoreactivity was visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies and Pierce SuperSignal ECL substrate (Thermo Fisher Scientific).

Western Blot Quantification

Evaluation of bands from Western blots (WBs) was performed using ImageJ. Three independent experiments were performed and blots imaged using a UVITEC Alliance Q9 Advanced system. The individual bands were selected and the integrated density was measured. All WB bands were normalized to β-Actin and the ratios averaged and tested for significance. Mean β-Actin signals were measured and compared between groups to exclude effects of the treatments on the proteins used for normalization. No significant differences in β-Actin levels were detected for any treatment group.

Microbiome Analysis

DNA Extraction

DNA extraction of murine fecal samples was performed using the Mo Bio PowerFecal DNA Isolation Kit according to the manufacturer′s protocol. The resulting DNA concentration was measured on a Nanodrop 2000. Purity was assessed by calculating the measured A260/A280 ratio. DNA samples with an A260/A280 ratio between 1.7 to 2.0 were considered pure and subsequently used for microbiome profiling.

Pyrosequencing of 16S rDNA Region V3–V5

Primers were designed to target conserved sequences around the variable region 3–5 (V3–V5) of bacterial 16S rDNA. All bacterial taxonomic profiling via Illumina MiSeq was performed by Eurofins Genomics (Ebersberg, Germany).

Pyrosequencing Data Processing and Taxonomic Classification

All reads with errors were removed from the data set. Processing of remaining reads was performed using minimum entropy decomposition (MED), while splitting up the marker gene dataset into Operational Taxonomic Units (OTUs). Assignment of taxonomic information to each OTU was performed by BLAST aligning of cluster representative sequences to the NCBI sequence database. As a minimal requirement for reference sequences, only sequences with a sequence identity of 80% across at least 80% of a representative sequence were chosen. For each OTU a specific taxonomic assignment was transferred, selected from a set of best matching reference sequences. Using the QIIME software (version 1.8.0), taxonomic assignments and OTUs were processed further.

Gene Expression Analysis (qRT-PCR)

Total RNA from murine brain tissue was isolated with the RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's protocol. Elution of total RNA was performed with sterile RNAse-free water. RNA concentration was measured with the Take3 plate on the Synergy H1 plate reader (Biotek). RNA purity was assessed by A260/A280 absorbance ratio. For each biological replicate the same amount of RNA was used per run. Quantitative RT-PCR was performed using the QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect Primers (Qiagen) in a total volume of 10 μl. Thermal cycling and fluorescent detection were performed using the QuantStudio^{TM} 7 Flex Real-Time PCR System (Applied Biosystems), measuring the SYBR Green I reporter dye signal. Transcript levels were normalized to the housekeeping gene Hmbs. All cycle threshold values (ct) were calculated by the QuantiStudio Real-Time PCR software. All PCR reactions were run in triplicates.

qRT PCR Quantification

Relative quantification is based on internal reference genes to determine virtual mRNA levels of target genes. Ct values were transformed into virtual mRNA levels according to the formula: virtual mRNA level = $10^{*}((ct_{(target)} - ct_{(standard)})/\text{slope of standard curve})$.

LAL Chromogenic Endotoxin Quantitation

Bacterial endotoxins in murine liver tissue samples were measured with the Pierce LAL (Limulus Amebocyte Lysate) Chromogenic Endotoxin Quantitation Kit (Thermo Fisher) according to the manufacturer′s protocol. All materials used for endotoxin measurement in samples were sterile and endotoxin-free (e.g., Fisherbrand DNase/RNase and pyrogen free 1.5 ml tubes, Eppendorf ep Dualfilter T.I.P.S® SealMax). Liver samples were homogenized in Hepes Sucrose buffer using a sonic dismembrator (Fisherbrand) and diluted in endotoxin-free water for the assay. Absorbance levels of standards and samples were measured at 405 nm with the Synergy H1 plate reader (Biotek). With the help of a standard curve, endotoxin levels [endotoxin units/ml (EU/ml)] in liver samples were calculated.

Statistics

Statistical analysis was performed with Sigmaplot 11.0 and GraphPad Prism 5. Data are shown as mean ± SEM. For multiple-group comparisons, analysis of variance (ANOVA) was performed. If groups showed significant differences *post hoc* tests for within-group comparisons were performed (Tukey test). For comparisons of two independent groups, student's *t*-tests were used. Statistically significant differences are indicated in the figures by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

RESULTS

Low Levels of Zinc and Low Zinc Bioavailability Both Lead to Acute Zinc Deficiency in Mice

To understand the relationship of zinc deficiency, microbiome, and brain physiology, female wild type C57BL/6 mice (10 weeks

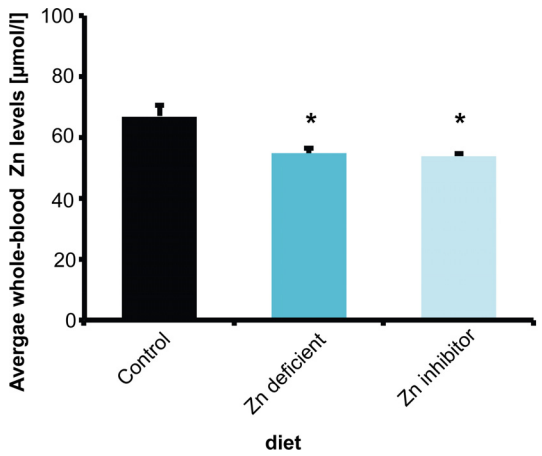

FIGURE 1 | Whole-blood Zn levels of mice measured by AAS in three animals per group. Animals on a control diet (35 ppm) show average zinc levels of 66.7 µmol/l. After 8 weeks on a zinc-deficient diet (<5 ppm), significantly lower zinc levels are measured. Mice on a diet with control zinc levels (35 ppm) but the bioavailability of zinc lowered due to the presence of antagonists of absorption (Zn inhibitor) similarly to mice on a zinc-deficient diet show a significant reduction in whole-blood zinc levels.

of age) were fed 3 different diets for 8 weeks. Mice received either a control diet with adequate supply of all necessary nutrients including zinc (Control diet), a diet low in zinc (Zn deficient diet) that was shown to produce mild zinc deficiency before (Grabrucker et al., 2016), or the control diet with increased levels of Zn uptake inhibitors (phytates, Ca and Fe, and folic acid) (Zn inhibitor diet). Average whole-blood zinc levels were investigated in three animals per group (**Figure 1**). A reduction of zinc in whole-blood does not only reflect decreased zinc levels of a fast exchanging pool of plasma zinc but indicates a zinc deficiency affecting also intracellular zinc levels of blood cells and most likely several other tissues.

The results show that animals on a standard control diet had average whole-blood zinc levels around 67 µmol/l. As expected from previous studies (Grabrucker et al., 2014, 2016), a zinc-deficient diet significantly reduced zinc levels compared to mice on the control diet (one way ANOVA, $F_{(2,6)} = 8.739$, $p = 0.017$, *Post hoc* analysis: Control vs. Zinc deficient, $p = 0.0461$). Interestingly, the presence of additional phytates, folic acid, and Ca and Fe ions (zinc uptake antagonists) also led to a significant reduction in zinc levels (**Figure 1**) (Control vs. Zinc inhibitor, $p = 0.0307$). Thus, the presence of high phytate levels such as found in a plant-rich diet and folic acid and mineral (Ca, Fe) supplements significantly lowers zinc bioavailability in the diet to a level comparable to a zinc depleted diet in these experiments.

Low Dietary Levels or Bioavailability of Zinc Result in Altered Microbiota Composition in Pregnant Mice

Next, to investigate whether low dietary zinc availability over a period of 8 weeks is sufficient to induce alterations in the gut microbiota composition of mice, we performed pyrosequencing of 16S rDNA of fecal samples. A microbiome profile of mice from all different groups was established (**Figure 2**). The results show that both Zn deficient diet and the Zn inhibitor diet lead to significantly different microbiota composition in pregnant mice (**Figure 2A**). However, the microbiota composition was also different between Zn deficient diet and the Zn inhibitor diet. Thus, in the presence of zinc uptake antagonists, some microbiota are still successfully able to compete for zinc. In general, on phylum level, Verrucomicrobia were the most prevalent in control mice. In mice on a zinc-deficient diet, Verrucomicrobia levels are dramatically reduced and Firmicutes become by far the most prevalent microbiota phylum. In mice on a diet with adequate zinc levels but the presence of zinc uptake antagonists, Verrucomicrobia levels are similar to control mice but Firmicutes increase as well. A significantly higher number of different species was detected in the microbiome of animals on a zinc-deficient diet compared to animals on control diet (one way ANOVA, $p = 0.002$, *Post hoc* analysis: Control vs. Zinc deficient, $p = 0.0167$) (data not shown).

In detail (**Figure 2B**), a significant difference was found in the phylum Actinobacteria (one way ANOVA, $p = 0.00003$). While no difference was found between control and Zn inhibitor diet, mice on Zn deficient diet had significantly higher levels of Actinobacteria compared to controls and mice on Zn inhibitor diet (Tukey *post hoc* analysis: Control vs. Zinc deficient, $p < 0.01$; Control vs. Zinc inhibitor, $p < 0.01$). In addition, levels of Bacterioidetes were significantly different (one way ANOVA, $p = 0.00004$). Mice on Zn deficient diet showed significantly increased levels compared to control and Zn inhibitor diet (Tukey *post hoc* analysis: Control vs. Zinc deficient, $p < 0.01$; Zinc deficient vs. Zinc inhibitor, $p < 0.01$). Further, both Zinc deficient and Zinc inhibitor diets significantly increase the level of Firmicutes compared to the control diet (one way ANOVA, $p = 0.00003$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p < 0.01$; Control vs. Zinc inhibitor, $p < 0.01$). The increase was significantly higher in mice on a zinc-deficient diet (Zinc deficient vs. Zinc inhibitor, $p < 0.01$).

In contrast, both Zinc deficient and Zinc inhibitor diets significantly decrease the level of Proteobacteria compared to the control diet (one way ANOVA, $p = 0.0003$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p < 0.01$; Control vs. Zinc inhibitor, $p < 0.01$). The decrease was significantly higher in mice on a zinc inhibitor diet (Zinc deficient vs. Zinc inhibitor, $p < 0.01$). Zinc depleted diet, but not the diet with adequate zinc levels and presence of zinc uptake inhibitors dramatically reduced the amount of Verrucomicrobia (one way ANOVA, $p = 0.0006$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p < 0.01$; Zinc deficient vs. Zinc inhibitor, $p < 0.01$). The number of unclassified reads was significantly higher in mice on a zinc-deficient diet (**Figure 2C**).

Based on the alterations observed on the phylum level, we conclude that the phylum Proteobacteria is very sensitive to zinc depletion as already lower bioavailability of zinc leads to a significant decrease of this phylum. However, within the phylum, not all classes respond similarly. For example, while Epsilonproteobacteria and Betaproteobacteria are highly reduced by zinc restriction, Deltaproteobacteria slightly increase

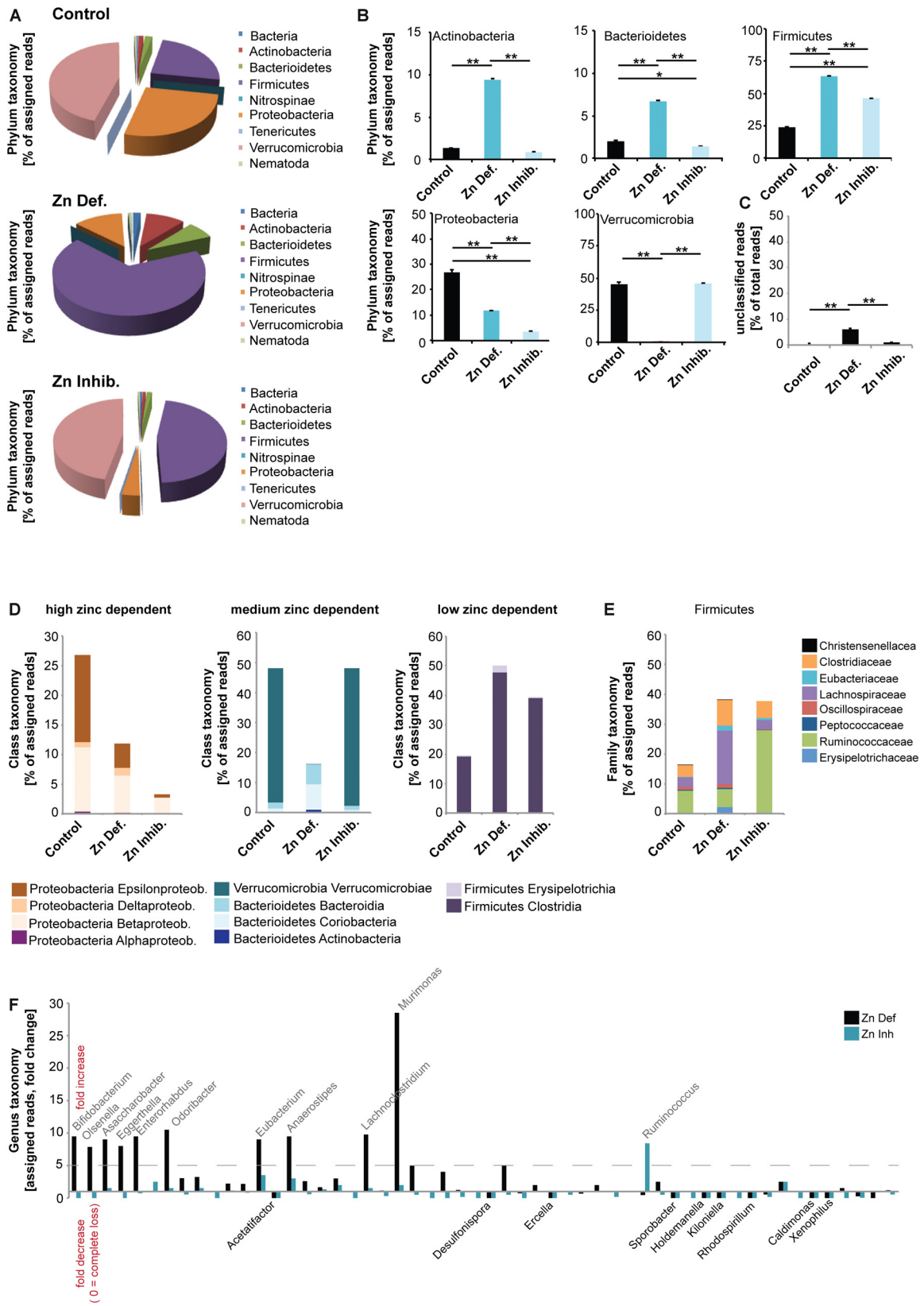

FIGURE 2 | Continued

FIGURE 2 | DNA was extracted from feces from three animals per group and microbiota composition analyzed using 16s microbiome profiling. **(A,B)** On phylum level, significant differences were detected. **(A)** Using the obtained sequence information, single species and their relative amounts were identified. Each phylum was assigned a color. Both, relative abundance and the composition of species are different between the three groups. Verrucomicrobia is the most prevalent phylum in control mice. On a zinc-deficient diet, Verrucomicrobia levels are reduced and Firmicutes are the most prevalent phylum. Mice on the Zn inhibitor diet show an intermediate microbiome with Verrucomicrobia levels similar to controls but also an increase in Firmicutes. **(B)** While no difference was found between the control and Zn inhibitor diet, mice on Zn deficient diet had significantly higher levels of Actinobacteria compared to controls and mice on the Zn inhibitor diet. Levels of Bacterioidetes were significantly increased in mice on Zn deficient diet compared to the control and Zn inhibitor diet. Zn deficient and Zn inhibitor diets significantly increase the level of Firmicutes. The increase was significantly higher in mice on a Zn deficient diet. The level of Proteobacteria significantly decreases in mice on Zn deficient and Zn inhibitor diet. The decrease was significantly higher in mice on a Zn inhibitor diet. Zn deficient but not Zn inhibitor diet significantly reduces the amount of Verrucomicrobia. **(C)** The number of unclassified reads was significantly higher in mice on a zinc-deficient diet. **(D)** Left panel: Proteobacteria are highly sensitive to zinc depletion with a significant decrease occurring both on Zn deficient and Zn inhibitor diet. Although Epsilonproteobacteria and Betaproteobacteria are significantly reduced by zinc restriction, Deltaproteobacteria increase. Middle panel: Verrucomicrobia are not altered under the Zn inhibitor diet, but significantly reduced in mice on a Zn deficient diet. Bacterioidetes, especially Bacteriodia, significantly increase on a Zn deficient diet. Right panel: Firmicutes significantly increase under Zn deficient and Zn uptake inhibition. **(E)** Lachnospiraceae are significantly increased on a Zn deficient diet. Ruminococcaceae are more characteristic of a Zn inhibitor diet. **(F)** A more than fivefold increase in mice on Zn deficient or Zn inhibitor is found in the genus Bifidobacterium (*Bifidobacterium pseudolongum),* Olsenella, Asaccharobacter (*Asaccharobacter WCA-131-CoC-2*), Eggerthella (*Eggerthella YY7918*), Enterorhabdus (*Enterorhabdus mucosicola*), Odoribacter (*Odoribacter laneus*), Eubacterium *(Eubacterium plexicaudatum*), Anaerostipes (*Anaerostipes butyraticus*), and Lachnoclostridium (*Lachnoclostridium scindens*). The most significant and largest increase was seen in the genus Murimonas (*Murimonas intestine*) under Zn deficient conditions. Ruminococcus (*Ruminiclostridium cellobioparum*) display the largest increase under Zn uptake inhibition. Bacteria of the genus Acetatifactor, Desulfonispora, Ercella, Sporobacter, Holdenmanella, Kiloniella, Rhodospirillum, Caldimones, and Xenophilus were highly reduced or absent in mice on Zn deficient and Zn inhibitor diet.

(**Figure 2D**). In contrast, the phylum Firmicutes thrives under low zinc conditions (**Figure 2D**). Verrucomicrobia can cope with low bioavailability of zinc and seem to have mechanisms of zinc intake that successfully compete with the presence of uptake inhibitors. However, under zinc-depleted conditions, the presence of the phylum in the gut microbiome is drastically reduced. Bacterioidetes, especially the class Bacteriodia, are more successful in populating the gut microbiome under zinc depletion (**Figure 2D**).

Given that the loss of Proteobacteria and an increase in Firmicutes occurs in both zinc restricted diets, we closer analyzed the composition of Firmicutes to investigate, which bacteria increase in numbers or are newly found in the GI tract of these mice (**Figure 2E**). We found that especially the Firmicutes family Lachnospiraceae benefits from zinc depleted conditions, while Ruminococcaceae are more characteristic for a diet high in zinc uptake antagonists. Changes in the occurrence of members of the Lachnospiraceae have been associated with chronic inflammation of the gut (Manichanh et al., 2006; Berry et al., 2012).

Finally, we analyzed the different microbiomes on genus level (**Figure 2F**). Among 223 different genera, we highlight those that show a more than 5-fold increase in mice on zinc-deficient or zinc inhibitor diet and those being lost from the microbiome in both zinc deficient or zinc inhibitor diets. We found an increase in the genus Bifidobacterium mostly due to an increase in *Bifidobacterium pseudolongum*, Olsenella, Asaccharobacter due to an increase in *Asaccharobacter WCA-131-CoC-2,* Eggerthella due to an increase in *Eggerthella YY7918,* Enterorhabdus due to an increase in *Enterorhabdus mucosicola*, Odoribacter due to an increase *Odoribacter laneus*, Eubacterium due to an increase in *Eubacterium plexicaudatum*, Anaerostipes due to an increase *Anaerostipes butyraticus*, and Lachnoclostridium due to an increase *Lachnoclostridium scindens*. A very large increase was found in the genus Murimonas, especially *Murimonas intestine* under zinc-deficient conditions, which may be a potent biomarker for zinc deficiency (**Figure 2F**). The genus Ruminococcus, especially *Ruminiclostridium cellobioparum* shows a specific increase in mice on the zinc inhibitor diet (**Figure 2F**).

The genus Turicibacter, Allobaculum, Marvinbryantia, and Butyrivibrio only appeared in the microbiome of mice on zinc-deficient or zinc inhibitor diets (not shown).

Low Dietary Levels or Bioavailability of Zinc Result in Altered Gut Physiology

Enterorhabdus mucosicola was originally isolated from inflamed ileal samples of $TNF^{deltaARE}$ mice (Clavel et al., 2009). In addition, both *E. plexicaudatum* and *E. mucosicola* are associated with inflamed gut mucosa and intestinal epithelial barrier dysfunction. Further, *Egerthella YY7918* is closely related to the type strain *Eggerthella lenta VPI0255*. *Eggerthella lenta* has been to be part of the normal human intestinal microbiome and has been associated with infections of the gastrointestinal tract (Gardiner et al., 2015). Therefore, next, we evaluated markers of intestinal physiology and "leakiness".

We selected three markers, FABP2 (Intestinal fatty acid-binding protein 2), CLAUDIN3, and ZONULIN1 (HP1) (**Figures 3A–C**). FABP2 is a cytosolic protein found in small intestine epithelial cells where it participates in the uptake, intracellular metabolism, and transport of long-chain fatty acids. CLAUDIN3 is a cell adhesion protein found at tight junctions between gut epithelial cells. ZONULIN1 is a physiological modulator of intercellular tight junctions and alterations in the ZONULIN regulated pathways have been associated with both intestinal and extra-intestinal autoimmune and inflammatory disorders (Fasano, 2011). ZONULIN1 and FABP2 have been proposed as markers of gut dysbiosis and gut permeability integrity (Stevens et al., 2018), with a decrease in FABP2 and an increase in ZONULIN1 linked to increased gut permeability (Fasano, 2012).

While we found no significant differences in the lysate from the entire small intestine for FABP2 (**Figure 3A**), mice on a zinc-deficient diet had significantly reduced levels of

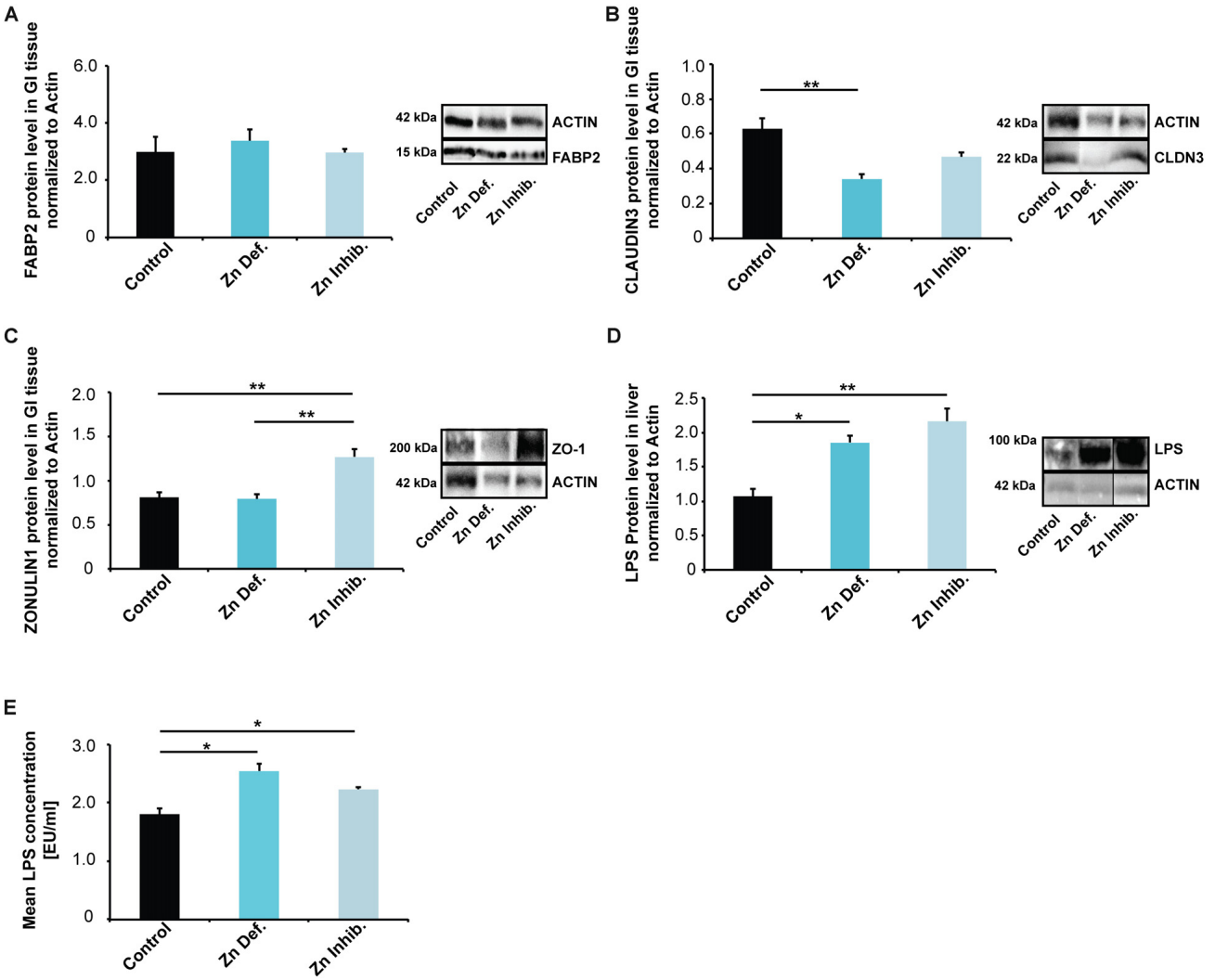

FIGURE 3 | (A–C) Gastro-intestinal epithelium was isolated from three mice per group and protein lysates analyzed using Western blotting. **(A)** No significant differences in FABP2 were detected. **(B)** Mice on a Zn deficient diet had significantly reduced levels of CLAUDIN3. **(C)** ZONULIN1 was significantly increased in mice on Zn inhibitor diet. **(D)** Liver tissue was isolated from three mice per group and protein lysates analyzed for the levels of *E. coli* lipopolysaccharide (LPS) using Western blotting. A significant increase in liver LPS is visible in mice on a Zn deficient and Zn inhibitor diet. **(E)** LPS levels were measured by LAL assay from three mice per group in triplicates. The results show significantly increased LPS levels in liver lysate from mice on Zn deficient and Zn inhibitor diet compared to controls.

CLAUDIN3 (**Figure 3B**) (one way ANOVA, $p = 0.0084$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p = 0.0068$), and mice on Zinc inhibitor diet showed a trend toward a reduction. ZONULIN1 was significantly increased in mice on Zinc inhibitor diet (**Figure 3C**) (one way ANOVA, $p = 0.0046$; Tukey *post hoc* analysis: Control vs. Zinc inhibitor, $p = 0.0082$) compared to controls and mice on zinc-deficient diet (Tukey *post hoc* analysis: Zinc deficient vs. Zinc inhibitor, $p = 0.0069$).

The detoxification of microbial products from gut-derived microbiota is a function of the liver. Analyzing liver tissue of mice for the levels of *E. coli* lipopolysaccharide (LPS), we found a significant increase in liver LPS in mice on a zinc-deficient and zinc inhibitor diet (one way ANOVA, $p = 0.0054$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p < 0.05$; Control vs. Zinc inhibitor, $p < 0.01$) (**Supplementary Figure S1A** and **Figure 3D**). To validate this data, we additionally measured *E. coli* LPS (endotoxin) levels in mouse liver using a Limulus Amebocyte Lysate (LAL) based assay. The results confirm significantly increased levels of LPS in liver of mice on a zinc-deficient and zinc inhibitor diet (one way ANOVA, $p = 0.047$; *post hoc* analysis: Control vs. Zinc deficient, $p = 0.041$; Control vs. Zinc inhibitor, $p = 0.0143$) (**Supplementary Figure S1B** and **Figure 3E**). Thus, altered microbiota composition together with an increased intestinal permeability may be responsible for increased translocation of bacterial LPS into the systemic circulation (Szabo et al., 2010).

Low Dietary Levels or Bioavailability of Zinc Result in Increased Inflammation in Pregnant Mice

Given that an increase of ZONULIN1 and loss of CLAUDIN3 have been associated with increased gut permeability and inflammation, we next analyzed whether we can detect signs of (neuro)inflammation in the brain of pregnant mice with low zinc status. To that end, we analyzed tissue for the expression levels of GFAP and IL-6. Glial fibrillary acidic protein (GFAP) is an established marker for the activation of astrocytes following injury or stress (Zhang et al., 2017). Expression of IL-6 was reported being induced in both astrocytes and microglia in response to LPS and increased levels in the brain are related to inflammatory and pathological situations (Erta et al., 2012). Our results show a significant increase in GFAP expression levels in brains of mice subjected to zinc deficiency and lowered bioavailability in the diet compared to control mice (one way ANOVA, $p = 0.0044$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p = 0.0447$; Control vs. Zinc inhibitor, $p = 0.0036$) (**Figure 4A**). The number of GFAP positive cells was not significantly altered (**Figure 4A**, lower panel).

The levels of IL-6 were significantly elevated in brain tissue of both mice on zinc-deficient diet and mice with sufficient dietary zinc levels but presence of zinc uptake inhibitors compared to controls (one way ANOVA, $p = 0.028$; Tukey *post hoc* analysis: Control vs. Zinc deficient, $p = 0.04547$; Control vs. Zinc inhibitor, $p = 0.038557$) (**Supplementary Figure S2A**). However, measuring IL-6 levels in brain sections by IHC suffers from specificity and sensitivity issues. Therefore, to further validate the data from IHC, we assessed IL-6 expression on the transcription level. The results show a significantly higher concentration of IL-6 mRNA in the brain of pregnant mice on a zinc-deficient diet (*t*-test, $p = 0.0041$) compared to controls (**Figure 4B**). Besides, we detected the increased expression of further inflammatory marker genes in pregnant mice on a zinc-deficient diet such as significantly higher levels of IL-1b ($p = 0.0034$), S100β ($p = 0.0185$), and CCL2 ($p = 0.0109$, *t*-tests) (**Supplementary Figure S2B**). The increased IL-6 transcription translates into increased IL-6 on protein level, further quantified by western blotting (*t*-test, $p = 0.0283$) (**Figure 4C**).

Although more extensive analyses need to be done in the future, the obtained data hint at a physiologic relevant impact of dietary zinc restriction during pregnancy on the brain of pregnant mice.

Supplementation of Maternal Diet With ZnAAs Prevents Several Alterations Induced by Low Bioavailability of Zinc

Recently, we have investigated the mechanisms of uptake and absorption of ZnAAs (Sauer et al., 2017). ZnAAs are zinc supplements with zinc stably conjugated in an amino acid backbone. These ZnAAs were taken up by cells, not through classical zinc transporter proteins but amino acid transporters. Therefore, ZnAAs showed a significant advantage compared to inorganic zinc salts such as $ZnCl_2$ as a supplement, since ZnAAs do not compete with other metals for zinc transporters and seem

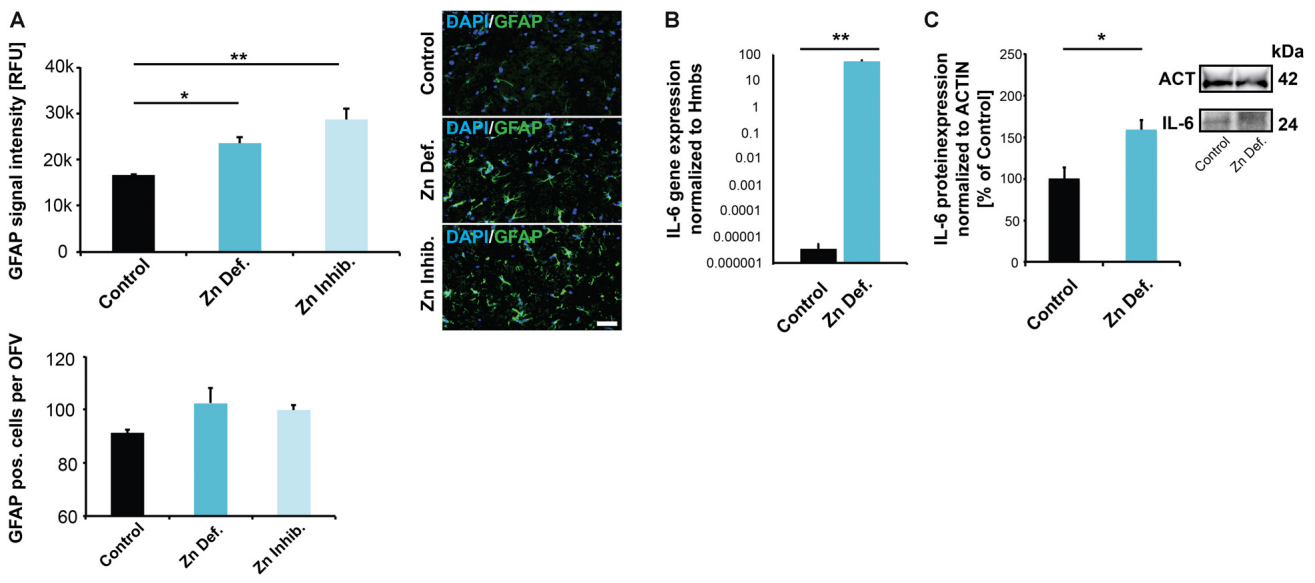

FIGURE 4 | (A) Brain sections from three animals per group were used for immunohistochemistry. DAPI (labeling cell nuclei) and GFAP was visualized and fluorescent signal intensities from 10 cells in the hippocampus from three sections per animal measured. Mean values show the average of three animals per group. Upper panel: A significant increase in GFAP expression levels can be detected in the brains of mice subjected to zinc deficiency and lowered bioavailability (Zn Inhibitor) compared to control mice. Lower panel: No significant difference in the number of GFAP positive cells per optic field of view (OFV) was found. Scale bar = 100 μm. **(B)** Whole-brain total RNA lysate from three animals per group was used to analyze the expression of IL-6 on gene level normalized to Hmbs. A significantly higher IL-6 expression was found in the brain of zinc-deficient mice. **(C)** Whole-brain protein lysate from three animals per group was used to analyze IL-6 expression on protein level normalized to ACTIN. Significantly higher IL-6 levels are found in the brain of zinc-deficient mice.

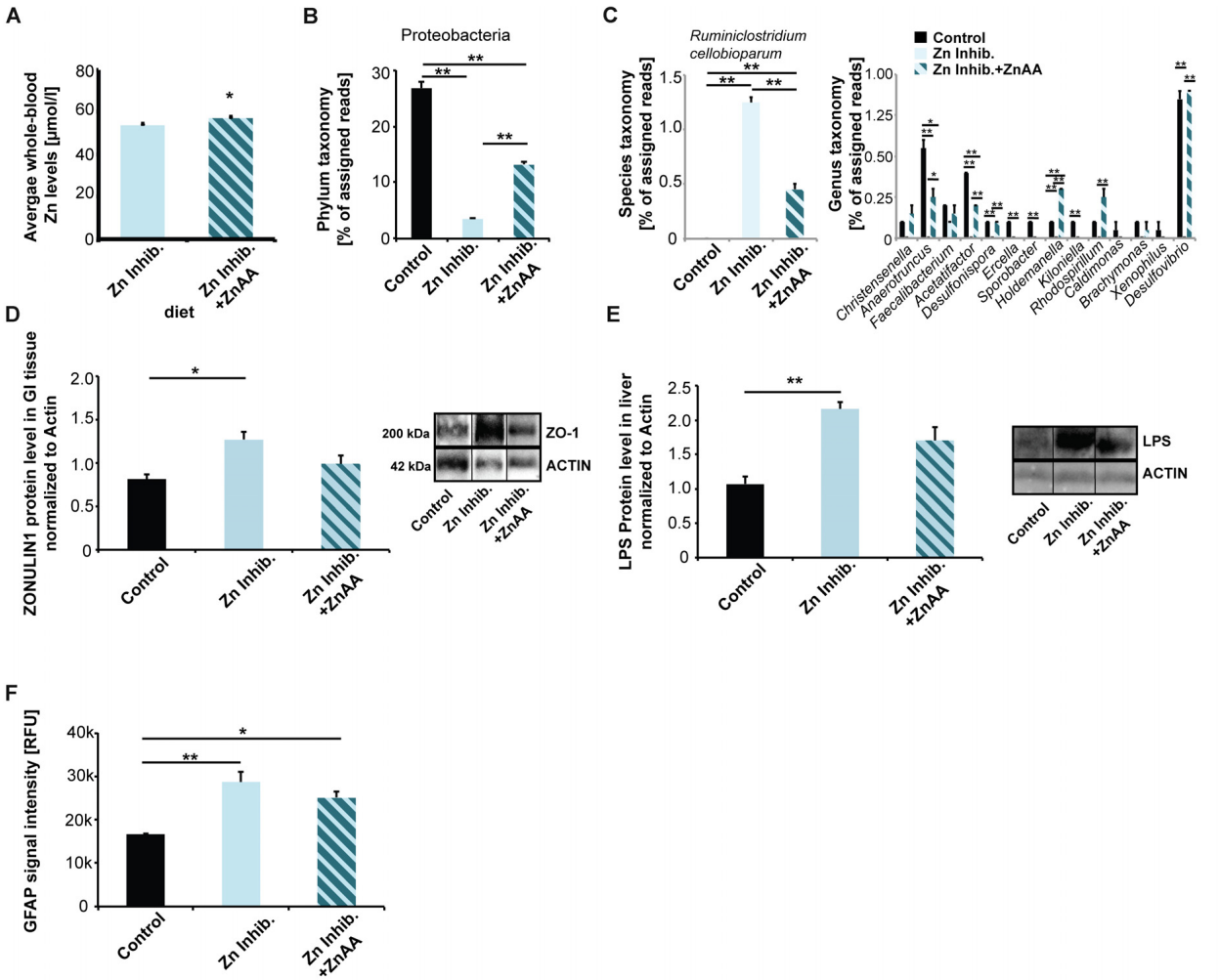

FIGURE 5 | (A) Whole-blood Zn levels of mice measured by AAS in three animals per group. Mice were fed ZnAA supplemented diet (diet 4, Zn Inhibitor + ZnAA). At the end of pregnancy (8 weeks treatment in total), mice on a diet with control zinc levels (35 ppm) but bioavailability of zinc lowered due to the presence of antagonists of absorption (Zn inhibitor) show significantly lower whole-blood zinc levels compared to mice on the same diet with the addition of ZnAAs. **(B)** Pyrosequencing of 16S rDNA of fecal samples shows that the abundance of Proteobacteria is significantly higher in mice on Zn inhibitor + ZnAA diet compared to mice on Zn inhibitor diet. The levels were still significantly lower compared to mice on the control diet. **(C)** Left panel: On species level, the almost 10-fold increase in *Ruminiclostridium cellobioparum* seen in mice on Zn Inhibitor diet was partially prevented. Right panel: Bacteria of the genus Christensiella, Anaerotruncus, Faecalibacterium, Acetatifactor, Desulfonispora, Ercella, Sporobacter, Holdenmanella, Kiloniella, Rhodospirillum, Caldimones, Brachymonas, Xenophilus, and Desulfovibrio were highly reduced or absent in mice on Zn inhibitor diet. Supplementation with ZnAA was able to prevent the reduction/loss for Christensiella, Anaerotruncus, Faecalibacterium, Acetatifactor, Desulfonispora, Holdenmanella, Rhodospirillum, Brachymonas, and Desulfovibrio. **(D)** GI epithelium was isolated from three mice per group and protein lysates analyzed using Western blotting. Supplementation with ZnAA was able to prevent the significant increase of ZONULIN1 observed in mice on Zn Inhibitor diet. Data for Control and Zn Inhibitor diet has been reused from **Figure 3C**. **(E)** Liver LPS is significantly increased in mice on Zn inhibitor diet, but not in mice on Zn inhibitor + ZnAA diet. **(F)** Brain sections from three animals per group were used for immunohistochemistry. DAPI (labeling cell nuclei) and GFAP or IL-6 were visualized and fluorescent signal intensities from 10 cells in the hippocampus from three sections per animal measured. Mean values show the average of three animals per group. **(F)** A significant increase in GFAP expression levels can be detected in the brains of mice on the Zn Inhibitor diet compared to control mice. Dietary supplementation with ZnAA slightly, but non-significantly decreased GFAP expression levels.

to be less accessible for folic acid or phytic acid. Therefore, here, we supplemented a diet rich in zinc uptake antagonists (diet 3, Zn Inhibitor) using ZnAAs to investigate whether the observed changes can be prevented by dietary zinc supplementation.

Mice were fed a ZnAA supplemented diet (diet 4, Zn Inhibitor + ZnAA) for 8 weeks. At the end of pregnancy, the average whole-blood zinc levels were investigated and compared to mice on the same diet with low bioavailability of zinc but without zinc supplementation and controls. The results show that the addition of ZnAAs to the diet leads to significantly higher zinc levels compared to mice on the diet with low bioavailability of zinc (*t*-test, $p = 0.0225$) (**Figure 5A**) and no significant difference can be seen compared to controls ($p = 0.0736$).

Next, we examined if alterations in microbiota composition are normalized by zinc supplementation. We again performed

pyrosequencing of 16S rDNA of fecal samples and investigated the microbiome of mice fed ZnAA supplemented diet (**Figure 5B**). The results show that on phylum level, the abundance of Proteobacteria was significantly higher in mice supplemented with ZnAA compared to mice on a diet with lowered bioavailability of zinc. However, the levels did not reach those of mice on a control diet (one way ANOVA, $p = 0.0004$; Tukey *post hoc* analysis: Control vs. Zinc Inhibitor, $p < 0.01$; Control vs. Zinc inhibitor + ZnAA, $p < 0.01$; Zinc Inhibitor vs. Zinc inhibitor + ZnAA, $p < 0.01$). We could not detect further rescue effects on phylum level. Instead, supplementation with ZnAA seemed to generate a new microbiota composition, which was significantly different from controls and mice on the Zn Inhibitor diet (**Supplementary Figure S3A**). Therefore, we focused on the most prominent alterations observed in mice on a diet with low bioavailability of zinc on genus and species level and compared these to mice on the same diet, but with supplementation of ZnAAs. An almost 10-fold increase in Ruminococcus (*R. cellobioparum*) was observed under the low bioavailability of zinc. Supplementation with ZnAAs was able to partially prevent this increase (**Figure 5C**, left panel) (one way ANOVA, $p = 0.0005$; Tukey *post hoc* analysis: Control vs. Zinc Inhibitor, $p < 0.01$; Control vs. Zinc inhibitor + ZnAA, $p < 0.01$; Zinc Inhibitor vs. Zinc inhibitor + ZnAA, $p < 0.01$). Bacteria of the genus Christensiella, Anaerotruncus, Faecalibacterium, Acetatifactor, Desulfonispora, Ercella, Sporobacter, Holdenmanella, Kiloniella, Rhodospirillum, Caldimones, Brachymonas, Xenophilus, and Desulfovibrio were highly reduced or absent in mice on Zn inhibitor diet. On genus level, supplementation with ZnAA was able to prevent many of these losses (**Figure 5C**, right panel) (one way ANOVA followed by Tukey *post hoc* analysis). For example, no decrease in the Faecalibacterium genus that may be beneficial to the host concerning inflammatory processes (Sokol et al., 2008) was seen.

Interestingly, supplementation with ZnAA was able to prevent the loss of ZONULIN1 observed in mice on Zn Inhibitor diet (**Figures 3C**, **5D**) (one way ANOVA, $p = 0.0219$; Tukey *post hoc* analysis: Control vs. Zinc Inhibitor, $p = 0.0186$; Control vs. Zinc inhibitor + ZnAA, $p = 0.34$). In addition, the levels of liver LPS decreased and were no longer significantly different from mice on control diet (**Figure 5E**) (one way ANOVA, $p = 0.0065$; Tukey *post hoc* analysis: Control vs. Zinc Inhibitor, $p < 0.01$; Control vs. Zinc inhibitor + ZnAA, $p = 0.05738$).

In the brain, we could not observe a significant effect on astrocyte activation, as animals on Zinc inhibitor + ZnAA diet still showed a significant increase in GFAP expression, although slightly less compared to mice on Zinc inhibitor diet (one way ANOVA, $p = 0.0044$; Tukey *post hoc* analysis: Control vs. Zinc Inhibitor, $p = 0.0039$; Control vs. Zinc inhibitor + ZnAA, $p = 0.0219$) (**Figure 5F**). In contrast, while mice on Zinc inhibitor diet had significantly increased IL-6 brain tissue levels, we detected no difference between Controls and mice on Zinc inhibitor diet supplemented with ZnAAs (**Supplementary Figure S3B**). Thus, zinc supplementation was able to prevent an increase in IL-6 brain levels.

DISCUSSION

Zinc deficiency plays a role in the etiology of depressive disorders in mouse models and humans. Several studies have reported an inverse relationship between low zinc levels and higher Hamilton Depression Rating Scale scores in patients (Maes et al., 1994). Interestingly, zinc deficiency also impairs the efficacy of several antidepressants (Tassabehji et al., 2008; Młyniec and Nowak, 2012; Młyniec et al., 2012). However, the mechanisms behind are not fully understood.

Further abnormalities have been independently reported in animal models and human patients with depression such as alterations in the gut microbiota composition and increased inflammatory responses and chronic inflammation. Several studies in the past revealed a link between depression and altered gut microbiota composition. From these a motif emerged, where significant alterations in the abundance of gut microbiota within the phyla *Bacteroidetes, Firmicutes, Proteobacteria*, and *Actinobacteria* were reported in patients diagnosed with major depressive disorder, but also in relevant rodent models (Winter et al., 2018).

With regards to inflammation, it was found that patients with major depressive disorder show all of the key features of an inflammatory response such as increased expression of pro-inflammatory cytokines and chemokines, cytokine receptors, and soluble adhesion molecules in peripheral blood and cerebrospinal fluid (CSF) (Miller and Raison, 2016). Especially, increased expression of IL-1β, IL-6, TNF, Toll-like receptor 3 (TLR3) and TLR4, has been found in post-mortem brains (Maes, 1995; Brambilla et al., 2014; Drago et al., 2015) and consistent with this, activation of IL-6, IL-8 and type I IFN-induced signaling pathways has been reported (Brambilla et al., 2014). A meta-analysis found that IL-1β, IL-6, TNF and C-reactive protein (CRP) in peripheral blood are the most reliable biomarkers of inflammation in patients with depression (Miller et al., 2009).

Here, we sought to establish a link between maternal zinc deficiency, altered microbiota composition, and inflammation. Zinc deficiency was reported before to affect microbiota composition. For example, similar to the result reported here, a decrease in Veruccomicrobia and an increase in Firmicutes has been observed (Mayneris-Perxachs et al., 2016). The low relative abundance of Verrucomicrobia populations and a decrease in beneficial bacteria was correlated with zinc deficiency in further studies (Lopez and Skaar, 2018). In addition, low zinc status as well as zinc supplementation were reported to affect gut microbiota in chicken (Reed et al., 2015, 2018). However, to our knowledge, the effects of zinc deficiency on the microbiome of pregnant mice in light of the observed behavioral alterations in the offspring of zinc-deficient mothers has not been investigated so far.

Using pyrosequencing of 16S rDNA of fecal samples, we obtained microbiota profiles from animals on four different diets: a control diet, a diet low in zinc, a diet with low bioavailability of zinc induced by elevated concentrations of other dietary components such as Fe, Ca, and folic acid that are commonly prescribed to pregnant women, and a diet with

low bioavailability that was supplemented with zinc in the form of ZnAA to overcome inhibition by zinc uptake antagonists present in this diet (Sauer et al., 2017). Although no clear cut-off values between hypozincemia and zinc deficiency are established for mice, we consider the status of mice on a zinc-deficient diet and diet with zinc uptake inhibitors as mild zinc deficient. This is based on the fact that in many cases, in human studies, hypozincemia cannot be picked up in blood samples and blood/plasma zinc content is generally considered a poor measure of marginal zinc deficiency in humans (King, 1990; Wood, 2000). However, in our study, both animals on the zinc-deficient diet and diet with uptake inhibitors show significantly reduced zinc levels in blood. On the other hand, severe zinc deficiency was shown to induce gross anatomical malformation in pups from rats with severe zinc deficiency (Hurley et al., 1971). The pups born from pregnant mice in this study did have similar birth weight, no malformations and no statistically significant difference in the number of pups was detected in the different treatment groups compared to controls. Therefore, we conclude that the zinc deficiency we created was not severe but mild.

While both mice on a zinc-deficient diet and mice on a diet low in the bioavailability of zinc showed low tissue zinc levels and alterations in gut microbiota composition, the observed alterations in microbiota were not identical.

Mice on a zinc-deficient diet showed an increase in the phylum *Actinobacteria* and *Bacteroidetes*. *Actinobacteria* belong to the dominant commensal communities in humans and mice (Qin et al., 2010) and are generally regarded as pathobionts. Under certain circumstances, they are known to promote disease. In particular, *Actinobacteria* are associated with chronic inflammatory conditions and, for example, an increase in *Actinobacteria* has been associated with Inflammatory Bowel Disease (Frank et al., 2007; Morgan et al., 2012). However, mice on a diet with low bioavailability of zinc showed no such increase in *Actinobacteria* and *Bacteroidetes*.

Both groups of mice, however, showed an increase in *Firmicutes* and a decrease in *Proteobacteria*. In previous studies using mouse models for stress and depression-like behavior, an increase in *Actinobacteria* (Bangsgaard Bendtsen et al., 2012), both an increase and a decrease in *Bacteroidetes* (Aoki-Yoshida et al., 2016; Bharwani et al., 2016), an increase in *Firmicutes* (Aoki-Yoshida et al., 2016), as well as a decrease in *Proteobacteria* (Galley et al., 2014; Aoki-Yoshida et al., 2016) has been reported. Therefore, although different in some aspects both mice on a zinc-deficient diet and mice on a diet low in the bioavailability of zinc show alterations similar to those reported in models for stress and depression-like behavior.

The differences may originate in the competition between gut microbiota and enterocytic zinc uptake transporters for zinc. In mice on a zinc-deficient diet, a low amount of zinc is available for both. In contrast, in the diet with low bioavailability of zinc, zinc levels are normal but zinc uptake by enterocytic zinc transporters is inhibited through the antagonists present in the diet. Some microbiota may have an advantage over enterocytic zinc transporters with respect to inhibition by antagonists and may still be able to access sufficient amounts of zinc. For example, the phylum *Verrucomicrobia* was hardly affected by low bioavailability of zinc but reacted strongly to low general zinc levels. Further, due to low zinc levels, some families of bacteria may gain an advantage either by less demand for zinc, more sufficient intake mechanisms, or lack of competition through more zinc sensitive bacteria. For example, bacteria in the phylum *Firmicutes* were significantly increased in both zinc restricted diets. In addition, the presence of the zinc uptake inhibitors phytic acid, Ca, Fe, and folic acid may additionally influence microbiota. Therefore, it is not expected that a zinc-deficient diet and a diet with low bioavailability will alter microbiota composition in identical ways. However, besides shared and unique features in microbiota composition, both groups of animals show a reduction in tight junction markers and increased liver LPS levels. Thus, the shared aberrations from an established gut microbiota composition and/or low availability of zinc for GI cells are associated with pathological changes, such as increased permeability in the GI system in these mice.

Several studies support the idea that intestinal barrier dysbiosis leads to inflammatory responses in peripheral tissues and may ultimately drive inflammation in the brain. Therefore, we investigated the brain for characteristic alterations using GFAP and IL-6 as markers. We detected significantly higher GFAP expression in the brain of mice on zinc-restricted diets. Increased GFAP expression is a marker for activation on astrocytes and inflammation (Zhang et al., 2017). Our results are in line with previous reports that acute stress increases GFAP expression in the hippocampus of rodents (Lambert et al., 2000). Zinc deficiency may physiologically act as an acute stressor.

IL-6 plays a key role in the development of stress-associated depression-like behaviors in mice (Chourbaji et al., 2006). IL-6 signaling can result from activation of inflammatory pathways and alterations in IL-6 levels in the brain were demonstrated contributing to depression symptomatology (Hodes et al., 2016). Indeed, IL-6 is consistently reported as elevated in the blood of patients with depression (Haapakoski et al., 2015) and has been proposed as a predictive biomarker. Therefore, here, we assessed IL-6 levels in the brain mice. Our data show increased IL-6 tissue levels on mRNA and protein levels in response to low zinc status in pregnant mice. These results are in line with previously reported results showing up-regulation of cell activation markers in THP1 cells, a model for human monocytes, that coincided with increased IL-6 responses following LPS stimulation (Wong et al., 2015). In addition, a decreased zinc status in aged mice was associated with increased IL-6 expression levels (Wong et al., 2015).

Finally, supplementation of the diet with low bioavailability of zinc with ZnAAs was investigated to validate the contribution of zinc deficiency to the observed alterations and to understand the usability of ZnAAs for zinc supplementation during pregnancy. Supplementation with ZnAAs was able to prevent a significant drop in zinc levels in the maternal blood. In terms of microbiota composition, the presence of the ZnAA supplement is not expected to create a similar condition as observed in controls due to the presence of zinc uptake antagonists and

increased zinc levels. However, supplementation with ZnAA may reverse some effects caused by lowered bioavailability of zinc due to the presence of the antagonists. Indeed, ZnAA supplementation ameliorated the decrease in *Proteobacteria.* In addition, supplementation with ZnAAs prevented the decrease in *Actinobacteria* and *Bacteroidetes,* which was specific for this diet. However, it leads to an increase of both phyla as observed in the zinc-deficient diet before. Both phyla, therefore, seem to respond very sensitive to zinc levels and possibly contain species that thrive in low zinc conditions and others that strive with high zinc levels. Therefore, it is more important to investigate alterations on the genus and species level. Here, ZnAA supplementation ameliorated alterations observed before in several genera such as *Anaertruncus, Acetifactor, Desulfonispora, Holdemanella, Rhodospirillum,* and *Desulfovibrio*.

In terms of GI pathology, we could no longer detect an increase in ZONULIN1 levels in ZnAA supplemented mice and, in line with this, no significant increase in liver LPS. Thus, effects on gut physiology seem indeed to be dependent on zinc availability much more than on microbiota composition and it can be assumed that alterations in microbiota composition are a consequence of altered GI function or dependent on dietary factors only, or both. The reduction in GI abnormalities and liver LPS are expected to decrease pro-inflammatory processes in the mice. While we could not detect a normalization of GFAP expression, IL-6 protein levels in brain tissue were indeed normalized. While IL-6 levels in the brain of humans are difficult to measure, a reduction of IL-6 levels in plasma after zinc supplementation has been reported also in humans before (Bao et al., 2010). The data further confirms that ZnAAs are not only increasing zinc levels in animals but that they are biologically active.

Taken together, we conclude that both low levels of zinc or the presence of zinc uptake inhibitors that are commonly found in western diets and supplements for pregnant women alter the microbiome of pregnant mice. This may not only play a role in the observed autism-like phenotype of the offspring of mice with zinc deficiency during pregnancy but may also directly influence brain functionality through altered gut-brain signaling. Low zinc status was associated with changes in the intestinal epithelial barrier and an increase in liver LPS hints at increased leakiness of the gut in response to these changes. Finally, this may contribute to increased inflammation as we have observed higher GFAP and IL-6 levels in the brain of mice. Acute zinc deficiency was linked to depression and a role of microbiota dysbiosis and inflammation suggested. Our results obtained from pregnant mice do not exclude that similar alterations may occur independent of pregnancy in response to low zinc status. Based on our results, as low availability of zinc during pregnancy influences both microbiota and inflammatory status, a link between maternal zinc deficiency and postpartum depression seems plausible.

DATA AVAILABILITY STATEMENT

Additional datasets generated for this study are included in the **Supplementary Material**, and all datasets are available on request.

ETHICS STATEMENT

All animal experiments were performed in accordance with the guidelines and regulations for the welfare of experimental animals issued by the Federal Government of Germany and by the local ethics committee (Ulm University). The protocol used was approved by the Regierungspräsidium Tübingen, state of Baden-Württemberg, and the Ethics Committee of Ulm University (ID Number: 1257).

AUTHOR CONTRIBUTIONS

AS carried out the analysis of mice and revised the manuscript. AG conceived the study, participated in its design, coordination, and data analysis, and drafted the manuscript. All authors read and approved the final 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/fnins.2019.01295/full#supplementary-material

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PERSPECTIVE
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Facilitation of Gastrointestinal (GI) Tract Microbiome-Derived Lipopolysaccharide (LPS) Entry Into Human Neurons by Amyloid Beta-42 (Aβ42) Peptide

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Human gastrointestinal (GI)-tract microbiome-derived lipopolysaccharide (LPS): (i) has been recently shown to target, accumulate within, and eventually encapsulate neuronal nuclei of the human central nervous system (CNS) in Alzheimer's disease (AD) brain; and (ii) this action appears to impede and restrict the outward flow of genetic information from neuronal nuclei. It has previously been shown that in LPS-encased neuronal nuclei in AD brain there is a specific disruption in the output and expression of two AD-relevant, neuron-specific markers encoding the cytoskeletal neurofilament light (NF-L) chain protein and the synaptic phosphoprotein synapsin-1 (SYN1) involved in the regulation of neurotransmitter release. The biophysical mechanisms involved in the facilitation of the targeting of LPS to neuronal cells and nuclei and eventual nuclear envelopment and functional disruption are not entirely clear. In this "Perspectives article" we discuss current advances, and consider future directions in this research area, and provide novel evidence in human neuronal-glial (HNG) cells in primary culture that the co-incubation of LPS with amyloid-beta 42 (Aβ42) peptide facilitates the association of LPS with neuronal cells. These findings: (i) support a novel pathogenic role for Aβ42 peptides in neurons *via* the formation of pores across the nuclear membrane and/or a significant biophysical disruption of the neuronal nuclear envelope; and (ii) advance the concept that the Aβ42 peptide-facilitated entry of LPS into brain neurons, accession of neuronal nuclei, and down-regulation of neuron-specific components such as NF-L and SYN1 may contribute significantly to neuropathological deficits as are characteristically observed in AD-affected brain.

Keywords: Alzheimer's disease (AD), brain microbiome, dysbiosis, gastrointestinal (GI) tract, lipopolysaccharide (LPS), neurofilament light (NF-L), synapsin-1 (SYN1), the thanato-microbiome (the post-mortem microbiome)

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OVERVIEW

The highest known density of microorganisms anywhere in the biosphere is in the human GI-tract microbiome at about $\sim 10^{11}$ microorganisms per gram of GI-tract content (Angelucci et al., 2019; Castillo-Álvarez and Marzo-Sola, 2019; Fox et al., 2019). This vast number represents a remarkably complex and highly dynamic source of microbes; of the approximate ∼1,800 different microbial phyla that make up the GI-tract microbiome, the overwhelming majority are facultative anaerobic bacteria with archaea, fungi, microbial eukaryotes, protozoa, viruses, and other microbes making up the remainder (Bhattacharjee and Lukiw, 2013; Fox et al., 2019; Tierney et al., 2019). One major species of bacteria in the human GI-tract microbiome, about ∼100-fold more abundant than *Escherichia coli* in certain GI-tract regions is *Bacteroides fragilis*, an obligate, anaerobic, non-spore forming Gram-negative, rod-shaped enterotoxigenic bacterium. *B. fragilis*: (i) generates a remarkable array of highly neurotoxic exudates; and (ii) produces a particularly virulent, pro-inflammatory LPS glycolipid subtype (BF-LPS) that accumulates in Alzheimer's disease (AD) brain (Sears, 2009; Fathi and Wu, 2016; Lukiw, 2016a,b; Wexler and Goodman, 2017; Zhao et al., 2017a,b,c; Allen et al., 2019). Besides BF-LPS, *B. fragilis*-derived neurotoxins include small non-coding RNA (sncRNA), bacterial amyloids, endo-, exo-, and enterotoxins such as *fragilysin*, and truncated LPS molecules known as lipooligosaccharides (LOS). These neurotoxins have recently been shown to be capable of transversing normally restrictive gastrointestinal (GI) tract and blood-brain barriers (BBBs) in transgenic murine models of AD (Varatharaj and Galea, 2017; Sweeney et al., 2018; Tulkens et al., 2018; Barton et al., 2019; Erdö and Krajcsi, 2019; Panza et al., 2019; Sweeney and Lowary, 2019). Both the GI-tract and BBB may become weakened with aging or following surgery, disease or trauma (Sweeney et al., 2018; Sweeney and Lowary, 2019). For example, BF-LPS and the *B. fragilis*-derived enterotoxin *fragilysin* very effectively disrupt cell-cell adhesion, in part by E-cadherin cleavage and/or the action of LPS binding protein and Toll-like receptor 4 (TLR4), and subsequent LPS internalization, followed by translocation of neurotoxins into the systemic circulation, past the BBB and on into the parenchyma of the brain [Wu et al., 1998; Holton, 2008; Tsukamoto et al., 2018; Barton et al., 2019; Jeon et al., 2019; Lukiw, 2019 (submitted)].

LPS ACCUMULATION IN AD BRAIN

Multiple, independent research laboratories have reported: (i) the association of LPS and microbial-derived amyloid with AD brain (Zhao and Lukiw, 2015; Zhao et al., 2015); (ii) the remarkable affinity of specific LPS isoforms with AD brain parenchyma (Lukiw, 2016a,b); (iii) that Gram-negative bacterial molecules associate with AD neuropathology (Zhan et al., 2016); (iv) that microbiome-derived *E. coli* LPS and *B. fragilis* LPS associate the hippocampal CA1 region of AD brain (Zhao et al., 2017a,b,c); (v) of LPS accumulation within neocortical neurons of the AD brain that impair transcriptional output (Zhao et al., 2017a,b,c); (vi) that there is a strong association of LPS with neuronal nuclei and the specific LPS-mediated impairment of expression of the neurofilament light (NF-L) chain gene expression (Lukiw et al., 2018); (vii) of LPS association with the amyloid plaques, neurons and oligodendrocytes in AD brain (Zhan et al., 2018); and (viii) a significantly reduced expression of the AD-relevant synaptic components such as synapsin-1 (SYN1) in LPS-treated human neuronal-glial (HNG) cells in primary culture (Zhao et al., 2019). Most recently, it has been shown that LPS has a very strong affinity for, and association with, the neuronal nuclear envelope of the HNG cells in primary culture. This is also observed in the superior temporal lobe neocortex (Brodmann area A22; Wernicke's area) and the hippocampal CA1 region of AD-affected brain (Lukiw, 2016a,b; Zhao et al., 2017a,b,c, 2019; Lukiw et al., 2018; Ticinesi et al., 2019; **Figure 1**). Interestingly in moderate-to-late-stage AD LPS totally encapsulates neuronal nuclei in the AD brain with the subsequent restriction in the output of genetic information from those neuronal nuclei (Lukiw et al., 2018; Zhao and Lukiw, 2018a,b; Zhao et al., 2019). Interestingly, gene expression profiling showed a long-lasting deficit in neuron- and synaptic-specific gene expression and signaling in the hippocampus and neocortex of both transgenic murine models for AD and in patients with mild cognitive impairment or AD (Colangelo et al., 2002; Counts et al., 2014; Jaber et al., 2019; Parra-Damas and Saura, 2019).

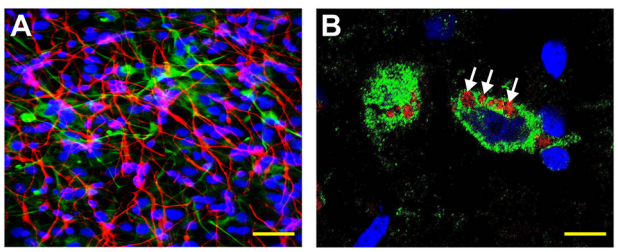

FIGURE 1 | Human neuronal-glial (HNG) cells (transplantation grade) in primary co-culture were used to study the dynamics of amyloid-beta 42 (Aβ42) peptide-mediated entry of lipopolysaccharide (LPS) into neurons (Bhattacharjee and Lukiw, 2013; Zhan et al., 2018; Zhao and Lukiw, 2018a,b; Zhao et al., 2019). **(A)** HNG cells are a primary co-culture of neuronal [β-tubulin III (βTUBIII)-stained; red; λ_{max} = 690 nm] and glial (GFAP-stained; green; λ_{max} = 520 nm) human brain cells; HNG cells are also stained for nuclei (DAPI-stained; blue; λ_{max} = 470 nm); cells shown are ∼2 weeks in culture; HNG cells are about ∼60% neurons (red) and about ∼40% astroglial (green) at ∼65% confluence; human primary neuronal and glial "support" cell co-cultures are utilized, because human neuronal cells do not culture well by themselves (Cui et al., 2010; Zhao et al., 2017c); HNG cells were exposed to 50 nM LPS for 36 h in the presence or absence of 10 nM Aβ42 peptides; other LPS concentrations at similar times displayed analogous trends; yellow scale bar (lower right) ∼50 μm. **(B)** Affinity of LPS for the neuronal nuclear envelope (white arrows); LPS (red; λ_{max} = 690 nm); β-tubulin III (βTUBIII)-stained (green; λ_{max} = 520 nm) and nuclei (blue; λ_{max} = 470 nm) stained HNG cells; white arrows indicate punctate and perinuclear clustering of LPS and LPS affinity for the nuclear envelope as has been previously reported (Hill and Lukiw, 2015; Zhan et al., 2016, 2018; Yang and Chiu, 2017; Zhao et al., 2017a,b); yellow scale bar (lower right) = 20 μm.

Aβ42 PEPTIDES AND LPS IN AD NEURONS

Recent evidence shows that LPS-type glycolipids not only have an unusually high affinity for neuronal nuclear membranes but also that amyloid-β 42 (Aβ42) peptides significantly facilitate LPS access and translocation into human neuronal cells and across neuronal nuclear envelopes in HNG cells in primary co-culture (**Figures 2A–L**). There are several possible explanations for the facilitation of LPS entry into, and their association with, neuronal membranes and neuronal nuclei by Aβ42 peptides or Aβ42 oligomers and these include:

Pore Formation by Aβ42 Peptides

Amyloid peptides (both Aβ40 and Aβ42) form a remarkable number of heterotypic structures and configurations under pathological conditions. These include: (i) the self-assembly and deposition of multiple types of fibrillar and globular structures and polymorphic assemblies both in solution and on membrane surfaces; and (ii) the formation of heterogeneous ionic pores spanning the lipid bilayer that is linked to the pathogenicity of these molecules. These later findings are supported by multiple independent reports regarding the capability of Aβ42 peptides [2 hydrophobic amino acid residues (isoleucine and alanine) longer (at the C-terminal) than Aβ40] to form up to 2.4-nm diameter pores through lipid bilayer membranes (Lashuel et al., 2002; Connelly et al., 2012; Sciacca et al., 2012; Ullah et al., 2015; Di Scala et al., 2016; Jang et al., 2016; Davidson, 2019; Hicks et al., 2019; Nguyen et al., 2019; Sun et al., 2019; Österlund et al., 2019). Interestingly, the slightly longer and more hydrophobic Aβ42-based peptide assemblies in oligomeric preparations have been observed to form voltage-independent, non-selective ion channels in contrast to Aβ40 peptide-based oligomers, fibers, and monomers which do not generally support pore structure formation (Bode et al., 2017, 2019; Nguyen et al., 2019). Although LPS is intrinsically heterogeneous and over time tends to form aggregates of ∼1–4 mDa or greater, smaller LOS or LPS monomers in the range of ∼50 to ∼100 kDa appear to have little difficulty in transversing ∼2.4 nm diameter pores to reach their final destination within the nucleoplasm (Zimmer et al., 1988; Millipore Sigma; Lipopolysaccharides[1]).

Membrane Disruption by Aβ42 Peptide Oligomers

As recently visualized by atomic force, transmission electron microscopy, mobility-mass spectrometry and liquid surface X-ray scattering there is a remarkable influence of Aβ monomers, short fibrillar Aβ oligomers, globular non-fibrillar Aβ oligomers and full-length Aβ fibrils on lipid bilayer membrane integrity and stability (Bode et al., 2019; Nguyen et al., 2019; Österlund et al., 2019; Vander Zanden et al., 2019). Abundant evidence indicates an Aβ oligomeric fibril-induced reorganization of membrane lipid packing and the induction of membrane destabilization and lipid disorganization by globular non-fibrillar Aβ oligomers (Di Lorenzo et al., 2019; Vander Zanden et al., 2019). Scanning electron microscopy (SEM) and thioflavin-T fluorescence assay have revealed: (i) that LPS and/or LPS-binding protein (LBP) have strong disruptive effects on the structural and biophysical organization of Aβ peptides and amyloidogenesis in Parkinson's disease (Montagne et al., 2017; Pretorius et al., 2018); and (ii) that LPS strongly induces NF-kB signaling, inflammatory responses, neuroinflammation, the generation of Aβ42 peptides and amyloidogenesis in transgenic murine models of AD (Gu et al., 2018; Jeon et al., 2019; Sheppard et al., 2019). Conversely, as evidenced by atomic force and electron microscopy imaging, short fibrillar Aβ42 oligomers appear to have a profound detergent-like, highly-localized, solubilizing effect on lipid membrane bilayers and this may predispose to hydrophobic interaction with LPS already present in the parenchyma of AD brain (Bode et al., 2019).

Highly Specialized Features of Neuronal Nuclear Membranes

Used for highly regulated nucleocytoplasmic transport, the nuclear envelope of typical neuronal cells contain about ∼10,000 nuclear pore complexes/transporters (significantly more than the ∼3,000 nuclear pores of a typical eukaryotic cell), and each ∼110 MDa nuclear pore complex (NPC) consists of about ∼1,000 nucleoporin proteins (Cooper, 2000; Kabachinski and Schwartz, 2015; Davidson, 2019; Lin and Hoelz, 2019; Sun et al., 2019). The affinity of LPS for any NPC component or any nucleoporin protein is not well understood and is an understudied area of both the neurobiology, microbiology and neuropathology of the human central nervous system (CNS). The perinuclear accumulation of LPS and LPS-mediated envelopment of human neuronal nuclei (Zhao et al., 2017a,b,c), and the restriction of the outflow of neuron-specific information, such as those mRNAs encoding the neuron-specific neurofilament light (NF-L) chain protein and SYN1 (Zhao et al., 2019), underscore the novel pathogenic potential of LPS in supporting dysfunction in neuronal cytoarchitecture and the capacity for efficient inter-neuronal signaling by disrupting SYN1 availability and hence synaptic integrity. In addition, LPS strongly associates with amyloid plaques (Zhan et al., 2018) and perinuclear LPS, and encasement of neuronal nuclei by LPS may also contribute to the biophysical blockage of exit of mRNA through the NPC into the cytoplasm in AD brain (Zhao et al., 2019). Interestingly, using stable isotope labeling of amino acids in cell culture and quantitative proteomics, it has recently been shown that the interactome of the 695 amino acid beta-amyloid precursor protein βAPP695, which is the direct precursor to Aβ42 peptide, interacts strongly with the NPC and nucleoporin proteins in neuronal cells (Andrew et al., 2019). This suggests some novel roles for both βAPP695 and Aβ42 peptide in both NPC function and amyloid peptide processing and generation. The unique phospholipid composition of the inner nuclear neuronal membrane (that encases the genome) and the outer neuronal nuclear membrane that together form the nuclear envelope, their extremely high ratio of phospholipid to cholesterol, the biophysics of nuclear lipid membrane remodeling and lipid raft formation may predispose the neuronal

[1]https://www.sigmaaldrich.com/technical-documents/protocols/biology/lipopoly-saccharides.html (last accessed November 5, 2019)

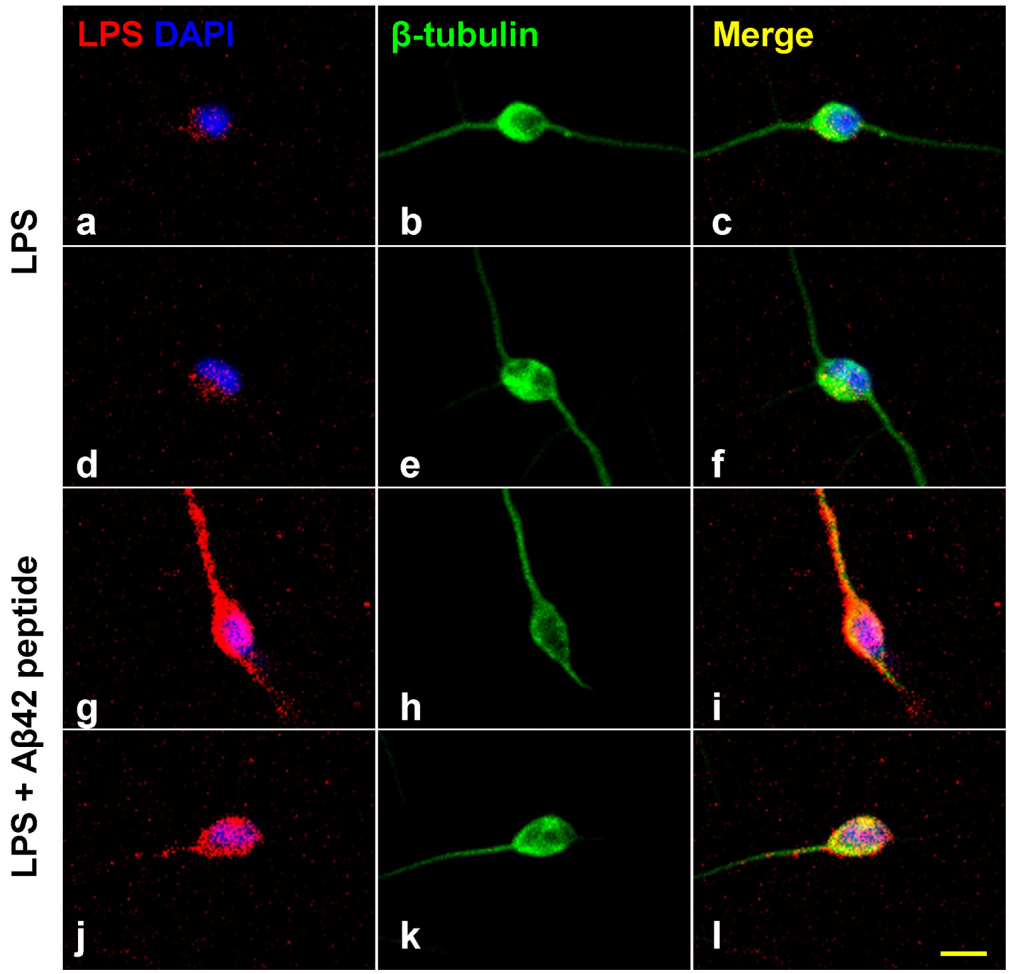

FIGURE 2 | Increased affinity of LPS for neuronal nuclei in the presence of Aβ42 peptide. Panels **(A–L)** show LPS-neuronal interactions in the presence or absence of Aβ42 peptide; **(A–F)** in the absence of Aβ42 peptide and **(G–L)** in the presence of Aβ42 peptide. LPS preferentially associates with human neuronal nuclei both in Alzheimer's disease (AD) and in LPS-addition experiments (Zhao et al., 2017a,b,c; Zhao and Lukiw, 2018a,b). Panels **(A,D)** show LPS (red) affinity for a polar region of a single DAPI-stained neuronal nucleus (blue). Panels **(B,E)** show single neuronal nucleus stained with neuron-specific β-tubulin III (green). Panels **(C,F)** show merged stain indicating LPS affinity for the polar region involving a single DAPI-stained neuronal nucleus. Panels **(G,J)** show the presence of Aβ42 significantly increases the affinity of LPS for single DAPI-stained neuronal nucleus. Panels **(H,K)** show single neuronal nucleus stained with neuron-specific β-tubulin III (green). Panels **(I,L)** show merged stain indicating LPS affinity for the neurite and soma of a single DAPI-stained neuronal nuclei. The results suggest that LPS is stimulated to associate with DAPI-stained neuronal nuclei in the presence of the hydrophobic Aβ42 peptide; neither Aβ40 peptide or β-actin showed comparable "association" effects (Zhao et al., 2017a,b,c; Lukiw et al., 2018); yellow scale bar (lower right) = 50 μm.

nuclear envelope to the potential interaction between amyloid peptides and LPS, and with NPC nucleoporin proteins.

Other Interactions Between LPS and Aβ42 Peptides

Lipopolysaccharide (LPS) is a type of prokaryotic glycoconjugate-glycolipid comprised of three major domains: (i) an "O" antigen consisting of an "O polysaccharide"; (ii) a "core" polysaccharide domain (the innermost hydrophilic domain of the three regions of LPS); and (iii) a hydrophobic "lipid A" domain. The "core" polysaccharide domain contains an oligosaccharide covalently attached directly to the "lipid A" moiety and commonly contains sugars such as heptose, 3-deoxy-D-mannooctulosonic acid as well as non-carbohydrate components that include phosphate, amino acids linkages and ethanolamine components characteristic of each Gram-negative bacterial genus and species (Whitfield and Trent, 2014; Tulkens et al., 2018). Both the 50–100 kDa LPS monomer (especially the "lipid A" domain responsible for much of the toxicity of Gram-negative bacteria) and the 4.5 kDa Aβ42 peptide monomer are highly hydrophobic and this alone may favor their mutual interaction with the lipid bilayer of neuronal membranes (National Institutes of Health, PubChem, 2019). As mentioned earlier, Aβ42-based oligomers are highly disruptive toward lipid bilayer membranes, but whether the chaotropic actions of LPS and Aβ42 peptide are additive or synergistic is unknown as their specific interactions are currently not well understood.

UNANSWERED QUESTIONS

While Aβ42 peptides clearly support LPS entry into neurons it is not clear why neuronal membranes are specifically targeted for these actions and/or why other cellular plasma membranes (lipid bilayers) are not preferred or involved to a lesser extent; perhaps this has something to do with the unique neuronal membrane proteolipid composition and/or the electrical activity of these cell types or other unique neuronal features including Aβ42 peptide-mediated pore formation (see above; Nguyen et al., 2019; Österlund et al., 2019). While LPS has been shown to completely envelope neuronal nuclei in the superior temporal lobe neocortex (Brodmann area A22) in both aging, and especially in AD brain, there is emerging evidence that the end result of this biophysical occlusion of nuclear pores is the restriction of outflow of genetic information, i.e., messenger RNA (mRNA) through these nuclear pores, however some other pathogenic mechanism may be involved (Clement et al., 2016; Lukiw et al., 2018; Zhao et al., 2019; Cornelison et al., 2019). Very recently it has been demonstrated that there is an LPS-induced translocation of cytosolic NF-κB into the cell nucleus (Bagaev et al., 2019) and LPS-induced neuronal hypertrophy (Tellez-Merlo et al., 2019) but these potentially pathogenic and neuroinflammatory outcomes require further investigation. It would be very useful to know at what point an induced disruption along the gut-brain axis and LPS-signaling pathways might be beneficial in the clinical management of AD.

A HUMAN BRAIN MICROBIOME?

There exists the intriguing and enigmatic possibility, as has been suggested for other major organ groups, that the human brain and/or CNS might have its own, as yet poorly characterized microbiome (Bhattacharjee and Lukiw, 2013; Hill et al., 2014a,b; Köhler et al., 2016; Emery et al., 2017; Zhao et al., 2017b,c; Roberts et al., 2018; Zhao and Lukiw, 2018a,b; Zhou and Bian, 2018; Javan et al., 2019; Mazmanian, 2019). Currently, it is understood that microbes can enter the brain and CNS through the BBB which becomes leaky *via* physical damage, disease and/or aging, and/or *via* nerves that innervate both the brain and the gut (Roberts et al., 2018; Javan et al., 2019). Very recent studies indicate the presence of bacteria in the human and mouse brain at the BBB under noninfectious or non-traumatic conditions. Microbes have been identified *via* morphological criteria and ultrastructural imaging analysis with high bacterial counts found in the human hippocampus and prefrontal cortex, but low bacterial counts in other brain anatomical regions such as the striatum (Roberts et al., 2018; Javan et al., 2019). Significantly increased bacterial populations have been observed in association with neurological deterioration in AD brain tissues compared with controls (Emery et al., 2017). Other supportive studies come from investigations involving the human thanatomicrobiome—the microbiome of death—that reflects the post-mortem microbial changes which vary by organ and as a function of time and temperature (Zhao et al., 2017a,b,c; Zhou and Bian, 2018; Javan et al., 2019). Further support for the idea that a microbiome may already be present in the brain arises when considering that microbes (or microbial-derived neurotoxins) in the GI tract would need to travel a significant distance to reach the brain compartments post-mortem vs. the extremely rapid proliferation of bacteria in the brain shortly after death. This might contribute locally to the presence of bacterial-derived neurotoxins such as LPS and other microbial-derived molecules in brain tissues (Hill et al., 2014a,b; Lukiw, 2016a,b; Zhan et al., 2016; Emery et al., 2017). Put another way, post-mortem microscopic examination of the post-mortem brain routinely detects bacteria far more rapidly than the bio-physiological capability of microbes to transit from the GI-tract across the systemic circulation into the brain or other CNS compartments (Roberts et al., 2018; Javan et al., 2019).

FUTURE DIRECTIONS

The remarkable affinity of the glycoconjugate LPS, the major component of the outer membrane of Gram-negative bacteria such as *B. fragilis* and *E. coli*, for the neuronal nuclear envelope in human brain cells and tissues was first described just ∼2 years ago (Zhao et al., 2017a,b,c; Zhan et al., 2018). The mean abundance of GI-tract-sourced LPS can be increased by either an up-regulation in the biosynthesis of LPS itself or *via* an increase in the number of Gram-negative bacteria capable of generating and releasing LPS in part through the process of dysbiosis. Because both the *de novo* induction of LPS and bacterial division times of ∼15–20 min are relatively rapid microbiological-pathological events, it seems also that the production of LPS can be a rapidly undertaken and perhaps even exponential biological event (Raetz and Whitfield, 2002; Whitfield and Trent, 2014; Sweeney and Lowary, 2019). Interestingly, growth rates in the GI-tract microbiome for Gram-negative bacteria have been shown to be dependent on ingested dietary fiber, and the relative proportion of *B. fragilis* in the GI tract can for example decrease 2–3-fold after a fiber-laden meal while a high-fat-cholesterol (HFC) meal has the opposite effect (Heinritz et al., 2016; Huang and Liu, 2019). Indeed, dietary modification by increasing both soluble and/or insoluble fiber intake has been shown to decrease the abundance of *B. fragilis* in the GI-tract microbiome on a time-scale of hours-to-days after the ingestion of the fiber-enriched meal itself (Simpson and Campbell, 2015; Chen et al., 2017; Dhillon et al., 2019; Huang and Liu, 2019; Parada Venegas et al., 2019). Dietary manipulation, probiotics and prebiotic supplementation and increased ingestion of fiber is one research area urgently requiring more study, because the management of diet could yield real and more effective therapies for both the treatment of neurodegeneration and malignancy (Rios-Covian et al., 2017; Poeker et al., 2018; Dhillon et al., 2019; Huang and Liu, 2019). It should be mentioned that under realistic physiological conditions the ∼1,800 phyla of bacteria of the GI-tract microbiome are together most likely capable of generating an extremely complex neurotoxic cocktail of exudates, and at this point in time we

are analyzing just a very small number of GI-tract derived neurotoxins from a vast neurotoxic pool of huge abundance and bewildering biological complexity (Hicks et al., 2019; Tierney et al., 2019).

SUMMARY

Over the last few years, the GI-tract microbiome-brain axis has emerged as a focus of increasing interest in the establishment of a neurophysiological and neurobiological basis for age-related, developmental, neurodegenerative, neuroinflammatory and psychiatric disease. The microorganisms which constitute the human GI-tract microbiome have potential to secrete some of the most neurotoxic and inflammation-inducing substances known, including bacterial glycolipid lipopolysaccharide (LPS) from abundant, anaerobic, GI-tract resident Gram-negative bacteria (Whitfield and Trent, 2014; Batista et al., 2019; Patrick et al., 2019; Ticinesi et al., 2019). As a particularly abundant commensal, non-motile, non-spore forming obligatory anaerobic, Gram-negative bacillus of the human GI-tract microbiome, *Bacteroides fragilis (B. fragilis)*, releases an intensely pro-inflammatory species of LPS (BF-LPS), amongst the most pro-inflammatory substances known, that in HNG cells in primary culture induces the pro-inflammatory transcription factor NF-kB (p50/p65) complex (Lukiw, 2016a,b; Zhao and Lukiw, 2018a,b; Batista et al., 2019; Sweeney and Lowary, 2019). LPS translocation into the nucleoplasm and access to neuronal nuclei are greatly facilitated in the presence of Aβ42 peptides (**Figures 1**, **2**). LPS-triggered NF-kB (p50/p65) up-regulation is associated with: (i) the induction of pro-inflammatory, pathogenic microRNA-regulated gene expression programs in the AD brain; these microRNAs have multiple NF-kB (p50/p65) recognition features in their immediate promoters (Pogue and Lukiw, 2018); and (ii) multiple independent laboratories have provided evidence that GI-tract derived glycolipids such as LPS associated with the pro-inflammatory, cytoarchitectural and/or synaptic neuropathology of AD brain and transgenic murine models of AD (Bhattacharjee and Lukiw, 2013; Hill and Lukiw, 2015; Zhan et al., 2016, 2018; Lukiw et al., 2018; Zhao et al., 2019). Many of these noxious biopolymers are potent enterotoxins which can neutralize cadherins and other cell-cell adhesion molecules, inducing leakage through the GI-tract epithelial barrier, which normally is largely impermeable, allowing neurotoxin access to the systemic circulation and subsequent translocation across the BBB (Leshchyns'ka and Sytnyk, 2016; Sweeney et al., 2018; Jeon et al., 2019; Sweeney and Lowary, 2019). Clinically, the detection of these GI-tract microbiome-derived neurotoxins in blood serum may be of prodromal, prognostic and/or diagnostic value as biomarkers for the onset and/or propagation of neurological disease or malignancy; or of forensic value in the determination of temporal aspects of the post-mortem interval (Li and Yu, 2017; Zhou and Bian, 2018).

Lastly, obligate anaerobic bacteria such as *Bacteroides fragilis* make up the largest proportion of Gram-negative microbes in the human GI-tract microbiome. In a recent study of 2,100 human donors, the most recent estimate is that all together microbial constituents of this microbiome harbor at least 22.3 million non-redundant prokaryotic genes in contrast to the 26.6 thousand protein-encoding transcripts of the human genome (Venter et al., 2001; Tierney et al., 2019). Hence, GI-tract microbial genes outnumber host genes by about 840-to-1, which represents staggering genetic complexity (Fields et al., 1994; Venter et al., 2001; Tierney et al., 2019). With this comes a GI-tract microbial proteome of remarkable proportion and speciation that includes highly neurotoxic and pro-inflammatory exudates such as LPS (Hicks et al., 2019; Roy Sarkar and Banerjee, 2019). It is tempting to speculate that we are just scratching the surface of our understanding of the potential impact of these prokaryotic GI-tract microbiome-derived genes and their extruded neurotoxic molecules on our own host gene signaling and expression systems which are likely to have a tremendous impact and relevance to both human health and disease.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by Louisiana State University (LSU) Ethics Committee, Louisiana State University, New Orleans, LA, USA.

AUTHOR CONTRIBUTIONS

WLi, WLu, TB and YZ performed and analyzed all experiments. WLu wrote the article.

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REVIEW
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Antidepressive Mechanisms of Probiotics and Their Therapeutic Potential

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The accumulating knowledge of the host-microbiota interplay gives rise to the microbiota-gut-brain (MGB) axis. The MGB axis depicts the interkingdom communication between the gut microbiota and the brain. This communication process involves the endocrine, immune and neurotransmitters systems. Dysfunction of these systems, along with the presence of gut dysbiosis, have been detected among clinically depressed patients. This implicates the involvement of a maladaptive MGB axis in the pathophysiology of depression. Depression refers to symptoms that characterize major depressive disorder (MDD), a mood disorder with a disease burden that rivals that of heart diseases. The use of probiotics to treat depression has gained attention in recent years, as evidenced by increasing numbers of animal and human studies that have supported the antidepressive efficacy of probiotics. Physiological changes observed in these studies allow for the elucidation of probiotics antidepressive mechanisms, which ultimately aim to restore proper functioning of the MGB axis. However, the understanding of mechanisms does not yet complete the endeavor in applying probiotics to treat MDD. Other challenges remain which include the heterogeneous nature of both the gut microbiota composition and depressive symptoms in the clinical setting. Nevertheless, probiotics offer some advantages over standard pharmaceutical antidepressants, in terms of residual symptoms, side effects and stigma involved. This review outlines antidepressive mechanisms of probiotics based on the currently available literature and discusses therapeutic potentials of probiotics for depression.

Keywords: microbiota-gut-brain axis, gut microbiota, major depressive disorder, probiotics, inflammation

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Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine (serotonin); 5-HTP, 5-hydroxytryptamine; BBB, blood-brain barrier; BDNF, brain-derived neurotropic factor; CgA, salivary chromogranin A; CORT, corticosterone; CREB, cAMP response element binding protein; CRP, C-reactive protein; CUMS, chronic unpredictable mild stress; DA, dopamine; DC, dihydroxyphenylacetic acid; EIF2, eukaryotic initiation factor 2; GABA, gamma-aminobutyric acid; GLP-1, glucagon-like peptide-1; GPx, glutathione peroxidase; GR, glucocorticoid; H_2O_2, hydrogen peroxide; HPC, hippocampus; HVA, homovanillic acid; IBS, irritable bowel syndrome; IDO, indolamine 2,3-dioxyhydrogenase; IFN, interferon; IgA, immunoglobin A; IL, interleukin; KA, kynurenic acid; KYN, kynurenine; LPS, lipopolysaccharides; MAOA, monoamine oxygenase A; MCP-1, monocyte chemotactic protein-1; MDD, major depressive disorder; MR, mineralocorticoid; MS, maternal separation model; NE, norepinephrine; PFC, prefrontal cortex; PGE2, prostaglandin E2; REM, rapid eye movement; SCFA, short-chain fatty acids; SNRI, serotonin-noradrenaline reuptake inhibitor; SOD, superoxide dismutase; SSRI, selective serotonin reuptake inhibitor; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; Tph1, tryptophan hydroxylase 1; TRANCE, TNF-related activation-induced cytokine; TRP, tryptophan.

INTRODUCTION

Approximately 10^{14} microbes, also known as gut microbiota, reside in the human gastrointestinal tract. The majority of these microbes belong to the Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla. The gut microbiota flourishes in a symbiotic alliance with the host and, as such, has eminent regulatory effects on the host physiology. The gut microbiota actively engages with the proper development and functioning of both the immune system and brain. This is mediated by the microbiota–gut–brain (MGB) axis that lays the foundation for the intricate communicative pathways between gut microbiota and the nervous, immune and endocrine systems. However, the diversity and richness of gut microbiota are susceptible to change based on the host's lifestyle. An adverse change induces a gut dysbiosis which disrupts the symbiosis maintained by the MGB axis. Indeed, a gut dysbiosis has been linked to various health conditions, such as obesity, IBS, schizophrenia, Parkinson's disease and MDD (Sherwin et al., 2016; Thursby and Juge, 2017; van de Guchte et al., 2018).

Major depressive disorder is currently the leading cause of disability worldwide and is expected to outrank heart diseases as the number one disease burden by 2030 (Reddy, 2010; Tucci and Moukaddam, 2017). According to the Diagnostic and Statistical Manual of Mental Disorders-5, MDD is diagnosed when a person experiences most of the following symptoms for at least 2 weeks: depressed mood, anhedonia, excessive guilt, suicidal ideation, changes in appetite and sleep, psychomotor retardation, poor concentration and fatigue. Among these criteria, either depressed mood or anhedonia (or both) must be present for a diagnosis of MDD (American Psychiatric Association, 2013). In this review, the term "depression" would be used to refer to symptoms that characterize MDD.

A causal relationship potentially exists between the gut microbiota and MDD. Germ-free (GF) rodents developed depressive-like behaviors following fecal microbiota transplantation from MDD patients, but not from healthy people (Kelly et al., 2016; Zheng et al., 2016). As compared to healthy individuals, MDD patients have a different gut microbiota profile. The decrease in *Faecalibacterium*, *Bifidobacterium*, *Lactobacillus* (Aizawa et al., 2016), and *Dialister* (Kelly et al., 2016), and increase in *Clostridium*, *Streptococcus*, *Klebsiella*, *Oscillibacter*, *Allistipes* (Naseribafrouei et al., 2014; Jiang et al., 2015; Lin et al., 2017; Rong et al., 2019), *Eggerthella*, *Holdemania*, *Gelria*, *Turicibacter*, *Paraprevotella,* and *Anaerofilum* (Kelly et al., 2016) genera have been found among MDD patients. This shift in the gut microbiota composition may contribute to a shift in the regulation of the host physiology (Luan et al., 2017). It is, thus, worthwhile to tackle MDD from the MGB axis standpoint, with an emphasis on the gut microbiota.

Probiotics are microbes (usually lactic acid bacteria such as Lactobacilli and Bifidobacteria) that benefit the host physiology upon ingestion. Probiotics are marketed in the form of capsules, powder or fermented products. The global market size of probiotics amount to billions and is increasing annually due to consumers' interest in optimizing their health with functional foods (Di Cerbo and Palmieri, 2015). Probiotics have been utilized to modulate the MGB axis in an attempt to treat diseases, including MDD. Meta-analyses and systematic reviews have already supported the efficacy of probiotics in reducing clinical depression and depressive-like symptoms in MDD patients and healthy individuals, respectively (Huang et al., 2016; Pirbaglou et al., 2016; Wang et al., 2016; McKean et al., 2017; Wallace and Milev, 2017).

To what extent are probiotics viable tools to treat MDD/depression? This review addresses this question by first outlining the workings of MGB axis and process by which this axis becomes maladaptive, leading to the development of depression. Antidepressive mechanisms of probiotics are further elucidated by drawing parallels between the physiological outcomes that accompanied the behavioral changes to the MGB axis from animal and human research. Lastly, in light of the heterogeneous nature of both the gut microbiota composition and depression subtypes in the clinical setting, challenges and potentials in translating probiotics for clinical use are discussed.

THE MGB AXIS AND DEPRESSION

Signaling Pathways of the MGB Axis: Neural and Humoral Routes

The first point of contact between the gut microbiota and host nervous system is likely via the enteric nervous system (ENS). The ENS has been described as "the second brain" due to its neuronal complexity on par with the brain and its ability to function as an independent, discrete unit to regulate gut-related activities and the immune system (Furness, 2012; Breit et al., 2018). Without gut microbiota, the excitability of enteric neurons would likely be attenuated, based on data observed in GF mice (McVey Neufeld et al., 2013). Through the ENS, gut microbiota and the brain communicate bidirectionally through neural and humoral (systemic circulation) pathways (Luan et al., 2017). Parasympathetic vagus afferents carry neural information from internal organs, including the gut, to the brain (Breit et al., 2018). The vagus nerve also consists of motor neurons that innervate nearly all enteric neurons (Powley, 2000). This enables the brain to influence the activity of ENS to some extent, particularly the state of intestinal permeability and gut inflammation. Sympathetic spinal nerves also connect enteric neurons to the brain, albeit to a lesser extent than vagal nerves (Lomax et al., 2010; Breit et al., 2018). Additionally, the humoral route allows microbial metabolites to enter the systemic circulation and exert its effects elsewhere, including the brain. Likewise, the brain also sends chemical messengers, such as cytokines and glucocorticoids, via the humoral route to regulate the gut physiology (Luan et al., 2017).

Signaling Mechanisms of the MGB Axis: Immune, Endocrine, and Neurotransmitter Systems

The gastrointestinal tract contains approximately 70% of the immune system (Vighi et al., 2008). Immune cells express TLRs

that respond to foreign antigens, such as LPS, as they penetrate the intestinal mucosal barrier. This promptly triggers production of inflammatory cytokines, mainly ILs, tumor necrosis factor (TNF)-α and IFN-γ (Sherwin et al., 2016). These cytokines enter the brain through various pathways. The humoral pathway enables cytokines to enter circumventricular organs or permeable regions of the BBB or bind to carrier proteins that cross the BBB. The neural pathway allows gut cytokines to stimulate specific brain areas such as the brainstem, hypothalamus and limbic structures via vagus and spinal afferents. The cellular pathway allows cytokines to be transported into the brain by the action of monocytes or macrophages. These cytokines could also bind to receptors on astrocytes and microglia, and subsequently trigger cytokine production within the brain (Schiepers et al., 2005; Miller and Raison, 2016).

When proinflammatory signals reach the brain, the hypothalamic-pituitary-adrenal (HPA) axis, a sympathetic-neuroendocrine system, is activated to restore homeostasis. In response to stress, the hypothalamic paraventricular nucleus (PVN) synthesizes and releases corticotropin-releasing factor (CRF) to stimulate the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH stimulates the adrenal cortex to release glucocorticoids (cortisol in humans and corticosterone in rodents) which inhibit the release of CRF, establishing a negative feedback loop. Glucocorticoids are core effectors of the HPA axis that travel by the humoral route to exert its adaptive effects elsewhere; for instance, to reduce gut inflammation (Tsigos and Chrousos, 2002; Schiepers et al., 2005).

Furthermore, neurotransmitters in the brain serve indispensable roles in maintaining proper brain functions. Neurotransmitters such as GABA, glutamate (Glu), serotonin (5-HT), DA, NE, histamine and acetylcholine (ACh) are known to be synthesized by the gut microbiota (Oleskin et al., 2016). Notably, *Lactobacillus*, a prominent probiotic genus, produces multiple neurotransmitters in a species-dependent manner *in vitro* (**Table 1**). It should be noted that gut-derived neurotransmitters are functionally different from brain-derived neurotransmitters (Mittal et al., 2017). The bioavailability of precursors for these neurotransmitters is also regulated by the gut microbiota. For example, carbohydrate-fermenting microbes secrete butyrate (a SCFA) that stimulates 5-HT synthesis from intestinal enterochromaffin cells (ECs) (Reigstad et al., 2015; Yano et al., 2015; Lund et al., 2018). In contrast, Clostridia metabolites, such as 4-cresol and 4-hydroxyphenylacetate (4-HPA), inhibit dopamine-β-hydroxylase (an enzyme that converts DA to NE in the brain) (Shaw, 2017). These microbial neuroactive molecules likely modulate local ENS signaling, which ultimately influence the MGB axis (Karl et al., 2018).

Dysregulated MGB Axis in Depression: Chronic Stress Response Loop

Acute psychological stress increases the release of ACh from cholinergic nerves (Saunders et al., 1997; Kiliaan et al., 1998) and glucocorticoids from the HPA axis (Alonso et al., 2012; Zheng et al., 2013; Vanuytsel et al., 2014), both of which loosen tight junctions of the intestinal barrier (**Figure 1**). Other stressors such as poor diet, sleep deprivation, antibiotics, environmental pollutants and excessive exercise also increase the intestinal permeability (Karl et al., 2018). Additionally, exposure to stress stimulates sympathetic spinal nerves to release NE into the gut which expedites quorum sensing systems and iron uptake of bacteria, leading to increased virulence and growth of pathogenic bacteria (e.g., *Escherichia coli*, *Salmonella*, *Campylobacter*, etc.) (Lomax et al., 2010; Freestone, 2013). These factors facilitate penetration of bacteria and their toxins, such as LPS, through the weakened intestinal barrier. Administration of LPS increased proinflammatory cytokines and caused anxiety and depression in healthy males in a dose-dependent manner (Grigoleit et al., 2011). This phenomenon is only transient due to the adaptive response of the immune system and HPA axis. However, chronic stress prevents this homeostatic restoration and causes prolonged inflammation and HPA axis overactivity, both of which aggravate the disrupted intestinal barrier. During this process, chronic inflammation renders the immune system insensitive to inhibitory signals from glucocorticoids (de Punder and Pruimboom, 2015). Excess proinflammatory cytokines, in turn, disrupt the negative feedback inhibition of circulating glucocorticoids of the HPA axis (Schiepers et al., 2005; Miller et al., 2009). Indeed, MDD patients often show increased intestinal barrier permeability (Stevens et al., 2018; Calarge et al., 2019; Ohlsson et al., 2019) and elevated serum antibodies against LPS (Maes et al., 2008).

Excessive glucocorticoids hyperactivate monoamine oxidases (MAOs; enzymes that degrade 5-HT, NE, and DA) (Grunewald et al., 2012). An overactive HPA axis can also induce gut dysbiosis (Murakami et al., 2017) and impairment of brain neurotransmitter systems (Pacak et al., 1993; Smith et al., 1995; Lopez et al., 1998; Hewitt et al., 2009). Higher baseline levels of cortisol, an indicator of an overactive HPA axis, were detected in more than 70% of MDD patients (Vreeburg et al., 2009; Lok et al., 2012). Proinflammatory cytokines and glucocorticoids upregulate indoleamine 2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) enzymes, respectively (Schimke et al., 1965; Young, 1981). Both enzymes metabolize TRP into KYN and quinolinic acid, which reduce the bioavailability of TRP to cross the BBB, thereby lowering 5-HT synthesis (Reus et al., 2015). This is evidenced by low plasma TRP levels that were also correlated to a heightened proinflammatory state found in MDD patients (Maes et al., 1993, 1994). Furthermore, proinflammatory cytokines can decrease levels of DA, 5-HT and NE in the brain by upregulating their reuptake via presynaptic transporters and downregulating enzymatic cofactors required for their synthesis (Miller and Raison, 2016). Indeed, administration of cytokines consistently induced neurotransmitter imbalances in the brain and behavioral changes that are reminiscent of depression in animals and humans (Miller et al., 2009). Similarly, higher levels of proinflammatory cytokines were observed in depressed individuals as reported using meta-analyses of the data available in the literature (Howren et al., 2009; Dowlati et al., 2010).

A stress-induced inflamed gut adversely alters the relative abundances of preexisting bacteria in the gut (**Figure 1**). Acute psychological stress stimulated the release of inflammatory

TABLE 1 | The neurotransmitters produced by probiotics and their regulatory functions.

Neurotransmitter	Regulatory functions	Probiotics	References
Gamma-aminobutyric acid (GABA)	• Hippocampal neurogenesis • HPA axis regulation • Mood	*L. brevis* *L. rhamnosus* *L. reuteri* *L. paracasei* *L. plantarum* *L. bulgaricus* *L. helveticus* *L. casei*	Komatsuzaki et al. (2005), Luscher et al. (2011), Stromeck et al. (2011), Barrett et al. (2012), Liao et al. (2013), Lin (2013), Oleskin et al. (2014), Yunes et al. (2016)
Serotonin (5-HT)	• Impulsivity • Aggression • Appetite • Circadian rhythm • Learning • HPA axis regulation • Mood	*L. plantarum* *L. helveticus*	Özogul (2011), Özoğul et al. (2012), Oleskin et al. (2014), Carhart-Harris and Nutt (2017)
Dopamine (DA)	• Motivation • Concentration • Psychomotor speed • Ability to experience pleasure • Mood	*L. plantarum* *L. helveticus* *L. casei* *L. bulgaricus*	Dunlop and Nemeroff (2007), Özogul (2011), Oleskin et al. (2014)
Norepinephrine (NE)	• Aggression • Cognitive function • Sleep • Sympathetic activity • HPA axis regulation • Mood	*L. helveticus* *L. casei* *L. bulgaricus*	Leonard (2001), Montgomery and Briley (2011), Oleskin et al. (2014)
Glutamate (Glu)	• Gastrointestinal reflexes • Intestinal motility • HPA axis regulation • Mood	*L. rhamnosus* *L. reuteri* *L. plantarum* *L. paracasei* *L. helveticus* *L. casei* *L. bulgaricus*	Weingand-Ziadé et al. (2003), Zalán et al. (2009), Stromeck et al. (2011), Zareian et al. (2012), Julio-Pieper et al. (2013), Oleskin et al. (2014)
Histamine	• Motivation • Learning • Memory • Appetite • Sleep • Sympathetic activity • Mood	*L. plantarum* *L. reuteri*	Kano et al. (2004), Özoğul et al. (2012), Thomas et al. (2012), Torrealba et al. (2012), Hemarajata et al. (2013)
Acetylcholine (ACh)	• Cognition • Synaptic plasticity • Analgesia • Sleep • HPA axis regulation • Mood	*L. plantarum*	Rowatt (1948), Girvin and Stevenson (1954), Pytka et al. (2016)

mediators that were correlated with the lowered abundance of *Coprococcus*, *Pseudobutyrivibrio, Dorea,* and *Lactobacillus* in mice. This, in turn, allowed the proliferation of *Clostridium* species in the gut (Bailey et al., 2011). The gut microbiota of chronic-stressed mice also deviated from the baseline, whereby an increase in proinflammatory bacteria, such as *Helicobacter* and *Streptococcus*, and a decrease in butyrate-producing bacteria, such as *Roseburia* and *Lachnospiraceae* species, were observed (Gao et al., 2018). Altered gut microbiota composition consequently exacerbates gut inflammation and further increases intestinal permeability and production of proinflammatory cytokines (van de Guchte et al., 2018). The precise mechanism underlying vulnerability of certain bacteria to inflammation remains poorly understood. It is hypothesized that inflammation disrupts β-oxidation of intestinal epithelial cells (IECs, both enterocytes and colonocytes) to increase oxygen content in the gut lumen. This promotes formate oxidation that favors the growth of facultative anaerobes, such as *E. coli*, that are pathogenic and inflammatory at the cost of obligate anaerobes, such as Bacteroides and Firmicutes (Hughes et al., 2017).

A dysregulated gut microbiota translates to a shift in the production of neuroactive metabolites and alters host neurotransmitter circuitry. This corresponds with disrupted levels of neurotransmitters in the brain of GF mice (Diaz Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2013; Pan et al., 2019). Altered neurotransmitter profile (e.g., GABA, Glu, 5-HT,

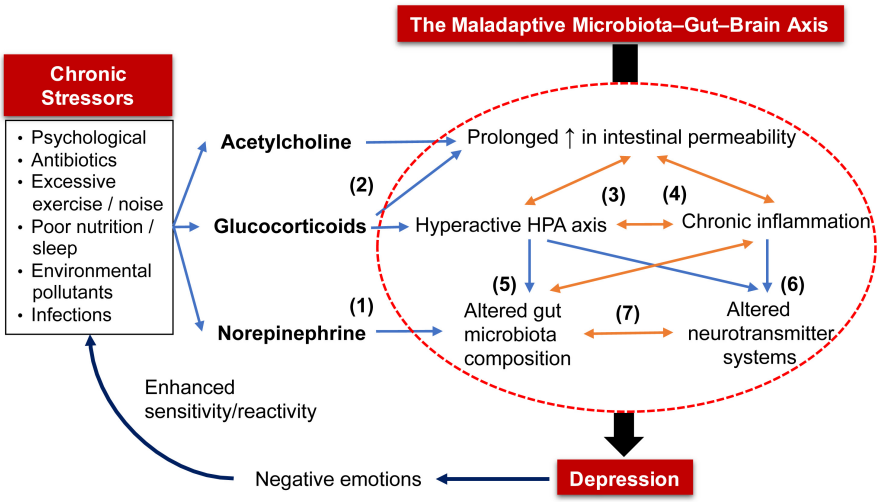

FIGURE 1 | The maladaptive microbiota–gut–brain (MGB) axis in the pathophysiology of depression. Chronic exposure to stressors (e.g., psychological, poor nutrition) triggers prolonged release of (1) norepinephrine that alters gut microbiota composition by shifting to one that is enriched with pathogenic bacteria, and (2) acetylcholine and glucocorticoids that increase intestinal barrier permeability. The increased intestinal permeability allows bacteria and their toxins to enter systemic circulation, triggering stress responses from the HPA axis and immune system that, when excessive; (3) leads to chronic inflammation and HPA axis overactivity; (4) aggravate intestinal permeability; (5) alter composition of gut microbiota; and (6) disrupt neurotransmitter systems. Altered gut microbiota also results in an inflamed gut and (7) a shift in the production of bioactive molecules that regulate host neurotransmitter systems and gut motor functions. As a proof of concept, these five factors (in the circle) that depict the maladaptive MGB axis are often detected in MDD patients. Lastly, the constant negative emotions displayed by depressed patients further trigger a stronger reaction or sensitivity to various stressors.

DA, and NE) has been associated with the pathophysiology of depression. Therefore, pharmaceutical antidepressants function to restore synaptic levels of neurotransmitters (Harald and Gordon, 2012). In addition, impaired neurotransmitter systems within the ENS may alter gut motor function. This has direct implications as gut motility is a determining factor in the size and diversity of gut microbiota (Quigley, 2011). Therefore, chronic stress sets up a vicious cycle of increased intestinal permeability, chronic inflammation, hyperactive HPA axis, altered gut microbiota profile and neurotransmitter imbalances – forming a maladaptive MGB axis (**Figure 1**). Furthermore, MDD patients perceive stress as more threatening and challenging to cope with compared to healthy individuals (Farabaugh et al., 2004; Salomon et al., 2009). These negative emotions can increase their sensitivity to stressors, such as an elevated cortisol response (Mendonca-de-Souza et al., 2007). To restore this malfunctioned axis, probiotics have been demonstrated by meta-analyses and systematic reviews as a potential treatment for MDD/depression (Huang et al., 2016; Pirbaglou et al., 2016; Wang et al., 2016; McKean et al., 2017; Wallace and Milev, 2017). Potential antidepressive mechanisms of probiotics are elucidated in the following section.

DELINEATING THE ANTIDEPRESSIVE MECHANISMS OF PROBIOTICS

Probiotics secrete a wide range of signaling molecules that operate via distinct pathways to exert their effects, be it antidepressive, immunomodulatory or modulation of neurotransmission (Luan et al., 2017). This review classifies probiotic-associated signaling molecules into four types: neurotransmitters, bacterial secreted proteins, butyrate and other bioactive molecules (**Figure 2**). Some probiotics can secrete signaling molecules of different types. In this regard, the mechanisms of individual probiotics will be presented in the order of pertinence and similarity to each other.

Lactobacillus rhamnosus

Lactobacillus rhamnosus JB-1, the typical experimental strain of *L. rhamnosus*, was formerly referred to as *Lactobacillus reuteri*. Orally administered *L. rhamnosus* reduced depressive-like behaviors in normal, healthy mice (Bravo et al., 2011) and chronic-stressed mice (McVey Neufeld et al., 2018). Postpartum women (Slykerman et al., 2017) and obese individuals (Sanchez et al., 2017) that were supplemented with *L. rhamnosus* reported lower depressive thoughts compared to the control group. In vagotomized rats, behavioral and physiological benefits of *L. rhamnosus* were abolished (Bravo et al., 2011). This substantiates the vagus nerve as an essential conduit in the signaling pathway of *L. rhamnosus*. Introduction of *L. rhamnosus* into the gut lumen heightened the firing rate of vagus nerve and enteric neurons in mice (Perez-Burgos et al., 2013, 2014). These findings suggest that *L. rhamnosus* signals to the brain via the neural route, which may influence the central GABAergic system and HPA axis to manifest an antidepressive effect (**Figure 2A**). However, it is unclear whether neurotransmitters, cytokines or other molecules are involved in the neural signaling of *L. rhamnosus*.

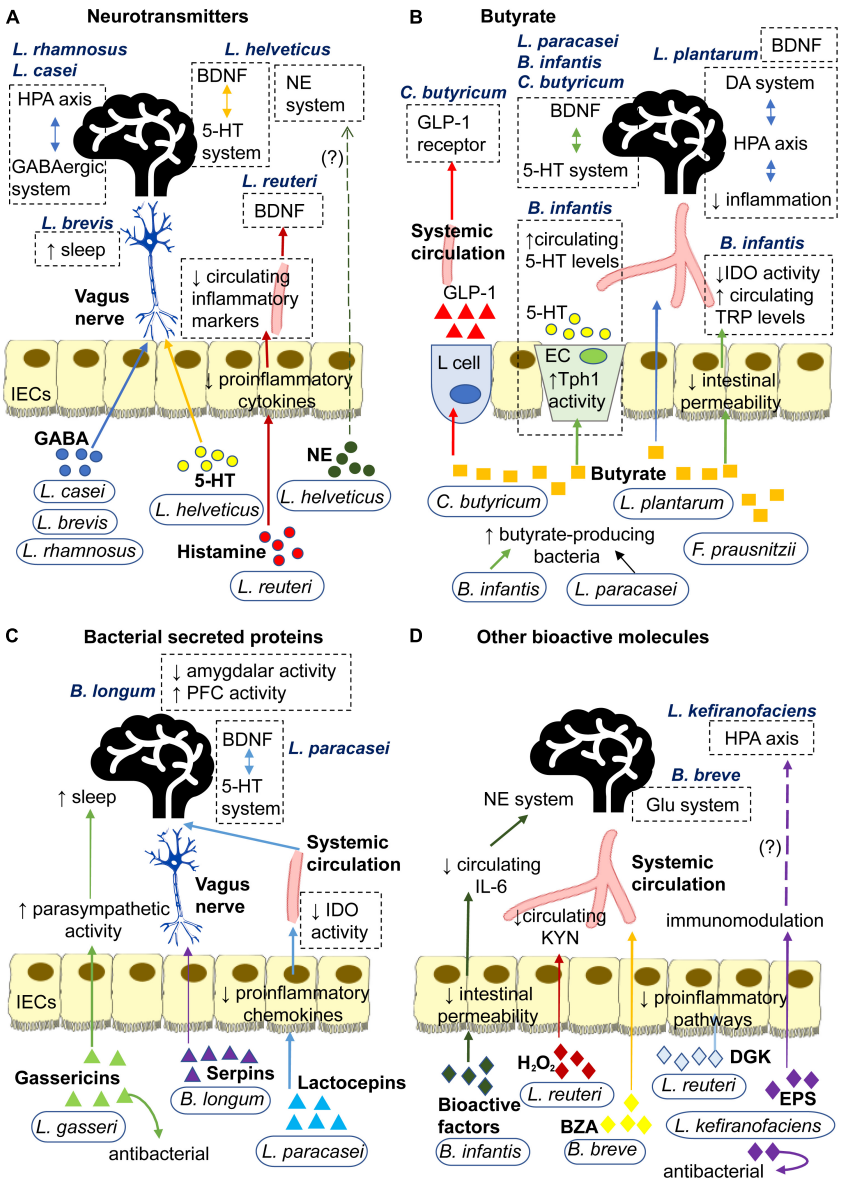

FIGURE 2 | Signaling mechanisms underlying antidepressive effects of probiotics mediated through secretion of **(A)** Neurotransmitters: *L. rhamnosus* and *L. casei* secrete GABA that may signal central GABAergic system and HPA axis via the neural route. *L. brevis* secretes GABA that enhances sleep. *L. helveticus* secrete 5-HT that may signal the central 5-HT system via the neural route. *L helveticus* also secretes NE that may affect the central NE system. *L. reuteri* secretes histamine that decreases secretion of proinflammatory cytokines by IECs. This may reduce circulating inflammatory markers, such as LPS, IL-6 and corticosterone, and subsequently prevent the inflammation-induced decrease in hippocampal BDNF. **(B)** Butyrate: *L. plantarum* produces butyrate that strengthens intestinal barrier and diffuses through the circulation to regulate BDNF expression and reduce inflammation in the brain. The latter consequently regulates the HPA axis and its regulator, the DA system. *C. butyricum* produces butyrate that influences central 5-HT and BDNF systems and stimulates L cell to secrete GLP-1 into the bloodstream which increases expression of GLP-1 receptors. *F. prausnitzii* produces butyrate that strengthens the intestinal barrier. *B. infantis* and *L. paracasei* promote growth of butyrate-producing bacteria. Through butyrate, *B. infantis* upregulates Tph1 activity of EC which increases circulating 5-HT and strengthens intestinal barrier to lower IDO activity and increase circulating TRP, both of which affect the central 5-HT system and BDNF expression. Through butyrate, *L. paracasei* may influence the central 5-HT system and BDNF expression. **(C)** Bacterial secreted proteins: *L. gasseri* secretes gassericins that increase parasympathetic activity to facilitate sleep and improves gut microbiota composition. *B. longum* secretes serpins that alter neural activities in the brain via the neural route. *L. paracasei* secretes lactocepins that decrease proinflammatory chemokines in IECs. This lowers IDO activity which, in turn, affects the central 5-HT system and BDNF expression. **(D)** Other bioactive molecules: *B. infantis* secretes bioactive factors (likely polysaccharides) that decrease circulating IL-6 which affects the central NE system. *L. reuteri* secretes H_2O_2 that decreases IDO activity and circulating KYN, and dgk that inhibits the initiation of proinflammatory pathways. *B. breve* converts albiflorin into BZA which affects the Glu system via the humoral route. *L. kefiranofaciens* secretes exopolysaccharides that have immunomodulatory and antibacterial properties, which may potentially prevent HPA axis overactivity. 5-HT, 5-hydroxytryptamine or serotonin; BDNF, brain-derived neurotrophic factor; DA, dopamine; BZA, benzoic acids; dgk, diacylglycerol kinase; ECs, enterochromaffin cells; EPS, exopolysaccharide; GABA, gamma-Aminobutyric acid; GLP-1, glucagon-like peptide-1; Glu, glutamate or glutaminergic; H_2O_2, hydrogen peroxide; HPA, hypothalamic-pituitary-adrenal; IECs, intestinal epithelial cells; IDO, indoleamine 2,3-dioxygenase; IL-6, interleukin-6; KYN, kynurenine; NE, norepinephrine; LPS, lipopolysaccharides; Tph1, tryptophan hydroxylase 1; TRP, tryptophan.

Microbial GABA, Central GABAergic System, and HPA Axis

Glutamine is a precursor to Glu while Glu is a precursor to GABA. Reduced levels of GABA and Glx (Glu + glutamine) have been consistently reported in cortical regions of MDD patients (Sanacora et al., 1999; Hasler et al., 2007; Bhagwagar et al., 2008; Moriguchi et al., 2018; Godlewska et al., 2019). A dysfunctional glutaminergic system, that is partly responsible by a decreased GABAergic tone, is also implicated in MDD (Murrough et al., 2017). *N*-acetyl aspartate (NAA) is regarded as a marker for neuronal vitality. In MDD patients, decreased NAA levels in the PFC and hippocampus have been detected (Gonul et al., 2006; Olvera et al., 2010; Lefebvre et al., 2017). These neurochemical (i.e., Glx, NAA, and GABA) levels in the PFC and hippocampus of mice increased when administered with *L. rhamnosus* (Janik et al., 2016), implicating its antidepressive potential.

Intake of *L. rhamnosus* altered the central mRNA expression of $GABA_A$ and $GABA_B$ receptors while reducing depressive- and anxiety-like behaviors in mice. These effects were also dependent on an intact vagus nerve (Bravo et al., 2011). With prebiotics, *L. rhamnosus* intake decreased hippocampal $GABA_{A\alpha2}$ mRNA expression in stressed mice (McVey Neufeld et al., 2017). *L. rhamnosus* produced GABA and Glu efficiently from microbial glutamate decarboxylase and glutaminase, respectively, *in vitro* (Stromeck et al., 2011; Liao et al., 2013; Lin, 2013). These biosynthetic machineries utilized by microbes to synthesize Glu and GABA are mutual in neurons (Mathews and Diamond, 2003), which support the interkingdom communication of microbial GABA (Lyte, 2011). It was demonstrated *in vitro* that gut microbial GABA can cross the intestinal barrier via H^+/GABA symporter (Thwaites et al., 2000; Nielsen et al., 2012). The microbial GABA may subsequently interact with GABA receptors and transporters that are widely expressed on enteric neurons and vagus afferents (Hyland and Cryan, 2010).

Administration of *L. rhamnosus* reduced stress-induced plasma corticosterone levels in mice that averted depression (Bravo et al., 2011; McVey Neufeld et al., 2018). This could be due to the innervation of PVN neurons by GABAergic synapses that can be desensitized by acute stress (Hewitt et al., 2009). Inhibited GABA signals allow continuous release of CRF by PVN neurons, which ultimately leads to cortisol overproduction and HPA axis overactivity (Cullinan et al., 2008). Impairment of GABA receptors also inhibits hippocampal neurogenesis, which has been shown to activate the HPA axis and induce depression in mice (Earnheart et al., 2007; Schloesser et al., 2009). Such effects may be possibly prevented by the production of GABA by *L. rhamnosus*.

Lactobacillus casei Strain Shirota

Individuals with low mood reported feeling happier after consuming milk containing *L. casei*, but not the placebo (Benton et al., 2007). Intake of mixed-species probiotics that included *L. casei* also reduced clinical depression and depressive-like symptoms in MDD patients (Akkasheh et al., 2016) and healthy individuals (Steenbergen et al., 2015; Mohammadi et al., 2016), respectively. Similar to *L. rhamnosus*, evidence suggests that *L. casei* may also regulate the HPA axis via the neural route (**Figure 2A**).

Microbial GABA and HPA Axis

Intake of *L. casei* stimulated vagus afferents and decreased both the activity and quantity of CRF-expressing cells in PVN of rats (Takada et al., 2016). Intragastric injection of *L. casei* downregulated the activity of sympathetic efferents to adrenal glands and liver, and this effect ceased upon vagotomy (Tanida et al., 2014). In clinical trials, *L. casei* supplementation lowered salivary cortisol levels, feelings of stress and frequency of abdominal- and flu-related symptoms in stressed individuals (Kato-Kataoka et al., 2016; Takada et al., 2016). These studies imply that *L. casei* prevents HPA axis overactivity via the vagus nerve, which may consequently lower stress-related feelings and illnesses. *L. casei* produced GABA *in vitro* (Oleskin et al., 2014), indicating a possibility that it may share an antidepressive mechanism of *L. rhamnosus*. Stressed individuals that consumed *L. casei* showed improvements in mental health and gut microbiota composition, characterized by increased *Lactobacillus* and *Bifidobacterium* populations (Rao et al., 2009; Kato-Kataoka et al., 2016). As most of the antidepressive probiotics belong to *Lactobacillus* and *Bifidobacterium* genera, the potential antidepressive capacity of *L. casei* is highly supported.

Lactobacillus brevis

Similar to *L. rhamnosus* and *L. casei*, *L. brevis* produces GABA via glutamate decarboxylase in substantial amounts (Yokoyama et al., 2002; Siragusa et al., 2007; Barrett et al., 2012; Ko et al., 2013; Yunes et al., 2016). This indicates that *L. brevis* may share a mutual mechanism of action with *L. rhamnosus* and *L. casei* (**Figure 2A**). Although *L. brevis* has been shown to influence neither the central GABAergic system nor the HPA axis, *L. brevis* appears to promote sleep.

Microbial GABA and Sleep

Milk fermented with *L. brevis* had increased GABA content. This *L. brevis*-fermented milk demonstrated an antidepressive potency on par with fluoxetine, a SSRI, in depressed rats (Ko et al., 2013). Intriguingly, intake of *L. brevis*-produced GABA improved sleep duration in mice (Han et al., 2017). Another study also showed that dietary *L. brevis* enhanced sleep quality and voluntary physical activity in mice (Miyazaki et al., 2014). GABA is the main inhibitory neurotransmitter that is widely associated with sleep, and GABA receptors are frequent targets for pharmaceutical drugs, such as benzodiazepine, to treat insomnia (Gottesmann, 2002). GABA-enriched foods and GABA extract have also been shown to improve sleep quality in insomniacs (Byun et al., 2018) and healthy individuals (Yamatsu et al., 2015). Therefore, *L. brevis* has therapeutic value for insomnia, which reflects one of the diagnostic criteria for MDD (American Psychiatric Association, 2013).

Lactobacillus reuteri

Treatment of *L. reuteri* ameliorated depressive-like behaviors in chronic-stressed (Marin et al., 2017) and immobilization-stressed mice (Jang et al., 2019). The former study further elucidated the mechanism of *L. reuteri* which involves regulation of IDO, a rate-limiting enzyme of immune cells that catabolizes TRP to KYN (Reus et al., 2015). It is also well documented that *L. reuteri* exhibits anti-inflammatory activities (Thomas et al., 2012; Gao et al., 2015; Ganesh et al., 2018). It is, thus, conceivable that *L. reuteri* may also prevent activation of IDO by proinflammatory cytokines (Reus et al., 2015).

Microbial Hydrogen Peroxide and Kynurenine Pathway

The etiology of depression is partly attributed to a dysregulated KYN/TRP pathway (Reus et al., 2015). An elevated ratio of plasma KYN/TRP often correlates positively with the depression severity in human (Maes et al., 2002; Gabbay et al., 2010; Baranyi et al., 2013; Zhou et al., 2019). It was demonstrated that *L. reuteri* intake improved behaviors of depressed mice by reversing the stress-induced (1) decrease in fecal H_2O_2 levels and *Lactobacillus* populations, and (2) increase in intestinal IDO1 expression and plasma KYN levels (Marin et al., 2017). KYN administration attenuated this antidepressive effect, which indicates that *L. reuteri* ameliorates depression by reducing plasma KYN levels. This study also showed that *L. reuteri* generated high amounts of H_2O_2 *in vitro*, and the author proposed that H_2O_2 is the key metabolite in mediating antidepressive effect of *L. reuteri* (Marin et al., 2017). This is because H_2O_2 catalyzes peroxidase-mediated reactions that inhibit IDO activity (Freewan et al., 2013). H_2O_2 is transported by aquaporin-3 transporters that are expressed on IECs (Thiagarajah et al., 2017) and immune cells (Moon et al., 2004). These findings suggest that microbial H_2O_2 can potentially cross the intestinal barrier to suppress IDO activity in immune cells, which would lower circulating KYN levels (**Figure 2D**).

Microbial Histamine, Diacylglycerol Kinase, and Brain-Derived Neurotrophic Factor (BDNF) Expression

Lactobacillus reuteri possesses histidine decarboxylase that converts dietary L-histidine to histamine, which inhibits the production of TNF-α *in vitro* (Thomas et al., 2012; Hemarajata et al., 2013). The microbial histamine suppressed proinflammatory cytokine activities in IECs via the histamine-2 receptor signaling pathway in mice. This effect disappeared when the histidine decarboxylase gene of *L. reuteri* was inactivated by mutagenesis (Gao et al., 2015). Intriguingly, microbial histamine also activated histamine-1 receptors to initiate downstream proinflammatory pathways in mice (Ganesh et al., 2018). However, the substrate for this pathway, diacylglycerol, is metabolized to phosphatidic acid by diacylglycerol kinase produced by *L. reuteri*. Thus, *L. reuteri* secretes both histamine and diacylglycerol kinase that act on histamine receptors to produce an anti-inflammatory effect (Ganesh et al., 2018). Orally administered *L. reuteri* simultaneously alleviated colitis and behaviors indicative of anxiety and depression in stressed mice. These effects were also accompanied by a decrease in colon inflammation and blood levels of LPS, interleukin-6 (IL-6) and corticosterone. In the same study, this reduction in peripheral inflammation prevented the infiltration of activated microglia into the hippocampus and increased hippocampal BDNF expression (Jang et al., 2019; **Figure 2A**). BDNF has been extensively studied for its vital role in neuronal function and its causal link to depression. Antidepressants such as SSRI and ketamine also increase hippocampal BDNF expression as part of their mechanism of action (Bjorkholm and Monteggia, 2016). Furthermore, this anti-inflammatory effect of *L. reuteri* may prevent IDO activation by proinflammatory cytokines (Reus et al., 2015).

Lactobacillus plantarum

Lactobacillus plantarum supplementation decreased depressive-like symptoms in chronic-stressed mice (Liu Y.W. et al., 2016; Dhaliwal et al., 2018) and stressed adults with mild depression (Lew et al., 2018), though the latter study did not reach statistical significance. Following *L. plantarum* intake, reduction in plasma corticosterone levels and inflammation were seen in mice with reduced depressive-like behaviors (Liu Y.W. et al., 2016). Another study reported that mice fed with *L. plantarum* displayed an increase in cecum SCFAs levels (acetic and butyric), and a decrease in intestinal permeability and level of MAOs in the brain (Dhaliwal et al., 2018). These physiological changes can be unified into a mutual mechanism that *L. plantarum* likely mitigates systemic inflammation (**Figure 2B**).

Butyrate, Intestinal Barrier, and BDNF Expression

Chronic-stressed mice fed with *L. plantarum* exhibited reduced depressive-like behaviors, coupled with an increase in butyrate and butyrate-producing bacteria, such as *Lactobacillus*, *Bacteroidetes,* and *Roseburia* (Dhaliwal et al., 2018). *L. plantarum* synthesizes butyrate via fatty acid synthase II–thioesterase, a glutamine-mediated butyrogenic pathway (Botta et al., 2017). Butyrate can enter IECs through cholesterol-rich microdomains and/or monocarboxylate transporter 1 protein (Suzuki et al., 2008; Goncalves et al., 2011; Nedjadi et al., 2014), and promote synthesis and assembly of tight junction proteins of IECs (Bordin et al., 2004; Ohata et al., 2005; Peng et al., 2009; Wang et al., 2012; Yan and Ajuwon, 2017). Butyrate also has anti-inflammatory properties; for instance, butyrate inhibited proinflammatory activities of IECs *in vitro* (Elce et al., 2017) and interacted with IECs to regulate host T cell responses (Lew et al., 2018; Xu et al., 2018). Butyrate may also diffuse into the systemic circulation to exert anti-inflammatory effects on various organs and tissues, including the brain (McNabney and Henagan, 2017; Matt et al., 2018). Indeed, butyrate has been shown to normalize behavior of depressed rodents through epigenetic regulations of hippocampal BDNF expression (Han et al., 2014; Wei et al., 2014; Sun et al., 2016). These outcomes are consistent with the finding that *L. plantarum* intake increased hippocampal BDNF expression and cecum butyrate levels in chronic stress-induced depressed mice (Dhaliwal et al., 2018).

HPA Axis and Central DA System

Lactobacillus plantarum supplementation decreased MAOs levels in brain tissues of mice with reduced depression (Dhaliwal et al., 2018). This is in line with another finding that *L. plantarum* intake in mice increased levels of DA and its metabolites (HVA and 3,4-dihydroxyphenylacetic acid, DOPAC) in the PFC, along with reduced depressive-like behaviors (Liu Y.W. et al., 2016). However, another study showed that *L. plantarum* increased DA levels in the striatum of mice while alleviating anxiety-like behaviors (Liu W.H. et al., 2016). These studies suggest that *L. plantarum* likely affects the central DA system in a context-dependent manner. It was also proposed that *L. plantarum* increases DA levels in the PFC to prevent HPA axis overactivation (Liu Y.W. et al., 2016). DA neurons in the PFC and ventral tegmental area (VTA) form the mesocortical pathway which regulates reward-seeking behaviors (Pariyadath et al., 2016) and the HPA axis (Sullivan and Dufresne, 2006). Glucocorticoids from the HPA axis can also influence the DA system either directly or indirectly, via epigenetic control and MAOs inhibition, respectively (Feenstra et al., 1992; Grunewald et al., 2012; Butts and Phillips, 2013). Taken together, *L. plantarum* may regulate both the DA system and HPA axis by attenuating glucocorticoid-induced MAOs activity.

Faecalibacterium prausnitzii (Previously Known as *Fusobacterium prausnitzii*)

Recently, it was discovered that oral gavage of *F. prausnitzii* exerted antidepressive and anxiolytic effects in chronic-stressed mice (Hao et al., 2019). *F. prausnitzii*, as the sole species of *Faecalibacterium* genera (Duncan, 2002), represents around 5% of the total human gut microbiota (Hold et al., 2003). Low populations of *F. prausnitzii* correlated with the disease severity of those with MDD (Jiang et al., 2015) and bipolar depression (Evans et al., 2017). In a recent large cohort study, fecal levels of *F. prausnitzii* correlated negatively with depressed mood and positively with quality of life (Valles-Colomer et al., 2019). Therefore, *F. prausnitzii* seems to have pertinent contributions to mental health.

Butyrate, Microbial Anti-inflammatory Molecules, and Peripheral Inflammation

Faecalibacterium prausnitzii produces butyrate in large quantities from fermenting glucose and fiber (Duncan, 2002; Hold et al., 2003). *F. prausnitzii* also secretes microbial anti-inflammatory molecules that suppress the proinflammatory nuclear factor (NF)-κB pathway in IECs (Sokol et al., 2008; Quevrain et al., 2016a,b). These immunomodulatory effects are consistent with neurochemical changes observed in *F. prausnitzii*-treated depressed mice, whereby cecum SCFAs and plasma IL-10 levels increased, while corticosterone and IL-6 levels decreased (Hao et al., 2019). Moreover, intragastric administration of *F. prausnitzii* decreased colonic cytokine levels and intestinal permeability in mice with colitis (Laval et al., 2015; Martin et al., 2015). Thus, butyrate produced by *F. prausnitzii* potentially strengthens the intestinal barrier (similar to *L. plantarum*; **Figure 2B**). However, whether local immunomodulatory effects of *F. prausnitzii* extend to the brain remains unknown. Nevertheless, the ability of *F. prausnitzii* to attenuate gut inflammation is sufficient to reduce depressive- and anxiety-like behaviors in mice (Hao et al., 2019).

Lactobacillus helveticus

Lactobacillus helveticus intake enabled the recovery of chronic- and subchronic-stressed rodents from their state of depression (Liang et al., 2015; Maehata et al., 2019). Probiotic sticks containing *L. helveticus*, in addition to *Bifidobacterium longum*, reduced clinical depression and depressive-like symptoms in MDD patients (Kazemi et al., 2019) and healthy individuals (Messaoudi et al., 2011), respectively. Most of the animal and human studies also showed that *L. helveticus* intake enhanced memory and, sometimes, attention and learning (Ohland et al., 2013; Chung et al., 2014; Luo et al., 2014; Liang et al., 2015; Ohsawa et al., 2018). Cognitive impairments, such as poor memory and concentration, represent one major cluster of MDD symptoms (Sharpley and Bitsika, 2014). Evidence suggests that *L. helveticus* may modulate the central NE system and HPA axis to improve cognition, and the central 5-HT system and BDNF expression to reduce depression (Liang et al., 2015) (**Figure 2A**).

Microbial NE, Central NE System, and HPA Axis

Supplementation of *L. helveticus* improved memory and cognitive performance in chronic-stressed rats, comparable to the SSRI citalopram-treated rats. This memory improvement correlated with increased plasma IL-10 and hippocampal NE levels, and reduced plasma corticosterone and ACTH levels (Liang et al., 2015). A previous study also showed that ingestion of *L. helveticus* enhanced memory and mitigated gut inflammation in neuroinflammation-induced rats (Luo et al., 2014). However, another study reported that memory improvement in *L. helveticus*-treated mice did not correlate with the state of gut inflammation (Ohland et al., 2013). Despite this discrepancy, it is well established that the hippocampal NE system and HPA axis both interact to regulate hippocampal glucose metabolism for memory consolidation (Osborne et al., 2015). This mechanism may be affected by microbial NE as *L. helveticus* produced NE *in vitro* in amounts that exceed the human bloodstream (Oleskin et al., 2014). It was also shown *in vivo* that gut bacteria are responsible for converting conjugated NE into its biologically active form (Asano et al., 2012). This neuroactive NE likely influences the MGB axis, but the exact mechanism remains unknown (Lyte, 2011).

Microbial 5-HT and Central 5-HT-BDNF System

Liang et al. (2015) showed that elevated hippocampal 5-HT levels correlated with reduced depression severity in *L. helveticus*-fed rats. The same study also demonstrated that treatment with SSRI citalopram alleviated depression and increased hippocampal BDNF expression and 5-HT levels (Liang et al., 2015). Hence, the antidepressive mechanism appears similar between *L. helveticus* and citalopram. Cultures of *L. helveticus* produced 5-HT at concentrations close to that in the human bloodstream (Oleskin et al., 2014). As shown *in vivo*, the gut microbiota has an indispensable function in deconjugating glucuronide-conjugated 5-HT to generate their free, biologically active counterparts in

considerable amounts (Hata et al., 2017). It is hypothesized that gut luminal 5-HT may sensitize 5-HT 3A receptors of enteric neurons by stimulating the glial cell-derived neurotrophic factor of IECs (Hata et al., 2017). 5-HT3 receptors are also expressed on IECs (Hasler, 2009) and vagal afferents (Hillsley and Grundy, 1998). Therefore, it can be speculated that *L. helveticus* influences the central 5-HT circuitry via the neural route. This is supported by a recent study showing that *L. helveticus* intake increased expression of 5-HT 1A receptors in the nucleus accumbens while restoring behaviors of depressed mice (Maehata et al., 2019).

Chronic-stressed mice that ingested *L. helveticus* displayed an increase in hippocampal BDNF levels (Liang et al., 2015) and neurogenesis in the nucleus accumbens (Maehata et al., 2019). Nucleus accumbens is a brain region implicated in reward behavior. The central BDNF and 5-HT systems are synergistic, whereby 5-HT upregulates hippocampal BDNF–TrkB signaling to increase expression and synthesis of BDNF. The elevated BDNF, in turn, facilitates neurogenesis of 5-HT neurons (Martinowich and Lu, 2008; Bjorkholm and Monteggia, 2016). Therefore, *L. helveticus* likely increases hippocampal BDNF levels via modulation of 5-HT circuitry, in a similar manner to SSRIs (Liang et al., 2015).

Lactobacillus paracasei

Dietary intervention of heat-killed *L. paracasei* prevented mood deterioration in times of stress in healthy individuals (Murata et al., 2018). In corticosterone-induced depressed mice, oral gavage of either live or heat-killed *L. paracasei* exhibited antidepressive efficacy equivalent to or better than fluoxetine. The same study also showed that live and heat-killed *L. paracasei* operated via different mechanisms. Live *L. paracasei* increased 5-HT levels whereas heat-killed *L. paracasei* increased DA levels in the brain (Wei et al., 2019). The signaling mechanism of *L. paracasei* appears independent of the HPA axis (Wei et al., 2019) or vagus afferents (Tanida and Nagai, 2011). The remaining evidence suggests that *L. paracasei* potentially functions via an immune-mediated humoral pathway.

Lactocepin, Butyrate, and Central 5-HT-BDNF System

Lactobacillus paracasei secretes lactocepin, a *PrtP*-encoded serine protease, that selectively degrades proinflammatory chemokines in inflamed ileal tissue of mice (von Schillde et al., 2012). Lactocepin is most likely a heat-labile cell surface protein unique to *L. paracasei* (Hoermannsperger et al., 2009; von Schillde et al., 2012). Mice fed with live *L. paracasei* exhibited lower inflammatory markers in serum, such as increased IL-10 and glutathione peroxidase and decreased TNF-α and MCP-1 (Huang et al., 2018). Another study showed that oral gavage of live *L. paracasei* with its bacterial products prevented adverse effect of stress on intestinal permeability in rats (Eutamene et al., 2007). This can be linked to a suppressed IDO activity, resulting in higher TRP bioavailability for 5-HT synthesis in the brain (Reus et al., 2015). Following this, it was shown that live *L. paracasei* delivered via gavage increased 5-HT and 5-HIAA (the main metabolite of 5-HT) levels in the hippocampus and striatum of mice (Huang et al., 2018; Wei et al., 2019). As 5-HT facilitates BDNF synthesis (Martinowich and Lu, 2008), the upregulated central 5-HT expression presumably explains the accompanying increase in hippocampal BDNF expression of mice alleviated of depression from *L. paracasei* intake (Wei et al., 2019). Therefore, *L. paracasei* may upregulate the central 5-HT-BDNF system (similar to *L. helveticus*; **Figure 2C**).

Treatment of live *L. paracasei* also increased fecal *Bifidobacterium* populations while normalizing behaviors of depressed mice (Wei et al., 2019). The gut microbiota profile, inflammatory markers and levels of acetate and butyrate were improved in IBS patients supplemented with live *L. paracasei* (Bertani et al., 2017; Cremon et al., 2018). Reduction in systemic inflammation, coupled with an improvement in hippocampal function, was also observed in obese rats fed with live *L. paracasei* (Chunchai et al., 2018). Thus, live *L. paracasei* may facilitate the colonization of butyrate-producing bacteria to reduce systemic inflammation (similar to *L. plantarum*) and increase 5-HT secretion from ECs (similar to *Bifidobacterium infantis*; **Figure 2B**).

Bifidobacterium infantis

In naïve rats, intake of *B. infantis* was shown to alter depression-related biomarkers (Desbonnet et al., 2008). The same group later showed that chronic-stressed mice no longer displayed depressive-like behaviors after *B. infantis* intake (Desbonnet et al., 2010). In flood victims with IBS, *B. infantis* consumption did not affect their IBS symptoms but improved their mental health instead (Murata et al., 2018). *B. infantis* did not influence corticosterone levels in mice (Desbonnet et al., 2008, 2010), implying that the effect of *B. infantis* is likely to be independent of the HPA axis. Evidence suggests that *B. infantis* has immunomodulatory effects that regulate the central NE system (Desbonnet et al., 2010). A recent study also provided support for the antidepressive mechanism of *B. infantis* that involves the hippocampal 5-HT system (Tian et al., 2019).

Bioactive Factors, IL-6, and Central NE System

Bifidobacterium infantis treatment manifested two physiological changes *in vivo*. First, *B. infantis* decreased plasma IL-6 levels in mice (Desbonnet et al., 2008, 2010) and patients with inflammatory conditions (Groeger et al., 2013). In depressed mice, the IL-6 release also correlated positively with the severity of depression (Desbonnet et al., 2010). Second, *B. infantis* increased NE levels in the murine brainstem (Desbonnet et al., 2010) containing the majority of NE neurons (Schwarz and Luo, 2015). Therefore, *B. infantis* likely regulates plasma IL-6 and central NE system to exert an antidepressive effect.

Bifidobacterium infantis secretes bioactive factors (probably polysaccharides) that enhance transepithelial resistance of IECs (Ewaschuk et al., 2008). Other studies involving rodents also showed that *B. infantis* treatment enhanced the intestinal barrier by strengthening the formation of tight junction proteins and anti-inflammatory activities of immune cells (Lomasney et al., 2014; Zuo et al., 2014; Javed et al., 2016). Indeed, bacterial DNA translocation from the gut lumen into the circulation was reduced in *B. infantis*-fed rodents (Osman et al., 2006; Gómez-Hurtado et al., 2012).

Bacterial DNA is a potent inducer of TLRs which facilitate the release of proinflammatory cytokines, including IL-6 (Gutierrez et al., 2016). Administration of IL-6 induced depression in mice, and this outcome was prevented by pharmaceutical blockage of NE neurons in the brainstem (Kurosawa et al., 2016). Hence, *B. infantis* potentially modulates the NE system via an immune-mediated humoral route to reduce depression (**Figure 2D**). This mechanism appears to be independent of the vagus nerve as oral gavage of *B. infantis* also decreased proinflammatory cytokine (including IL-6) levels in vagotomized mice with an inflamed colon (van der Kleij et al., 2008).

Butyrate, TRP, and Central 5-HT-BDNF System

Treatment of *B. infantis* upregulated mRNA expression of Tph1 in RIN14B cells, a cell line that mimics ECs (Tian et al., 2019). Tph1 converts TRP to 5-hydroxytryptophan (5-HTP) and aromatic amino acid decarboxylase subsequently converts 5-HTP to 5-HT. *B. infantis*-fed mice displayed reduced depressive-like behaviors, along with an increase in TRP biosynthesis and hippocampal 5-HT and 5-HTP levels. In the same study, *B. infantis* increased cecum butyrate levels and the abundance of butyrate-producing *Bifidobacterium*. The elevated butyrate levels also correlated with increased hippocampal 5-HTP and PFC BDNF levels (Tian et al., 2019). This could be due to the ability of butyrate and other SCFAs to increase Tph1 activity of ECs, thereby promoting 5-HTP and 5-HT secretions (Reigstad et al., 2015; Yano et al., 2015; Lund et al., 2018). This is consequential as ECs contribute about 95% of the bodily 5-HT (El-Merahbi et al., 2015), and that mice with a gut microbiota had 2.8-fold higher plasma 5-HT levels than GF mice (Wikoff et al., 2009). The evidence for the ability of 5-HT to cross the BBB is conflicting (Brust et al., 2000; Wakayama et al., 2002; Nakatani et al., 2008; El-Merahbi et al., 2015). In contrast, 5-HTP readily crosses the BBB and can be converted into 5-HT. Therapeutic 5-HTP has also been shown to treat clinical depression with a potency equivalent to or better than SSRIs (Birdsall, 1998; Jangid et al., 2013; Jacobsen et al., 2016).

Furthermore, *B. infantis* intake increased plasma TRP levels in healthy rats (Desbonnet et al., 2008), but another study with chronic-stressed rats reported otherwise (Desbonnet et al., 2010). The author then suggested that *B. infantis* regulates TRP metabolism differently, depending on the rat strain (Desbonnet et al., 2010). Therapeutic TRP can improve symptoms of mood, sleep and cognitive disorders as TRP readily passes through BBB to regulate numerous brain functions, such as 5-HT synthesis (Richard et al., 2009). The elevated plasma TRP levels from *B. infantis* intake is most likely a result of reduced proinflammatory cytokines (Desbonnet et al., 2008, 2010), which reduces IDO activity and prevents over-catabolism of TRP (Reus et al., 2015). Thus, *B. infantis* may upregulate the hippocampal 5-HT system via modulation of peripheral 5-HTP, 5-HT and/or TRP levels. As 5-HT promotes BDNF synthesis (Martinowich and Lu, 2008), this presumably explains the concomitant increase in BDNF levels in PFC of rats ameliorated of depression with *B. infantis* treatment (Tian et al., 2019). Taken together, *L. helveticus*, *L. paracasei* and *B. infantis* upregulate the central 5-HT-BDNF system as their mutual antidepressive mechanism, although via different pathways (**Figure 2B**).

Clostridium butyricum

Treatment of *C. butyricum* improved depressive-like behaviors in chronic-stressed mice. These treated mice also showed upregulated central 5-HT, BDNF and GLP-1 receptors in the brain (Sun et al., 2018). Remarkably, the combination of *C. butyricum* with antidepressants reduced depression in about 70% of treatment-resistant MDD patients, of which 30% achieved remission (Miyaoka et al., 2018). These studies support the antidepressive efficacy of non-pathogenic *C. butyricum*. It should be noted that certain strains of *C. butyricum* are pathogenic which may cause botulism and necrotizing enterocolitis (Cassir et al., 2016).

Butyrate, Central 5-HT-BDNF System, and GLP-1

Clostridium butyricum, as a resident of healthy gut microbiota, produces butyrate from carbohydrate fermentation (Araki et al., 2002; He et al., 2005; Liu J. et al., 2015). Treatment of *C. butyricum* increased central 5-HT levels and BDNF expression in mice with reduced depression (Sun et al., 2018). Another study also reported that *C. butyricum* intake upregulated neurogenesis-related pathways, such as BDNF, via butyrate production in mice (Liu J. et al., 2015). Additionally, intragastric inoculation of *C. butyricum* increased intestinal secretion of GLP-1 and the central expression of GLP-1 receptors in mice alleviated from depression (Sun et al., 2018). This effect may also be mediated by butyrate as SCFAs can bind to receptors expressed on intestinal L cells to stimulate GLP-1 secretion into the bloodstream (Tolhurst et al., 2012). GLP-1 is known for appetite and glucose control, but the activation of central GLP-1 receptors has been shown to regulate the central 5-HT system and reduce anxiety- and depressive-like behaviors in rats (Anderberg et al., 2016). Therefore, antidepressive mechanism of *C. butyricum* potentially involves a butyrate-mediated upregulation of central BDNF-5-HT system (similar to *L. paracasei* and *B. infantis*) and GLP-1 receptor expression (**Figure 2B**).

Lactobacillus kefiranofaciens

Lactobacillus kefiranofaciens is isolated from kefir, a type of fermented milk. Oral gavage of *L. kefiranofaciens* improved behaviors of chronic-stressed, depressed mice. These treated mice also showed several physiological alterations. Levels of circulating TRP, splenic IL-10 and beneficial gut bacteria (e.g., Lachnospiraceae, Bifidobacteriaceae, and Akkermansia) increased, and KYN/TRP ratio, splenic IL-6 and IFN-γ levels and Proteobacteria abundance decreased (Sun et al., 2019). What factors mediate such broad effects of *L. kefiranofaciens* on the TRP/KYN pathway, immune system, HPA axis and gut microbiota remain unclear, but exopolysaccharide is potentially a candidate (**Figure 2D**).

Exopolysaccharide, Peripheral Inflammation, and Gut Microbiota

The only known metabolite of *L. kefiranofaciens* is an exopolysaccharide called kefiran (Maeda et al., 2004; Xing et al., 2017). The intake of kefiran modulated the gut mucosal immune system of mice (Vinderola et al., 2006), which could potentially account for changes in splenic cytokines seen in depressed mice (Sun et al., 2019). Kefiran was also shown to protect human enterocyte cell lines from adhesion and damage inflicted by toxins of pathogenic bacteria (Santos et al., 2003; Medrano et al., 2008). A further study discovered that *L. kefiranofaciens* produces a novel exopolysaccharide (not kefiran) that is bactericidal toward enteropathogens *Listeria monocytogenes* and *Salmonella enteritidis* (Jeong et al., 2017a). It may be possible that the antibacterial effects of this exopolysaccharide extend to other species in the gut microbiota. This supports the finding that *L. kefiranofaciens* supplementation ameliorated depressive-like behaviors in chronic-stressed mice by regulating gut microbiota content, which included the decreased abundance of Proteobacteria, a phylum that includes pathogens such as *Salmonella* (Sun et al., 2019). Other mice studies also supported the role of *L. kefiranofaciens* in modulating gut microbiota composition (Jeong et al., 2017b; Xing et al., 2018). Collectively, these changes in gut microbiota profile prevent gut dysbiosis that could lead to chronic inflammation, HPA axis overactivity and depression (Jeong et al., 2017b).

Bifidobacterium breve

Bifidobacterium breve treatment improved symptoms of depression in innately anxious mice (Savignac et al., 2014), chronic-stressed mice (Tian et al., 2019) and schizophrenic patients with depression (Okubo et al., 2019). *B. breve* supplementation also improved mood and cognition in elderly people with mild cognitive impairment (Kobayashi et al., 2019). However, none of the accompanying physiological changes among these studies overlapped, making it difficult to identify an exact mechanism of *B. breve*. In spite of this, one study demonstrated that antidepressive mechanism of *B. breve* involves the generation of benzoic acid (Zhao et al., 2018; **Figure 2D**).

Benzoic Acid and Central Glu System

Among the 18 bacterial strains isolated from gut microbiota, *B. breve* was the most efficient converter of albiflorin to benzoic acid via microbial carboxylesterase, at the rate of 75% as compared to *L. casei*, *Lactobacillus acidophilus* and *B. longum* at about 5%. The same study further showed that orally administered benzoic acid alleviated depression in mice (Zhao et al., 2018). Benzoic acid readily crosses the intestinal barrier and BBB to inhibit D-amino acid oxidase that catabolizes D-serine, a co-agonist of *N*-methyl-D-aspartate receptor (NMDAR, a type of Glu receptor) (Zhao et al., 2018). Both D-serine and NMDARs are therapeutic targets in neuropsychiatric disorders, such as depression, schizophrenia and cognitive impairment (Durrant and Heresco-Levy, 2014). Indeed, a dysfunctional Glu system is linked to the pathophysiology of depression (Pytka et al., 2016). In line with this, *B. breve* intake increased Glu synapses in chronic-stressed mice while treating its depressive-like behaviors (Tian et al., 2019).

Bifidobacterium longum

Bifidobacterium longum treatment decreased depressive-like symptoms in innately anxious mice (Savignac et al., 2014) and IBS patients with mild to moderate depression and/or anxiety (Pinto-Sanchez et al., 2017). *B. longum* supplementation also presented anxiolytic efficacy in numerous human and animal studies (Bercik et al., 2010, 2011; Allen et al., 2016; Orikasa et al., 2016). However, *B. longum* did not affect the gut inflammatory state in animals and humans, indicating a lack of immunomodulatory function (Bercik et al., 2010, 2011; Pinto-Sanchez et al., 2017). Other physiological changes, such as BDNF expression and plasma KYN/TRP ratio, seen in *B. longum*-treated mice and humans were inconsistent (Bercik et al., 2010, 2011; Orikasa et al., 2016; Pinto-Sanchez et al., 2017). Collectively, these data suggest that brain neural activity and HPA axis are possible targets of *B. longum* signaling mechanisms (**Figure 2C**).

Serpin, Central Neural Activity, and HPA Axis

Both *in vitro* and *in vivo* studies showed that *B. longum* weakened the excitability of murine myenteric neurons (Bercik et al., 2011; Khoshdel et al., 2013). Mice with inflamed intestines that were fed with *B. longum* demonstrated reduced anxiety-like behaviors, and this effect ceased upon vagotomy (Bercik et al., 2011). Intriguingly, *B. longum* intake also alleviated anxiety in colon-inflamed mice that were vagotomized before treatment (Bercik et al., 2010). The author postulated that vagus afferents are an essential conduit when *B. longum* signals enterocytes, but not colonocytes (Bercik et al., 2011). The genome of *B. longum* encodes serpin, a serine protease inhibitor (Ivanov et al., 2006; Mkaouar et al., 2016). Serpin can inhibit the activation of enteric neurons by suppressing the secretion of elastase-like proteases from IECs (Ivanov et al., 2006; Buhner et al., 2018). These studies support the premise that *B. longum* interacts with the host via the neural pathway (similar to *L. rhamnosus*). Following this, the neural activity and HPA axis of the brain may be altered. Individuals consuming *B. longum* had increased neural activity in the PFC and decreased neural activity in the amygdala and fronto-limbic regions (Allen et al., 2016; Pinto-Sanchez et al., 2017). Anomalies in the anatomy and activity of the amygdala and PFC are also commonly observed among depressed patients (Liu W. et al., 2017). Furthermore, *B. longum* intake exerted simultaneous glucocorticoids-lowering and anxiolytic effects in humans and mice (Allen et al., 2016; Orikasa et al., 2016), suggesting that *B. longum* potentially modulates the HPA axis.

Lactobacillus gasseri

Supplementation of *L. gasseri* improved mood (Sashihara et al., 2013) and depressive-like symptoms (Sawada et al., 2017) in stressed individuals. However, no studies have evaluated the effect of *L. gasseri* on clinically depressed individuals. Interestingly, *L. gasseri* is the only dietary probiotic which showed consistent sleep-enhancing effects in humans (Nishida et al., 2017a,b; Sawada et al., 2017). Irregular sleeping patterns are frequently associated with MDD (American Psychiatric Association, 2013;

Wallace and Milev, 2017), supporting the use of *L. gasseri* as a potential treatment for MDD-related sleep disturbances.

Gassericins, Gut Microbiota, and Parasympathetic Activity in Sleep

Stressed individuals that were given probiotic-based milk containing either heat-killed or live *L. paracasei* showed alterations in the gut microbiota profile. Heat-killed *L. gasseri* decreased *Bacteroides vulgatus* and increased *Dorea longicatena* populations (Nishida et al., 2017a), whereas live *L. gasseri* decreased growth of inflammatory Enterobacteriaceae and *Veillonella* (Sawada et al., 2017). Both studies also showed that *L. gasseri* enhanced sleep quality of participants. Another study reported that heat-killed *L. gasseri* (in milk) increased the population of *Clostridium* cluster IV group and SCFAs levels in individuals with altered bowel movements (Sawada et al., 2016). Using a similar methodology, decreased *Clostridium* cluster IV and increased *Bifidobacterium* populations were found in another group of participants (Sugawara et al., 2016). Taken together, these results suggest that heat-killed *L. gasseri* does not have a specific microbial target, but rather modifies the preexisting gut microbiota that is unique to each individual. Nevertheless, these changes in the gut microbiota composition favor an anti-inflammatory state (Sawada et al., 2016; Sugawara et al., 2016; Nishida et al., 2017a). *L. gasseri* likely alters the gut microbiota profile through its unique, heat-resistant gassericins A and T with potent antibacterial properties against enteric pathogens (Pandey et al., 2013).

Heat-killed *L. gasseri* decreased expression of leukocytic stress-responsive microRNAs and salivary cortisol levels in stressed individuals (Nishida et al., 2017b). *L. gasseri* intake also prevented downregulation of EIF2-related genes in IBS patients (Nobutani et al., 2017). These studies suggest that *L. gasseri* confers protection against detrimental effects of stress. Moreover, heat-killed *L. gasseri* intake promoted parasympathetic nerve activity while improving sleep quality of stressed individuals (Nishida et al., 2017b). In healthy individuals, administration of either live or heat-killed *L. gasseri* increased their parasympathetic activity (Otomi et al., 2015; Sugawara et al., 2016). Therefore, *L. gasseri* may modify the gut microbiota profile in such a way that lowers gut inflammation and stress response, which may consequently promote parasympathetic activity to facilitate sleep (**Figure 2C**).

CHALLENGES AND PERSPECTIVES FOR PROBIOTICS AS TREATMENT FOR DEPRESSION

The existence of different gut microbiota compositions, depression subtypes and probiotic formulations complicate treatment outcomes and necessitate an individualized approach when using probiotics to treat depression. Despite these challenges, probiotics confer some benefits over antidepressant drugs, and there are more promising candidate probiotics that can potentially treat depression.

Heterogeneity of Gut Microbiota Composition

Several factors are known to influence the gut microbiota composition, such as diet, medications, genetics, age, geographical location and smoking (Thursby and Juge, 2017). Recently, approximately 1000 gut-derived putative bacterial species that do not belong to any existing genus were discovered in humans (Almeida et al., 2019). Such tremendous diversity complicates the understanding of how introduced probiotics affect the overall gut microbiota. One study showed that tolerability of individuals' gut microbiota toward the colonization of probiotics ranges from permissive to resistant (Zmora et al., 2018). This appears to depend on the baseline abundance of probiotic species in the host gut microbiota. For instance, those who were permissive toward the colonization of *Lactobacillus* had prior low levels of *Lactobacillus* populations before treatment (Zmora et al., 2018). Similarly, *B. longum* colonized the gut for a longer period in 30% of users who initially had low levels of *B. longum* (Maldonado-Gomez et al., 2016). Another study showed that the antidepressive effect of multi-species probiotics (MSP) only manifests when the administered MSP successfully colonized the gut of rats (Abildgaard et al., 2019). This is consistent with the observation that lower levels of two main probiotic genera, *Lactobacillus* and *Bifidobacterium*, are commonly found in individuals with MDD (Aizawa et al., 2016).

Despite most studies supported the effectiveness of probiotic supplements in reducing depression, not all randomized controlled trials reported the same outcome (**Table 2**). For instance, *L. rhamnosus* did not affect scores of anxiety, depressions, sleep, cognition, inflammatory and stress responses among healthy adults (Kelly et al., 2017). *L. rhamnosus* also did not affect perceptions of wellbeing, anxiety and stress among healthy older adults (Ostlund-Lagerstrom et al., 2016). In healthy individuals, *L. helveticus* exhibited no antidepressive effect (Chung et al., 2014; Ohsawa et al., 2018). These results imply that probiotics are less efficacious among the healthy population, which agree with a meta-analysis that reported an insignificant effect of probiotics on mood, particularly in healthy individuals (Ng et al., 2018). Therefore, probiotics could be generally more effective in colonizing gut microbiota of depressed individuals that are different from healthy people (Jiang et al., 2015; Zheng et al., 2016). In some cases, probiotic colonization may be optional for their effects to manifest. For instance, heat-killed *L. paracasei* benefited the human and animal host, in terms of neurochemical and behavioral changes (Corpuz et al., 2018; Murata et al., 2018; Wei et al., 2019). Some probiotics, such as *L. reuteri*, *L. paracasei*, *L. plantarum*, *L. gasseri*, *L. kefiranofaciens*, *B. breve,* and *B. infantis*, promoted the colonization of other beneficial microbes that contributed to the reduction of depressive-like symptoms in animals (Marin et al., 2017; Dhaliwal et al., 2018; Jang et al., 2019; Sun et al., 2019; Tian et al., 2019; Wei et al., 2019).

Heterogeneity of Depression

Major depressive disorder is characterized by depressed mood and/or anhedonia, in addition to excessive guilt, suicidal ideation,

TABLE 2 | Selected preclinical and clinical studies on the behavioral and physiological effects of single-species probiotics.

Probiotic species	Model	Behavioral changes	Physiological changes	References
Lactobacillus rhamnosus	Normal, healthy BALB/c male mice	↓ Anxiety ↓ Depression ↑ Memory No effect on locomotion	↓ Stress-induced ↓ in plasma CORT levels ↓ $GABA_{Aa2}$ mRNA expression in the PFC and amygdala ↓ $GABA_{B1b}$ mRNA expression in the HPC, amygdala and locus coeruleus ↑ $GABA_{Aa2}$ mRNA expression in the HPC ↑ $GABA_{B1b}$ mRNA expression in cortical regions (cingulate and prelimbic)	Bravo et al. (2011)
	BALB/c male mice subjected to MS	↓ Depression	↓ Stress-induced ↑ in plasma CORT levels ↑ Recovery toward basal corticosterone levels	McVey Neufeld et al. (2018)
	Healthy human males (aged 22–33, mean ≈ 23–25 years)	No effect on mood and anxiety	No changes in cortisol response to stress, plasma levels of IL10, IL1β, IL6, IL8 and TNFα, and whole blood levels of TLR-4	Kelly et al. (2017)
	Pregnant women (14–16 weeks gestation)	↓ Anxiety ↓ Depression	N/A	Slykerman et al. (2017)
	(With prebiotics) Obese individuals (aged 18–55, mean ≈ 35–58 years)	↓ Depression ↓ Food cravings ↑ Satiety	N/A	Sanchez et al. (2017)
Lactobacillus casei strain Shirota	Healthy middle-age human adults (aged 48–79, mean ≈ 62 years)	↓ Depression in those with low mood	N/A	Benton et al. (2007)
	Individuals with chronic fatigue syndrome (aged 18–65 years)	↓ Anxiety No effect on depression	↑ Fecal *Lactobacillus* and *Bifidobacteria* populations	Rao et al. (2009)
	Healthy students under stressful examination (aged < 40, mean ≈ 23 years)	↓ Stressful feelings No effect on anxiety	↓ Salivary cortisol levels ↓ Gastrointestinal symptoms ↓ Fecal *Bacteroidaceae* populations ↑ Diversity of the gut microbiota Prevented changes in expression of approx. 100 stress-responsive genes	Kato-Kataoka et al. (2016)
Lactobacillus brevis	Sprague–Dawley male depressed rats	↓ Depression	N/A	Ko et al. (2013)
	ICR male mice	↑ Sleep duration	N/A	Han et al. (2017)
	C3H-HeN male mice	↑ Sleep duration ↑ Wheel-running	N/A	Miyazaki et al. (2014)
Lactobacillus reuteri	C57BL/6J, C57BL/6N, and BALB/cJ male mice subjected to CUMS	↓ Depression	↓ Stress-induced ↑ in intestinal IDO1 expression ↓ Stress-induced ↑ in KYN levels ↑ Stress-induced ↓ in fecal H_2O_2 levels ↑ Stress-induced ↓ in *Lactobacillus* populations	Marin et al. (2017)
	C57BL/6 male mice subjected to immobilization stress	↓ Anxiety ↓ Depression	↓ Stress-induced ↑ in activated microglia infiltration into the HPC ↓ Stress-induced ↑ in colon shortening, myeloperoxidase activity and IL-6 expression in the colon ↓ Stress-induced ↑ in blood CORT, IL-6, and LPS levels ↓ Stress-induced colitis ↓ Stress-induced ↑ in Proteobacteria populations ↑ Stress-induced ↓ in HPC BDNF expression ↑ Stress-induced ↓ in Bacteroidetes, Firmicutes, and Actinobacteria populations	Jang et al. (2019)
Lactobacillus plantarum	MS vs. naïve male C57BL/6J mice	↑ Locomotion In naïve mice: ↓ Anxiety In MS mice: ↓ Depression ↓ Anhedonia	↓ Stress-induced ↑ in CORT in MS mice ↑ DA, DOPAC, and HVA in the PFC of MS and naïve mice ↓ 5-HIAA and no change in 5-HT levels in the PFC of MS mice ↑ 5-HT levels in the PFC of naïve mice ↓ 5-HIAA levels in the PFC of naïve mice ↑ IL-10, ↓ IL-6 and no effect on TNF-α levels in the serum of MS mice	Liu Y.W. et al. (2016)

(Continued)

TABLE 2 | Continued

Probiotic species	Model	Behavioral changes	Physiological changes	References
	Germ-free C57BL/6JN male mice	↓ Anxiety ↑ Locomotion No effect on depression	↑ 5-HT and DA levels in the striatum, but not the PFC or HPC No effects on serum GR levels	Liu W.H. et al. (2016)
	Swiss albino male mice subjected to CUMS or sleep-deprivation stress	↓ Anxiety ↓ Depression ↑ Memory ↑ Learning ↑ Locomotion	↓ Stress-induced ↑ in malonaldehyde, MAOs and nitrate levels in the brain ↓ Stress-induced ↑ in serum levels of TNF-α, CORT, and LPS ↑ Stress-induced ↓ in glutathione and HPC BDNF levels ↑ Abundance of *Lactobacillus* ↓ Stress-induced ↓ abundance of *Bacteroidetes* and *Roseburia* ↑ Fecal acetic and butyric acid levels Prevented stress-induced ↑ in permeability of BBB and intestinal barrier, and *Enterobacteriaceae* levels	Dhaliwal et al. (2018)
	MDD patients undergoing SSRI medications (mean age ≈ 39 years)	↑ Memory ↑ Attention ↑ Learning No effect on depression and stress	↓ Plasma KYN levels ↑ 3-hydroxykynurenine/KYN ratio No changes in plasma levels of TNF-α, IL-6, IL-1β, and cortisol	Rudzki et al. (2019)
	Stressed human adults with mild levels of depression (aged 18–60, mean ≈ 31 years)	↓ Anxiety ↓ Stress ↑ Memory ↑ Learning ↓ Depression (not stat. sig)	↓ Plasma IFN-γ and TNF-α levels ↓ Plasma IL-1β and cortisol levels (not stat. sig.)	Lew et al. (2018)
Faecalibacterium prausnitzii	Sprague–Dawley male rats subjected to CUMS	↓ Anxiety ↓ Depression	↓ Stress-induced ↓ in plasma levels of CORT, CRP, and IL-6 ↑ SCFAs levels in the cecum ↑ Stress-induced ↓ in plasma IL-10 levels	Hao et al. (2019)
Lactobacillus helveticus	Sprague–Dawley male rats subjected to chronic restraint stress	↓ Anhedonia ↓ Anxiety ↑ Locomotion ↑ Memory	↓ Stress-induced ↑ in CORT and adrenocorticotropic hormone levels ↓ Stress-induced ↓ in plasma IL-10 levels ↑ Stress-induced ↓ in HPC BDNF expression ↑ Stress-induced ↓ in 5-HT and NE levels in the HPC No changes in stress-induced ↓ in plasma IFN-γ and TNF-α levels	Liang et al. (2015)
	Sprague–Dawley male rats with hyperammonemia-induced neuroinflammation	↓ Anxiety ↑ Memory ↑ Learning	↓ Stress-induced ↑ in KA/KYN ratio ↓ Stress-induced ↑ in PGE2 levels in the cerebellum and HPC ↓ Stress-induced ↑ in IL-1β levels in the cerebellum, HPC, and PFC ↓ 5-HT levels in the cerebellum and HPC ↑ Stress-induced ↓ in KYN/TRP ratio No effect in stress-induced ↑ in 5-HIAA levels in the HPC, cerebellum, and PFC	Luo et al. (2014)
	C57BL/6J male mice subjected to sub-chronic social defeat stress	↓ Anhedonia ↓ Anxiety	↑ Stress-induced ↓ in dopamine D3 and serotonin 1A receptors expression Restore stress-induced changes in gene expression in the nucleus accumbens No effects on serum CORT levels and gut microbiota composition	Maehata et al. (2019)
	Healthy elderly humans (aged 60–75, mean ≈ 65 years)	↑ Memory ↑ Attention ↑ Learning No effects on stress levels and depression	No effects on plasma levels of BDNF and whole blood viscosity	Chung et al. (2014)
	Healthy middle-aged humans (aged 50–70, mean ≈ 58 years)	↑ Memory ↑ Attention No effects on depression	N/A	Ohsawa et al. (2018)

(Continued)

TABLE 2 | Continued

Probiotic species	Model	Behavioral changes	Physiological changes	References
Lactobacillus paracasei	CORT-induced depressed male C57BL/6J mice (live or heat-killed *L. paracasei)*	↓ Depression ↓ Anhedonia ↓ Anxiety	↑ Stress-induced ↓ abundance of *Bifidobacterium* (live) ↑ Stress-induced ↓ in 5-HT levels in the HPC, PFC, and striatum (live) ↑ Stress-induced ↓ DA levels in the HPC and PFC (heat-killed) ↑ Stress-induced ↓ in BDNF levels and MR and GR receptors expression in the HPC No effect on serum CORT levels, both basal and in response to stress	Wei et al. (2019)
	Senescence-accelerated female SAMP8 mice (heat-killed *L. paracasei*)	Prevented age-related cognitive decline	↓ 5-HT-degrading enzymes, particularly MAOA, levels in the HPC ↑ 5-HT levels in brain tissues and serum ↓ BDNF expression and CREB phosphorylation in the HPC No effect on the gene expression of 5-HT-synthesis-related enzyme	Corpuz et al. (2018)
	Senescence-accelerated male and female SAMP8 mice (live *L. paracasei*)	Prevented age-related cognitive decline and anxiety	↓ Serum TNF-α and MCP-1 levels ↑ Levels of DA, DC, 5-HT and 5-HIAA levels in the striatum and HPC ↑ Levels of serum BDNF, IL-10, SOD, and GPx	Huang et al. (2018)
	Healthy females under examination stress (heat-killed *L. paracasei*) (aged > 18, mean ≈ 21 years)	Prevented decline in mood and immunity	↓ Frequency of common cold in those susceptible No effect on salivary secretory IgA concentrations	Murata et al. (2018)
Bifidobacterium infantis	Naïve Sprague–Dawley male rats	No effect on depression	↓ Plasma IFN-γ, TNF-α, IL-10, and IL-6 levels ↓ 5-HIAA levels in the frontal cortex ↓ DOPAC levels in the amygdaloid cortex ↓ NE levels in the HPC (not stat. sig.) ↑ Plasma TRP and KYN levels No effects in baseline CORT levels	Desbonnet et al. (2008)
	Sprague Dawley male rats subjected to MS	↓ Depression	↓ Stress-induced ↑ in plasma IL-6 and corticotrophin-releasing factor mRNA expression in the amygdala ↑ Stress-induced ↓ in NE levels in the brainstem No effects on plasma TRP/KYN ratio and baseline CORT concentrations	Desbonnet et al. (2010)
	Male adult C57BL/6J mice subjected to CUMS	↓ Depression ↓ Anhedonia ↓ Anxiety	↓ Stress-induced ↑ Veillonellaceae and *Desulfovibrio* populations ↑ 5-HT and 5-HTP levels in the HPC ↑ Expression of Tph1 mRNA in RIN14B cells (*in vitro*) ↑ BDNF levels in the PFC ↑ Stress-induced ↓ in cecum butyrate levels ↑ Alpha diversity of gut microbiota ↑ Glutamatergic synapse ↑ Phenylalanine/tyrosine/TRP biosynthesis No effect on spleen regulatory T cells	Tian et al. (2019)
Clostridium butyricum	C57BL/6 male mice subjected to CUMS	↓ Depression No effect on locomotion	↑ Stress-induced ↓ in brain levels of 5-HT and BDNF ↑ Stress-induced ↓ in intestinal GLP-1 secretion and cerebral expression of GLP-1 receptor	Sun et al. (2018)
	(With SSRIs or SNRIs) Treatment-resistant MDD patients (mean age ≈ 42–44 years)	↓ Depression	N/A	Miyaoka et al. (2018)
Lactobacillus kefiranofaciens	Kunming male mice subjected to CUMS	↓ Depression ↓ Anhedonia	↓ Stress-induced ↑ in serum CORT levels and KYN/TRP ratio ↓ IL-6 and IFN-γ levels in the spleen ↓ Abundance of Proteobacteria ↑ Stress-induced ↓ serum TRP levels ↑ IL-10 levels in the spleen ↑ Abundance of anti-inflammatory Actinobacteria, Bacteroidetes, Lachnospiraceae, Coriobacteriaceae, Bifidobacteriaceae, and Akkermansia	Sun et al. (2019)

(Continued)

TABLE 2 | Continued

Probiotic species	Model	Behavioral changes	Physiological changes	References
Bifidobacterium breve	Innately anxious BALB/c male mice	↓ Depression ↓ Anxiety No effect on locomotion	No effect on CORT levels, both baseline and in response to stress	Savignac et al. (2014)
	Male adult C57BL/6J mice subjected to CUMS	↓ Depression ↓ Anhedonia ↓ Anxiety	↓ Chronic stress-induced CORT release ↓ Stress-induced ↑ Veillonellaceae populations ↑ Expression of Tph1 mRNA in RIN14B cells (*in vitro*) ↑ BDNF levels in the PFC ↑ Stress-induced ↓ in alpha diversity of the gut microbiota ↑ Glutamatergic synapse ↑ Phenylalanine/tyrosine/TRP biosynthesis No effect on spleen regulatory T cells	Tian et al. (2019)
	Schizophrenic individuals with anxiety and depression (aged > 20, mean ≈ 41–46)	↓ Depression ↓ Anxiety	↑ Relative abundance of Parabacteroides ↑ Serum IL-22 and TRANCE expression No effects on *Bifidobacterium* populations and serum levels of IL-6 and TNF-α	Okubo et al. (2019)
	Elderly humans with mild cognitive impairment (mean age ≈ 83 years)	↑ Mood ↑ Memory ↑ Attention ↑ Learning	N/A	Kobayashi et al. (2019)
Bifidobacterium longum	Innately anxious BALB/c male mice	↓ Depression ↓ Anxiety No effect on locomotion	No effect on CORT levels, both baseline and in response to stress	Savignac et al. (2014)
	Healthy human males (aged 18–40, mean ≈ 25 years).	↓ Stress ↓ Anxiety ↓ Memory ↓ Attention ↑ Learning	↓ Salivary cortisol output and anxiety scores in response to stressor ↑ Neural activity of the PFC	Allen et al. (2016)
	IBS patients with mild to moderate depression and/or anxiety (median age = 40 and 46.5 years)	↓ Depression ↑ Life quality No effect on anxiety	↓ Responses to negative emotional stimuli in the amygdala and fronto–limbic regions ↓ Urine levels of methylamines and aromatic amino acids metabolites No effect on fecal microbiota profiles, serum inflammatory markers (CRP, TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL12), BDNF, substance P and 5-HT levels	Pinto-Sanchez et al. (2017)
Lactobacillus gasseri	University male students with daily strenuous exercise (aged < 30, mean ≈ 20 years)	↑ Mood in depressed individuals	Prevent stress-induced ↓ in natural killer cell activity	Sashihara et al. (2013)
	Medical (cadaver dissection course) male students (aged 24)	↓ Depression ↓ Anxiety ↑ Sleep quality	↓ Salivary cortisol release ↓ Growth of inflammatory Enterobacteriaceae and *Veillonella* Prevented the downregulation of EIF2-related genes of peripheral leukocytes	Sawada et al. (2017)
	Medical (cadaver dissection course) students (heat killed *L. gasseri*) (aged 18–34, mean ≈ 21 years)	In men: ↓ Sleep latency ↑ Sleep duration In women: ↓ Somatic symptoms	↓ Diarrhoea-like symptoms (in men) ↑ Fecal *Bacteroides vulgatus* levels ↓ Fecal *Dorea longicatena* levels No effect on salivary stress markers (cortisol, CgA, and alpha amylase levels)	Nishida et al. (2017a)
	Medical students in pre-examination (heat-killed *L. gasseri*) (mean age ≈ 25 years)	↓ Sleep latency ↓ Sleep awakenings	↑ Ratio of parasympathetic/sympathetic nerve activity ↑ Stage N3 in the non-REM sleep period ↓ Stress-induced ↑ in salivary cortisol levels ↓ Stress-induced ↑ expression of stress-responsive microRNAs	Nishida et al. (2017b)

changes in appetite and sleep, psychomotor retardation, poor concentration and fatigue (American Psychiatric Association, 2013). From these diagnostic criteria, approximately a thousand combinations of symptoms (Ostergaard et al., 2011) and 19 depression subtypes (Harald and Gordon, 2012; Sharpley and Bitsika, 2014) can be derived. These subtypes of depression are often grouped as a single term, namely depression, which should not be the case when evaluating therapeutic potential of probiotics.

Some associations can be drawn by matching behavioral benefits of probiotics to the characteristics of depression subtypes (**Table 2**). For instance, the sucrose preference test in rodents reflects the anhedonia subtype (Dedic et al., 2011). Probiotics that have been shown to improve the outcome of this test include *L. helveticus* (Liang et al., 2015), *L. plantarum* (Liu Y.W. et al., 2016), *L. paracasei* (Wei et al., 2019), *L. kefiranofaciens* (Sun et al., 2019), *B. infantis* (Tian et al., 2019), and *B. breve* (Tian et al., 2019). Among these probiotics, *L. plantarum* (Liu Y.W. et al., 2016) and *L. paracasei* (Wei et al., 2019) also modulated the central DA system, whereas *B. infantis* and *B. breve* upregulated tyrosine (precursor to DA) biosynthesis (Tian et al., 2019). An impaired DA system represents the hallmark pathophysiology of anhedonia (Dunlop and Nemeroff, 2007). This provides a proof of concept that these probiotics may be effective in treating anhedonia.

Somatic depression subtype is characterized by psychomotor agitation/retardation (i.e., locomotion), changes in weight/appetite, insomnia/hypersomnia and fatigue without physical exertion (Sharpley and Bitsika, 2014). Probiotics that improved locomotor activity of rodents include *L. plantarum* (Liu W.H. et al., 2016; Dhaliwal et al., 2018), *L. helveticus* (Liang et al., 2015) and *L. brevis* (Miyazaki et al., 2014). Intake of *L. brevis* increased sleep duration in healthy mice (Miyazaki et al., 2014; Han et al., 2017), and *L. gasseri* enhanced sleep quality in medical students with mild depression (Nishida et al., 2017a,b). *L. rhamnosus* supplementation modulated appetite-associated genes and attenuated appetite in zebrafish (Falcinelli et al., 2016, 2017). In combination with prebiotics, *L. rhamnosus* exerted antidepressive effect and appetite control in obese individuals (Sanchez et al., 2017). Hence, symptoms of somatic depression are rather distinct and may be improved differently with different probiotics.

Cognitive depression subtype is distinguished by poor concentration and memory function as well as indecisiveness (Sharpley and Bitsika, 2014). Behavioral assessments for memory function in mice include the Morris water maze, Barnes maze and other behavioral tests (Dedic et al., 2011). Administration of probiotics including *L. helveticus* (Ohland et al., 2013; Luo et al., 2014; Liang et al., 2015), *L. plantarum* (Dhaliwal et al., 2018), and *L. paracasei* (Corpuz et al., 2018; Huang et al., 2018) enabled animals to perform these memory test more effectively. Attention, memory and learning behaviors in humans are assessed by cognitive tests, such as the Stroop, verbal-learning and digit-symbol tests. Improvements in these tests have been shown with the intake of (1) *L. helveticus* (Chung et al., 2014; Ohsawa et al., 2018) and *B. longum* (Allen et al., 2016) in healthy adults; (2) *L. plantarum* in MDD patients (Rudzki et al., 2019) and stressed adults with mild depression (Lew et al., 2018); and (3) *B. breve* in elderly with mild cognitive impairment (Kobayashi et al., 2019). Thus, some probiotics appear to improve cognition regardless of depression.

Anxious depression subtype refers to major depression that comorbid with high levels of anxiety (Harald and Gordon, 2012). In mice, anxiety can be measured by behavioral tests, such as the elevated plus maze and open field tests (Dedic et al., 2011). In humans, anxiety is generally assessed with questionnaires. Probiotics that exhibit anxiolytic effect include *L. rhamnosus* (Bravo et al., 2011; Bharwani et al., 2017; McVey Neufeld et al., 2017; Slykerman et al., 2017), *L. helveticus* (Ohland et al., 2013; Luo et al., 2014; Liang et al., 2015), *L. plantarum* (Liu W.H. et al., 2016; Liu Y.W. et al., 2016; Dhaliwal et al., 2018; Lew et al., 2018), *B. longum* (Bercik et al., 2010, 2011; Savignac et al., 2014; Allen et al., 2016) and *B. breve* (Savignac et al., 2014; Okubo et al., 2019; Tian et al., 2019). Moreover, MSPs intake often decreased depression and anxiety simultaneously in randomized controlled trials (Mohammadi et al., 2016; Kouchaki et al., 2017; Jamilian et al., 2018; Raygan et al., 2018; Ostadmohammadi et al., 2019; Salami et al., 2019).

Conventional SSRIs that target the 5-HT system often fail to treat anhedonic patients and, in some cases, worsen their symptoms (Dunlop and Nemeroff, 2007). Antidepressant drugs (e.g., SSRI and SNRI) are also ineffective against other depression subtypes, namely the somatic (Tylee and Gandhi, 2005), cognitive (Shilyansky et al., 2016) and anxious depression (Ionescu et al., 2014). Therefore, certain probiotics may serve as an adjuvant or alternative treatment for MDD and its subtypes. A pilot study showed that MSP, together with a magnesium supplement, decreased depression in SSRI treatment-resistant patients (Bambling et al., 2017). A clinical trial also reported that the combination of *B. longum* and *L. helveticus* decreased depression in MDD patients with prior use of standard antidepressants (Kazemi et al., 2019).

Single-Species and Multi-Species Probiotic

In studies that investigated behavioral effects of probiotics, about 60% of animal studies and 50% of human studies used single-species probiotics (SSPs) (Joseph and Law, 2019). Studies with SSPs promote a better understanding of the function and contribution of individual probiotic, which is difficult to measure in MSPs. However, MSPs may have higher potency in humans. In MDD patients, SSP (*L. plantarum*) did not reduce depression but improved cognition (Lew et al., 2018), whereas MSPs had repeatedly shown antidepressive efficacy (Akkasheh et al., 2016; Bambling et al., 2017; Ghorbani et al., 2018; Kazemi et al., 2019). MSPs often gave better therapeutic efficacy compared to that of SSPs in gut-related disorders and pathogen infections, which could be explained by an overall higher dosage (Chapman et al., 2011, 2012). Indeed, MSPs with a higher dosage improved symptoms of depression and anxiety in healthy individuals compared to that of a lower dosage (Tran et al., 2019). MSPs are also hypothesized to exhibit synergistic effects that would have an

expanded effect on the host physiology (Chapman et al., 2012). In contrast, SSPs are speculated to promote better colonization as it does not have to compete for nutrient or adhesion sites in the host (Chapman et al., 2011). This highlights the need for more studies to understand how probiotics in MSPs interact with each other and with existing gut microbiota, and which probiotic(s) is suitable in formulation of MSPs for antidepressive efficacy.

Advantages of Probiotics as Antidepressive Treatment

Probiotics are generally safe for consumption, except for immune-compromised and critically sick individuals wherein probiotics may cause sepsis, pneumonia, endocarditis and allergies (Didari et al., 2014). Still, it has been viewed by some that more human trials are required to establish the dosage efficacy and long-term safety profile of probiotics (Kothari et al., 2018). For antidepressant drugs such as SSRIs, side effects occur in 40-60% of users which include sexual dysfunction, suicidality, emotional numbness and addiction (Read and Williams, 2018). A meta-analysis data showed that users of antidepressant drugs were associated with a 33% increased risk of mortality (Maslej et al., 2017). On the other hand, probiotics possess fewer side effects than antidepressant drugs. For instance, rats fed with *L. brevis*-fermented milk exhibited comparable antidepressive efficacy to fluoxetine-treated rats, but without side effects of fluoxetine (decreased appetite and weight loss) (Ko et al., 2013).

Antidepressant usage is also associated with stigma, such as being perceived as emotionally weak and dependent on drugs, which contributes to the disease severity and poor adherence to treatment (Castaldelli-Maia et al., 2011). In a survey study, 77% of depressed patients prefer to hide their use of antidepressant medication from others (Martinez et al., 2018). However, the prevalence of perceived stigma against antidepressants differs based on the population studied (Castaldelli-Maia et al., 2011). To this end, probiotics may help as an alternative treatment for depression, given that probiotics have not been associated with any perceived social stigma (Wallace and Milev, 2017).

Candidate Probiotics With Potential Antidepressive Effect

Bifidobacterium pseudocatenulatum is known for its regulation of obesity-related changes in metabolism and the immune system (Cano et al., 2013; Moya-Perez et al., 2014, 2015; Sanchis-Chorda et al., 2018). *B. pseudocatenulatum* intake reversed diet-induced obesity, depression, high corticosterone and low hippocampal 5-HT levels in mice (Agusti et al., 2018). However, a high-fat diet model is meant to study the pathophysiology of obesity and type 2 diabetes (Winzell and Ahren, 2004; Wang and Liao, 2012). It is, thus, unclear if *B. pseudocatenulatum* would decrease depression in mice without obesity. Another study showed that anxiety-like behaviors diminished in chronic-stressed mice fed with *B. pseudocatenulatum*, but depressive-like behaviors were unevaluated (Moya-Perez et al., 2017). Therefore, further studies are required to determine whether *B. pseudocatenulatum* has an independent antidepressive effect.

Bacillus coagulans supplementation relieved symptoms of both IBS and depression in patients diagnosed with IBS and MDD. This clinical recovery is accompanied by a decrease in serum myeloperoxidase, an inflammatory marker (Majeed et al., 2018). However, patients might have experienced less depression as a result of reduced IBS symptoms. Interestingly, *B. coagulans* intake increased levels of circulating IL-10, fecal *F. prausnitzii* and SCFAs in older adults (Nyangale et al., 2014, 2015). As *F. prausnitzii* and butyrate are associated with antidepressive properties (Hao et al., 2019), *B. coagulans* may also indirectly reduce depression and improve gut health.

Bifidobacterium bifidum and *L. acidophilus* were often included in the formulation of MSPs to treat depressive symptoms in patients with MDD (Akkasheh et al., 2016; Bambling et al., 2017; Ghorbani et al., 2018) and other health conditions, such as polycystic ovarian syndrome, multiple sclerosis and IBS (Kouchaki et al., 2017; Ostadmohammadi et al., 2019; Zhang et al., 2019). Surprisingly, *B. bifidum* and *L. acidophilus* have not been tested independently for its antidepressive effect. *B. bifidum* intake improved mood and reduced symptoms of abdominal pain, diarrhea and constipation in patients with gastrointestinal disorders (Urita et al., 2015). However, the mood elevation could be due to recovery of gastrointestinal symptoms rather than effect of probiotics solely. Both *in vitro* and *in vivo* models showed that *L. acidophilus* protects the intestinal barrier integrity by preventing pathogen adherence and release of proinflammatory cytokines (Chen et al., 2009; Justino et al., 2015; Alamdary et al., 2018; Lepine et al., 2018; Najarian et al., 2019). Taken together, *B. bifidum* and *L. acidophilus* potentially exhibit antidepressive effect and their direct influence on depression warrants further investigation.

Bacteroides fragilis has been proposed as a potential probiotic, although its pathogenicity needs to be taken into consideration. *B. fragilis* secretes polysaccharide A and expresses sphingolipids that benefit the host gut health and immune system (Troy and Kasper, 2010; Tan et al., 2019). *Bacteroides* genus is likely to be the largest GABA producer amongst human gut microbiota, with *B. fragilis* produces GABA at low pH. The same study also found that neural patterns of a typical MDD patient correlated with low fecal levels of *Bacteroides* (Strandwitz et al., 2019). Hence, antidepressive potential of *B. pseudocatenulatum*, *B. coagulans*, *B. bifidum*, *L. acidophilus,* and *B. fragilis* warrants further investigation. It is also worth noting that *Bifidobacterium adolescentis*'s antidepressive capability may be a new probiotic candidate (Jang et al., 2019). Evidently, an increasing number of probiotics are being presented as a potential treatment for depression. This provides a wide repository of available probiotics, with different species combinations, that can be assessed for clinical efficacy against depression.

CONCLUSION

The MGB axis enables the bidirectional communication between the gut microbiota and the brain. When this axis becomes

maladaptive, the host physiology is adversely affected which may lead to the development of depression. Probiotics have shown clinical efficacy in the treatment of depression by modulating the MGB axis. Yet, the complexity of gut microbiota and heterogeneity of depression presents a challenge to explain the underlying mechanisms that contribute to this clinical efficacy. Nonetheless, cumulative evidence suggests the therapeutic potential of probiotics for certain depression subtypes, with fewer side effects and less stigma compared to standard antidepressants.

Limitations of this review include: (1) inferences of probiotic mechanisms were derived from preclinical and *in vitro* data; (2) interactions of probiotics with other members of gut microbiota were unexplored, therefore the mechanisms of MSPs was unable to be explored; (3) strain-specific effects of bacterial species were neglected; (4) potential applications for probiotics for depression subtypes are hypothesized, however, clinical evidence is limited; (5) effect sizes of probiotics as antidepressants was not evaluated. Notwithstanding these caveats, this review adds further understanding to the potential antidepressive effects and therapeutic potentials of probiotics. Venema (2017) stated that it is imperative to grasp the underlying molecular mechanisms of the MGB axis, and which microbial populations are pertinent for this intervention, to advance the marketability of probiotics.

AUTHOR CONTRIBUTIONS

SY wrote and edited the manuscript. TT, JC, and WL conceptualized and edited the manuscript. All authors contributed to the intellectual input and critical revision of the manuscript.

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Structural and Functional Characterization of the Gut Microbiota in Elderly Women With Migraine

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Migraine is a very common, multifactorial, and recurrent central nervous system disorder that causes throbbing headache, photophobia, phonophobia, nausea, and disability. Migraine occurs more often in females, and its complex physiopathology is not yet fully understood. An increasing number of gastrointestinal disorders have been linked to the occurrence of migraine suggesting that gut microbiota might play a pivotal role in migraine through the gut–brain axis. In the present work, we performed a metagenome-wide association study (MWAS) to determine the relationship between gut microbiota and migraine by analyzing 108 shotgun-sequenced fecal samples obtained from elderly women who suffer from migraine and matched healthy controls. Notably, the alpha diversity was significantly decreased in the migraine group at species, genus, and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologous levels. Firmicutes, especially the "unfriendly" *Clostridium* spp., were significantly enriched in the migraine group. Conversely, the healthy controls held more beneficial microorganisms, such as *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, and *Methanobrevibacter smithii*. For functional modules, the migraine group was enriched in gut–brain modules (GBMs) including kynurenine degradation and γ-aminobutyric acid (GABA) synthesis. However, the healthy controls held higher gut metabolic modules (GMMs) including glycolysis, homoacetogenesis, and GBMs including quinolinic acid degradation and *S*-adenosyl methionine (SAM) synthesis. The differences in gut microbiota composition and function between the migraine and healthy groups provided new information as well as novel therapeutic targets and strategies for migraine treatment, which could help to improve the early diagnosis of the disease, as well as the long-term prognosis and the life quality of patients suffering from migraine.

Keywords: migraine, gut microbiota, elderly women, metagenome-wide association study, structural characterization, functional modules

INTRODUCTION

Migraine can cause a severe unilateral throbbing headache or a pulsing sensation, which are typically accompanied by nausea, vomiting, and extreme sensitivity to light and sound, thereby adversely affecting daily activities (MacGregor, 2017). Constipation and mood changes also frequently occur together with migraine (Vacca, 2019). Migraines usually begin in adolescence or early adulthood, it is three times more common in women (17%) than in men (5–8%), and it accounts for over 90% of patients with recurrent headache (MacGregor, 2017). Multiple factors, including inheritance (Ulrich et al., 1999), hormones (Sacco et al., 2012), dietary habits (Nazari and Eghbali, 2012), mental stress (Lipton et al., 2014), and gastrointestinal disorders (Camara-Lemarroy et al., 2016), have been reported as triggers of migraine. For the prognosis, some patients remit, some experience recurrent episodes, and in others the condition evolves into a chronic and more refractory state.

Human gut microbiome, which is considered as the second genome and brain of human body (Li et al., 2014), is thought to be closely related to migraine (Gonzalez et al., 2016). The recent gut–brain axis theory (Carabotti et al., 2015) has proposed a bidirectional communication between the central and enteric nervous systems, linking the emotional and cognitive centers of the brain with the peripheral intestinal functions. Studies have found that irritable bowel syndrome (IBS) occurs in over half of migraine patients (Lau et al., 2014), and inflammatory bowel disease (IBD) patients are 2.7 times more likely to have migraines (Dimitrova et al., 2013). The fact that both IBD and IBS are severe gut disorders associated with gut permeability and inflammation caused by gut microbes through the gut–brain axis (Aggarwal et al., 2012) suggests an important role of gut bacteria in migraine. In an uncontrolled observational study on 1,020 patients, researchers found that multispecies probiotic formulations can reduce the intensity and the frequency of migraine attacks (Straube et al., 2018). However, another randomized placebo-controlled study conducted on 63 patients showed that the use of multispecies probiotics, compared with placebo, does not significantly affect intestinal permeability or inflammation (de Roos et al., 2017). These paradoxical conclusions need further confirmation.

The diagnosis of a migraine is based on signs and symptoms (Bartleson and Cutrer, 2010), such as with or without aura, duration, unilateral, pulsating headache, inability to work, nausea and vomiting, photophobia, and phonophobia. Diagnostic uncertainty has been associated with diagnostic variation (physicians giving different diagnoses to the same patient), over-testing, suboptimal management, more hospitalizations and referrals, and increased health care expenditure. In contrast to traditional pharmacotherapy, which is marked by unavoidable and often dangerous side effects (e.g., analgesics might cause nervous disorders; Do et al., 2019), dietary intervention is thought to be a promising safe therapeutic strategy to prevent migraine through the regulation of gut microbes (Camara-Lemarroy et al., 2016). Both the ketogenic diet (Gross et al., 2019) and the traditional Chinese medicinal plant *Gastrodia elata*, which is also used as food in China (Hua et al., 2019), have been reported to regulate gut microbiota and to promote remission from migraine. The few publications available, which are based on probiotic treatment, however, are with different conclusions about the effects of probiotics on migraine in humans (de Roos et al., 2017; Straube et al., 2018).

In this work, we performed a metagenome-wide association study (MWAS) based on shotgun-sequenced fecal samples obtained from 108 elderly women consisting of 54 migraineurs and 54 healthy subjects in order to shed some light over the connections between gut microbiota composition and function and migraine. The study aimed at discovering differences in gut microbiota that could help to design strategies based on the modulation of the gut microecology that could improve the long-term prognosis of migraine and provide a guidance for early diagnosis and management of this recurrent disease.

MATERIALS AND METHODS

Statement of Human Rights

The data used in this study were obtained from a previous study (Xie et al., 2016) whose ethics statement was approved by the local ethics committee, and an informed consent was collected from each subject. All the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (BGI-Shenzhen, China, Ethics Approval No. BGI-IRB 14074).

Materials

The 108 stool samples, from 54 migraineurs and 54 matched 201 healthy controls, were shotgun sequenced during a former study (**Table S1**) (Xie et al., 2016). All the samples and clinical indexes were collected by Prof. Spector's group at King's College London. Subjects were excluded if they had a history of chronic serious infection, any current infection, and any type of malignant cancer; individuals who had received antibiotic treatment within 1 month before participating in this study were also excluded.

Quality Control and Host Genome Filtering

The raw reads that had 50% low-quality bases (quality $\leq$ 20) or more than five ambiguous bases were excluded. The remaining reads were mapped to the human genome (hg19) by SOAP v2.22 (-m 100 -x 600 -v 7 -p 6 -l 30 -r 1 -M 4 -c 0.95), and the matching reads were removed (Fang et al., 2018). The high-quality non-human reads were defined as clean reads.

Acquisition of Gene Abundance and Taxonomic Profiles From Metagenomic Samples

The clean reads were aligned against the latest 11.4 M human gut microbial gene catalog (Xie et al., 2016) through SOAP v2.22 (-m 100 -x 600 -v 7 -p 6 -l 30 -r 1 -M 4 -c 0.9) to generate the gene abundance profile. To obtain the taxonomic profiles, metaphlan2 (Truong et al., 2015) (–input_type fastq –ignore_viruses –nproc 6) was used to generate phyla, genera, and species profiles from the clean reads.

Calculation of Gut Microbiome Functional Profiles

Putative amino acid sequences were translated from the gene catalog (Xie et al., 2016) and aligned against the proteins or domains in the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (release 79.0, with animal and plant genes removed) using BLASTP (v2.26, default parameter, except -m 8 -e 1e-5 -F -a 6 -b 50). Each protein was assigned to a KEGG orthologous (KO) group on the basis of the highest scoring annotated hit(s) containing at least one segment pair scoring over 60 bits. The relative abundance profile of KOs was determined by summing the relative abundance of genes from each KO using the mapped reads per sample (Xie et al., 2016). The abundance of each gut metabolic module (GMM) (-a 2 -d GMM.v1.07.txt -s average) and gut neuroactive module (GBM) (default parameter) were calculated as shown in the former article (Vieira-Silva et al., 2016; Valles-Colomer et al., 2019).

Permutational Multivariate Analysis of Variance of the Effect of Related Factors on Gut Microbiome

To evaluate the effects of the clinical and lifestyle factors on the microbiome, we performed the permutational multivariate analysis of variance (PERMANOVA) of the gene abundance of the samples. The Bray–Curtis distance and 9,999 permutations in R (3.2.5, vegan package) were used.

Richness and Diversity Analysis

Alpha diversity (within samples) at species, genus, and KO levels of the two groups was quantified by the Shannon index on the basis of the relative gene abundance profile.

Differential Analysis of the Gut Microbiome Between the Two Groups

MWAS was used to investigate the differences in taxon composition between fecal microbiomes of healthy controls and migraineurs. To investigate the specific differences in the gut microbiome composition and function between the migraine group and the healthy controls, first, the top 15 species, the top 10 genera, and the top 5 phyla of each group were selected according to their average relative abundances, and each of the taxon was compared by Wilcoxon rank-sum test to compare their differences between two groups ($p < 0.05$). Second, the significantly different species, genera, phyla (**Table S4**), GMMs (**Table S5**), and GBMs (**Table S6**) between two groups were tested by Wilcoxon rank-sum test ($p < 0.05$). Third, the significantly changed species analyzed above were further analyzed by Spearman's rank correlation ($p < 0.05$) according to their relative abundances in all samples. Then the software Cytoscape 3.4.0 was used to visualize the co-occurrence network of these species.

RESULTS

Characterization of the Gut Microbiomes

To investigate the characteristics of gut microbiome in migraine patients, a metagenomic shotgun-sequencing study was performed on a total of 108 fecal samples from 54 individuals with migraine and 54 healthy controls with matched age and body mass index (BMI) (**Figure S1**). After removal of low-quality and human DNA reads, an average of 7.27 gigabase pairs per sample were aligned to a gut microbiome gene catalog comprising 11.4 million genes (Xie et al., 2016), achieving an average of 77.6 ± 1.6% matched reads per sample (**Table S2**).

PERMANOVA revealed significant differences in the gut microbiome of the two groups ($p = 0.0066$, $R^2 = 0.014$, **Table S1b**). The alpha diversity was evidently decreased in the migraine group at both genus ($p = 0.036$, Wilcoxon rank-sum test, **Figure 1A**) and species ($p = 0.048$, Wilcoxon rank-sum test, **Figure 1B**) levels, whereas the species richness was not significantly different in the two groups at either level (genus, $p = 0.64$; species, $p = 1.0$; Wilcoxon rank-sum test, **Figures S2A**,**B**). Similarly, the KO analysis showed an evident decrease of the alpha diversity in the migraine group ($p = 0.045$, Wilcoxon rank-sum test, **Figure S2C**), whereas the difference in species richness between the two groups was not significant ($p = 0.085$, Wilcoxon rank-sum test, **Figure S2D**). The data used in **Figure 1** and **Figure S1** are included in **Table S3**.

Differences of the Gut Microbiome Between Two Groups

To investigate the differences in highly abundant bacteria in the gut of the two groups, we chose the top 15 species, top 10 genera, and top 5 phyla. Interestingly, the migraine group showed significantly higher levels of the phylum Firmicutes ($p = 0.0023$, **Figure S3**) and a reduction in the level of the beneficial genus *Faecalibacterium* ($p = 0.0029$, **Figure 1C**) relative to the control group. At the species level (**Figure 1D**), *Faecalibacterium prausnitzii* ($p = 0.0029$), *Bifidobacterium adolescentis* ($p = 0.041$), and *Methanobrevibacter smithii* ($p = 0.012$) were significantly enriched in the healthy controls.

To further illustrate the differences between the two cohorts, 21 and 22 significantly enriched species (**Figure 2**, $p < 0.05$, false discovery rate [FDR] = 0.14, **Table S4**) were identified for the migraine group and the healthy controls, respectively. The species enriched in the migraine group were *Blautia hydrogenotrophica*, *Clostridium asparagiforme*, *Clostridium clostridioforme*, *Clostridium bolteae*, *Clostridium citroniae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium spiroforme*, *Clostridium symbiosum*, *Eggerthella lenta*, *Flavonifractor plautii*, *Lachnospiraceae bacterium*, and *Ruminococcus gnavus*. *B. hydrogenotrophica* metabolizes H_2/CO_2 to acetate and contributes to the breakdown of plant polysaccharides and proteins in the host (Bernalier et al., 1996). Except for *C. bolteae*, an autism-associated bacterium (Pequegnat et al., 2013), all the other *Clostridium* spp. enriched in the migraine group have been reported to be correlated with solid tissue inflammation (Elsayed and Zhang, 2004b), infection (Finegold et al., 2005), and bacteremia (Elsayed and Zhang, 2004a). *E. lenta* and *F. plautii* can cause bacteremia (Wong et al., 2014) and bloodstream infection (Berger et al., 2018), respectively. *R. gnavus*, which degrades mucin (Crost et al.,

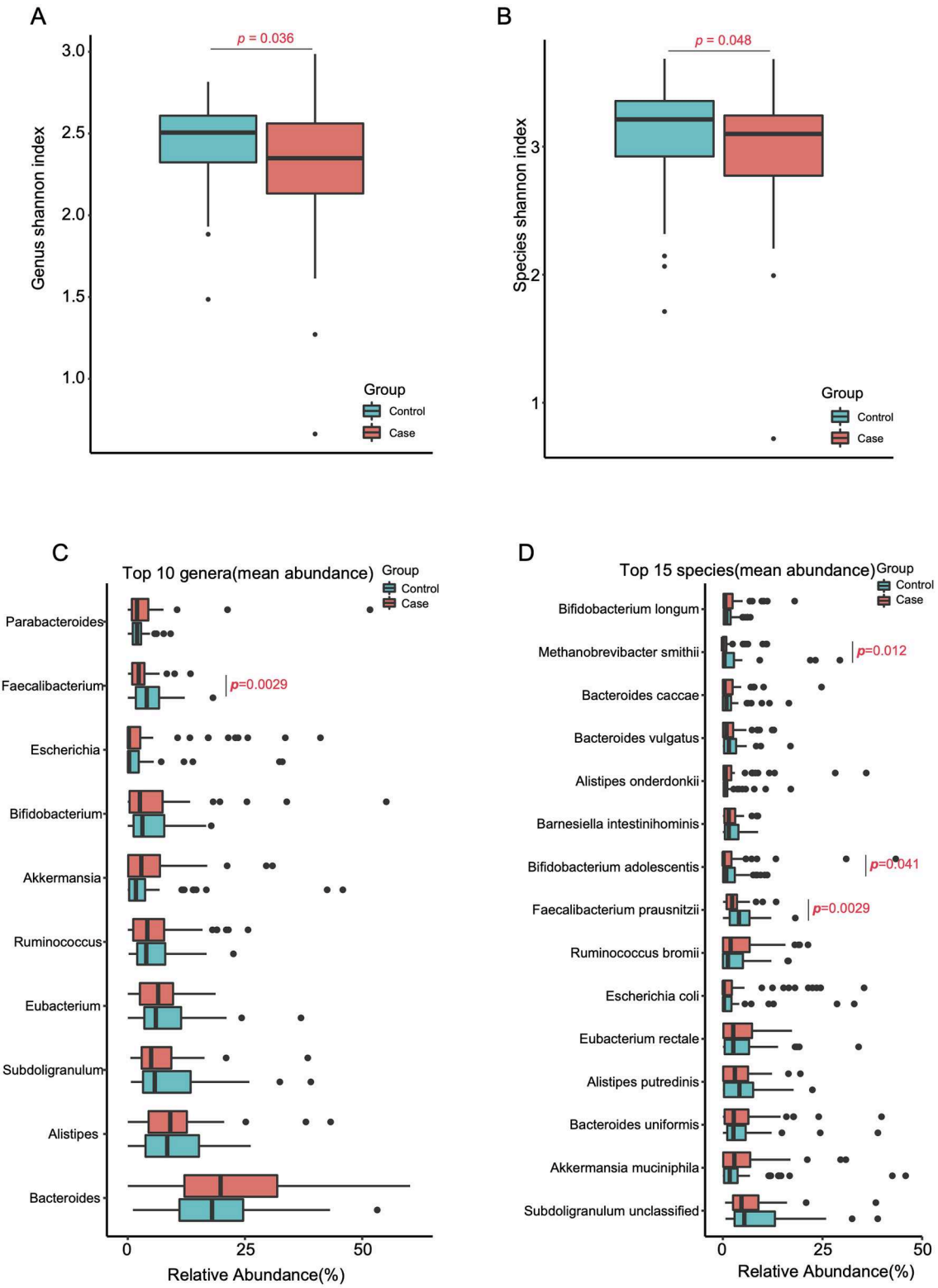

FIGURE 1 | Reduced gut microbial diversity in migraineurs. Alpha-diversity (Shannon index) at the genus **(A)** and species **(B)** levels of the two cohorts (Tested by two-tailed Wilcoxon-rank sum test). The top 10 genera **(C)** and top 15 species **(D)** (mean relative abundance higher than 3.09 and 1.83%, respectively) in the migraine patients and the control individuals (two-tailed Wilcoxon-rank sum test, **Tables S4B**,**C**).

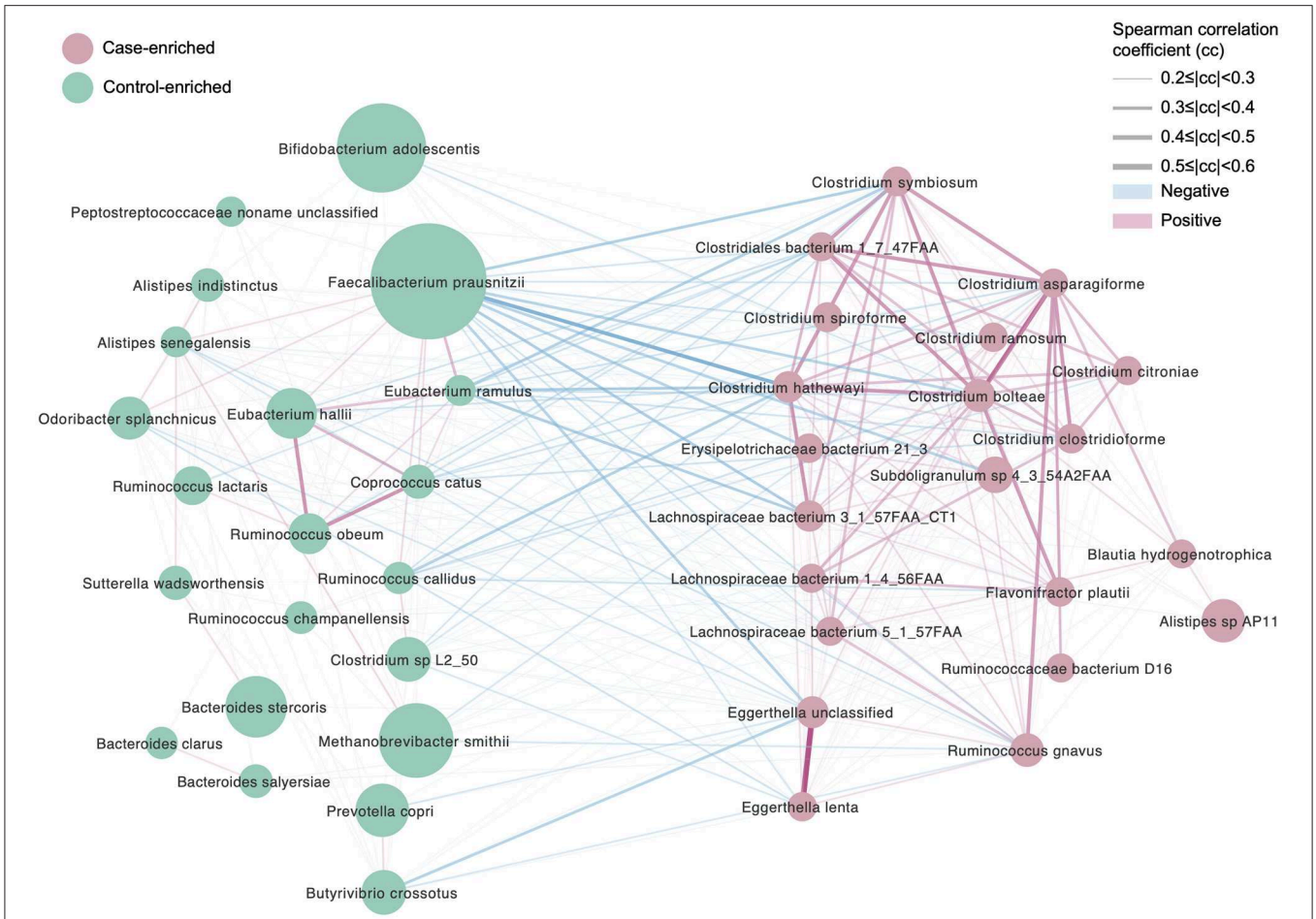

FIGURE 2 | Differentially enriched species, control vs. migraine. The orientation of enrichment was determined by two-tailed Wilcoxon-rank sum test (*U* statistic of two-tail Wilcoxon rank-sum test, $p < 0.05$, FDR< 0.29, **Table S4C**). The size of the nodes is consistent with the relative abundance of the species. Species were colored according to the extent of enrichment. The edges between the nodes indicate positive (red) and negative (blue) Spearman's correlation, respectively, the line width represents the correlation coefficient calculated for the samples under comparison.

2016), is enriched in IBD patients (Hall et al., 2017) and can cause bacteremia (Hansen et al., 2013).

The species significantly enriched in the controls were *Bacteroides clarus*, *Bacteroides intestinalis*, *Bacteroides salyersiae*, *Bacteroides stercoris*, *Butyrivibrio crossotus*, *Clostridium* sp. L2_50, *Coprococcus catus*, *Eubacterium hallii*, *Eubacterium ramulus*, *Odoribacter splanchnicus*, *Peptostreptococcaceae noname* unclassified, *Prevotella copri*, *Ruminococcus callidus*, *Ruminococcus champanellensis*, *Ruminococcus obeum*, and *Sutterella wadsworthensis*. In this list, at least one bacterium regarded as "unfriendly" can be found: *O. splanchnicus*, which has been isolated from the crevicular spaces of dogs with periodontitis (Hardham et al., 2008). However, many more species regarded as beneficial were found in the healthy subjects compared with the migraineurs. *B. clarus* is significantly decreased in colorectal cancer (Watanabe et al., 2010; Liang et al., 2017), and its enrichment in controls may be beneficial. *S. wadsworthensis* protects against IBD (Wexler et al., 1996); *C. catus* ferments fructose, lactate, and pyruvate to short-chain fatty acids (SCFAs) (Holdeman and Moore, 1974); *E. hallii* contributes to the intestinal propionate formation (Engels et al., 2016) and improves insulin sensitivity (Udayappan et al., 2016); *E. ramulus* produces propionic acid and dihydroxyphenylacetic acid (Schneider and Blaut, 2000; Braune et al., 2001); *R. obeum* plays an important role in the recovery process from *Vibrio cholerae* infection (Lawson and Finegold, 2015). In addition, species involved in polysaccharide degradation were also enriched in the healthy controls. These were *B. intestinalis*, which degrades arabinoxylan for energy acquisition (Wang et al., 2016); *B. crossotus*, which metabolizes polysaccharides into simpler sugars (Kelly et al., 2010); and *R. callidus* and *R. champanellensis*, which degrade various plant hemicelluloses and cellulose (Chassard et al., 2012).

Functional Alterations in the Gut Microbiome of Migraineurs

Significantly different GMMs and GBMs between two groups were analyzed ($p < 0.05$, Wilcoxon rank-sum test). Seven

significantly changed GMMs between two groups were observed (**Figure 3A**), and *glutamate degradation II* is the only GMM that is significantly higher in the migraine group. *Serine degradation*, *homoacetogenesis*, *glycerol degradation*, *mannose degradation*, *glycolysis (preparatory phase)*, and *pyruvate:ferredoxin oxidoreductase* were the six GMMs found to be enriched in the healthy controls. *Serine* is a major energy and SCFAs contributor in the human body and can be degraded to pyruvate by *Escherichia coli* (Su et al., 1989). *Homoacetogenesis* produces acetate by consuming hydrogen (Ni et al., 2011). Higher *glycerol degradation* can reduce the *triglycerides synthesis* and *phospholipids synthesis*. *Mannose*, which has been reported to impair tumor growth and to enhance chemotherapy, can be degraded into fructose, which is then catabolized to lactate, a precursor of SCFAs (Gonzalez et al., 2018). *Glycolysis (preparatory phase)* consumes energy to convert glucose into two 3-carbon molecules (Kathagen et al., 2013). *Pyruvate:ferredoxin oxidoreductase* is a key enzyme in metabolism that catalyzes pyruvate to acetyl-CoA and CO_2 (Furdui and Ragsdale, 2000). Taken together, these results indicated that the gut microbiota of the healthy controls were more active in energy metabolism and SCFA synthesis, which might be beneficial in maintaining their health.

Five significantly changed GBMs between two groups were observed (**Figure 3B**). *Glutamate degradation I*, *quinolinic acid degradation*, and *S-adenosyl methionine* (SAM) *synthesis* were predominant in the control group, which meant that there was a decrease in the levels of glutamate and quinolinic acid while an increase in those of the SAM in the healthy controls. Glutamate is a neurotransmitter in the healthy brain. Quinolinic acid is an endogenous *N*-methyl-D-aspartate (NMDA) receptor agonist and possesses neuroactive activity (Heyes et al., 1992). SAM is a major methyl donor in the brain. Conversely, *kynurenine degradation* and *γ-aminobutyric acid (GABA) synthesis III* were enriched in the migraine group. Kynurenine is a metabolite of tryptophan and can be degraded to quinolinic acid and kynurenic acid, an NMDA antagonist, and is thought to be involved in the pathophysiology and pathogenesis of schizophrenia. GABA is the chief inhibitory neurotransmitter in the developmentally mature mammalian central nervous system.

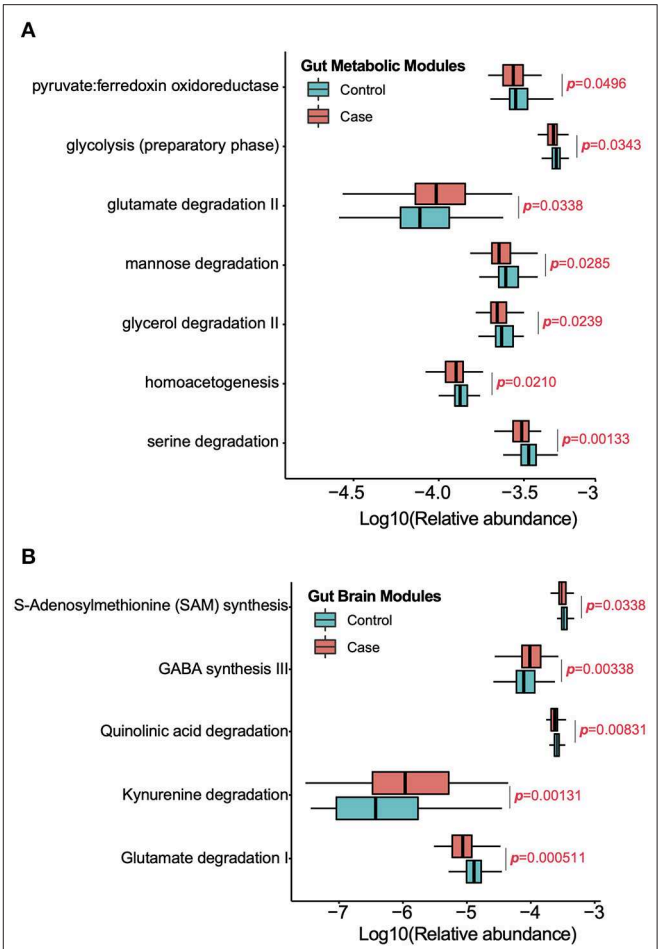

FIGURE 3 | Significantly enriched GMMs **(A)** and GBMs **(B)** in each group were analyzed by Wilcoxon rank sum test ($p < 0.05$). The Y-axis is the significantly enriched GMMs and GBMs in two groups and X-axis is the logarithm of relative abundance for each GMM and GBM. *P*-value was labeled.

DISCUSSION

Migraine susceptibility is multifactorial with genetic, hormonal, and environmental factors. The physiopathology of migraine is complex and still not fully understood. Recent reports demonstrate an increased frequency of gastrointestinal disorders, such as *Helicobacter pylori* infection, IBS, gastroparesis, hepatobiliary disorders, celiac disease, and alterations in the microbiota have been linked to the occurrence of migraine (Camara-Lemarroy et al., 2016). However, several case–control studies based on probiotic treatment have different conclusions (de Roos et al., 2017; Straube et al., 2018). Up to now, the precise characteristics and changes of gut microbiome in migraine were not fully elucidated. In this study, we presented the first set of evidence obtained from human cohorts for a significant gut microbiome dysbiosis from both compositional and functional aspects in migraine.

Significant differences in the gut microbiota composition were observed in our study. First, the migraine group showed a significantly lower alpha diversity at species, genus, and KO levels than did the healthy controls, which might be caused by a significant depletion in some highly abundant bacteria in migraine, such as *Faecalibacterium*, *F. prausnitzii*, *B. adolescentis*, and *M. smithii*. *F. prausnitzii* is a major producer of butyrate (Machiels et al., 2014), *B. adolescentis* exhibits strain-specific effects in the alleviation of constipation (Wang et al., 2017), and *M. smithii*, a predominant archaeon in the human gut, can affect the specificity and efficiency of dietary polysaccharides to influence host calorie harvest and adiposity (Samuel et al., 2007). Notably, Firmicutes, the main bacterial phylum in the gut, was significantly enriched in migraine. Second, some species thought to be detrimental to human health, especially *Clostridium* spp., were significantly enriched in migraineurs. Third, the controls held more beneficial microorganisms, such as *B. adolescentis*,

F. prausnitzii, and *Bacteroides intestinalis*, suggesting that the control subjects held a healthier gut microenvironment than did migraineurs. Notably, some "unfriendly" species, such as *Odoribacter splanchnicus* and *Prevotella copri*, were also elevated in controls, suggesting the likelihood of susceptibility to intestinal inflammation (Hardham et al., 2008) and arthritis (Scher et al., 2013) in the seemingly healthy control subjects.

From a functional point of view, significant changes in GMMs and GBMs between two groups were observed. Interestingly, the migraineurs had less GMMs and GBMs enriched than did the controls. The healthy controls held more modules related to substrates metabolism, glycolysis (preparatory phase), and SCFA production, whereas *glutamate degradation II* is the only significantly changed GMM enriched in migraine. These results suggested that migraineurs might suffer from metabolic dysfunctions and insufficient SCFA synthesis.

For GBMs, *kynurenine degradation* and *GABA synthesis III* were significantly higher in the migraine group. The higher *kynurenine degradation* module observed in the migraine group suggested the potential presence of elevated concentration of its catabolites, that is, the neuroexcitatory quinolinic acid and neuroinhibitory kynurenic acid, which can cause diseases in the nervous and immune systems (Heyes et al., 1992). The higher GABA synthesis observed in the migraine group indicated the potential presence of higher levels of GABA in the brain, which might be beneficial for health. Oral GABA administration was reported to relieve anxiety (Abdou et al., 2006), improve mood (Sakashita et al., 2019), and reduce symptoms of premenstrual syndrome (Rapkin and Akopians, 2012). In addition, GABA also supports the physiologic adjustment of pituitary gland function and controls growth hormone secretion from the pituitary gland (Acs et al., 1987), promotes muscle protein synthesis (Olarescu et al., 2000), stabilizes blood pressure (Ma et al., 2015), and relieves pain (Jasmin et al., 2004). On the contrary, the higher capability of *glutamate degradation I* was observed in the healthy group. Glutamate is the principal excitatory neurotransmitter in the healthy brain and provides energy for normal brain function, whose depletion is a common feature of many neuropsychiatric conditions such as schizophrenia (Zhou and Danbolt, 2014). In addition, higher quinolinic acid degradation was observed in the control group. Quinolinic acid is an NMDA antagonist, has a potent neurotoxic effect, and may be involved in many psychiatric disorders and neurodegenerative processes in the brain. These GBM changes reminded us that the healthy volunteers might also have a risk in getting mental disorders, although they have no significant signs. Higher synthesis of SAM in controls indicated higher levels of SAM, an antidepressant with few side effects (Young and Shalchi, 2005). The functional redundancy, which was reported by Kang et al. (2015), was also found in both migraine group and healthy controls in our study, which meant that the gut microbiome can self-regulate to relieve symptoms when their hosts suffered from migraine. In addition, changes in some unhealthy gut microbiome composition and function in the healthy group might also indicate that migraine may still happen in seemingly healthy people in the future.

In summary, our results revealed a significant decrease in species diversity and metabolic functions in the gut microbiota of migraine sufferers, which highlighted the importance of maintaining species diversity to improve the gut microecosystem stability. Additionally, the monitoring of harmful bacteria such as *Clostridium* spp. could be a new strategy for early diagnosis and timely prevention of migraine. In addition, proper probiotics could be supplemented to migraineurs to treat their intestinal dysbiosis or prevent them from gut disorders, which may reduce the occurrence of migraine attacks. Our findings revealed that gut microbiota can be a potential target for migraine management and offered not only novel promising treatment strategies but also an important functional basis for future research on this disease.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this study can be found in the European Bioinformatics Institute (EBI) with the accession ID ERP010708 or in the China National Genebank (CNGB) with a program ID CNPhis0000107.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Health Authority NRES Committee London - Westminster. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QW and JC conceived and designed the study. QW and AW were in charge of data analysis and graphics presentation. JC wrote the paper. QW and ZL reviewed and revised the manuscript. All authors read and approved the manuscript.

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PERSPECTIVE
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Gastrointestinal (GI) Tract Microbiome-Derived Neurotoxins—Potent Neuro-Inflammatory Signals From the GI Tract via the Systemic Circulation Into the Brain

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The microbiome of the human gastrointestinal (GI)-tract is a rich and dynamic source of microorganisms that together possess a staggering complexity and diversity. Collectively these microbes are capable of secreting what are amongst the most neurotoxic and pro-inflammatory biopolymers known. These include lipopolysaccharide (LPS), enterotoxins, microbial-derived amyloids and small non-coding RNA (sncRNA). One of the major microbial species in the human GI-tract microbiome, about ~100-fold more abundant than *Escherichia coli,* is *Bacteroides fragilis,* an anaerobic, rod-shaped Gram-negative bacterium that secretes: (i) a particularly potent, pro-inflammatory LPS glycolipid subtype (BF-LPS); and (ii) a hydrolytic, extracellular zinc metalloproteinase known as *B. fragilis* toxin (BFT) or *fragilysin*. Ongoing studies support multiple observations that BF-LPS and BFT (*fragilysin)* disrupt paracellular barriers by cleavage of intercellular proteins, such as E-cadherin, between epithelial cells, resulting in '*leaky*' barriers. These defective barriers, which also become more penetrable with age, in turn permit entry of microbiome-derived neurotoxic biopolymers into the systemic circulation from which they can next transit the blood-brain barrier (BBB) and gain access into the brain. This short communication will highlight some recent advances in this extraordinary research area that links the pro-inflammatory exudates of the GI-tract microbiome with innate-immune disturbances and inflammatory signaling within the human central nervous system (CNS) with reference to Alzheimer's disease (AD) wherever possible.

Keywords: Alzheimer's disease (AD), *Bacteroides fragilis* and BFT (fragilysin), dysbiosis, lipopolysaccharide (LPS), microbiome and microbial genetics, neurofilament light (NF-L), neuroinflammation, synapsin-2 (SYN2)

OVERVIEW–THE HUMAN GASTROINTESTINAL (GI) TRACT MICROBIOME—*BACTEROIDETES*

The gastrointestinal (GI)-tract microbiome, the largest reservoir of microbes in the human body, containing about 10^{14} microbial cells, is a complex, dynamic and abundant source of bacteria, methanogenic archaea, fungi, microbial eukaryotes, protozoa, viruses, and other microorganisms. Together the GI-tract microbiome possesses a remarkable microbiological diversity and staggering genetic complexity of at least 1000 major bacterial species. In the most recent estimate analyses of the GI-tract metagenomes of ∼2100 donors well over 22.3 million non-redundant prokaryotic genes were detected, and at least half of all the genes identified were unique to an individual (Tierney et al., 2019). When compared to the established human genome content of 26.6 thousand protein-encoding transcripts of the human genome sequencing project obtained about ∼18 years ago (Fields et al., 1994; Venter et al., 2001; Hicks et al., 2019) the number of microbial genes in the human GI-tract microbiome alone outnumbers human genes by about 837 to 1 (Tierney et al., 2019). Another interesting fact is that of the 52 major divisions of bacteria identified to date, only 2 phyla are known to predominate in the human GI-tract microbiome—the Gram-negative *Bacteroidetes* (representing about ∼20–30% of all GI-tract resident bacteria) and the Gram-positive *Firmicutes* (representing ∼70–80% of the total) with relatively minor contributions by *Actinobacteria* (∼3%), *Proteobacteria* (∼1%), *Fusobacteria* (∼0.1%) and *Verrucomicrobia* (0.1%). Collectively these microorganisms represent: (i) "the microbial core" of the human GI-tract microbiome (Sarkar and Banerjee, 2019; Ticinesi et al., 2019); (ii) an extremely active, dynamic, and changing ecosystem dependent on the host's age, diet, environment, ethnicity, and health and/or disease status (Sender et al., 2016; Zhao and Lukiw, 2018a,b; Rinninella et al., 2019); (iii) a rich source of commensal bacteria usually beneficial to human health because of their abilities to metabolize and/or biosynthesize complex sugars, polysaccharides, and dietary fiber into volatile short chain fatty acids (SCFAs; including acetate, propionate, butyrate, valerate and lactate and other nutrients). SCFAs (i) normally function in the development, maintenance, and homeostasis of the host immune, neuro-endrocrine and digestive systems; and (ii) play important regulatory roles in glucose homeostasis, lipid metabolism and anti-inflammatory signaling in endothelial cells of the lining of the GI-tract, sometimes known as the intestinal endothelium. Interestingly, there is recent evidence that SCFAs can signal through G-protein coupled receptors (GPCRs) at the cell surface, including GPCR41, GPCR43, and GPCR109a and these activate signaling cascades that control multiple immune functions. Recent transgenic mouse studies support a key role of these GPCRs in the regulation of intestinal inflammation (Sears, 2009; Fathi and Wu, 2016; Lukiw, 2016a,b; Castillo-Álvarez and Marzo-Sola, 2019; Fox et al., 2019; Parada Venegas et al., 2019).

Over 99% of the microbes in the human GI-tract are facultative and obligate anaerobic bacteria; the most abundant Gram-negative bacterial Phylum in the human GI-tract microbiome are the *Bacteroidetes,* with a major Genus-species being represented by the obligate Gram-negative anaerobe *Bacteroides fragilis.* In some intestinal tract regions *B. fragilis:* (i) are present at about ∼100-fold the abundance of the *Proteobacteria Escherichia coli*; (ii) colonize the human GI-tract at densities up to 8×10^{10} CFU per cm^3, the highest density of any microbial colonization known in nature (Sears, 2009; Fathi and Wu, 2016; Rios-Covian et al., 2017; Patrick et al., 2019; Rinninella et al., 2019); and (iii) reside and proliferate exclusively in the GI-tract of mammals, suggesting a strong adaptation to the pH, biophysical and microbial composition of the gut environment (Bhattacharjee and Lukiw, 2013; Wexler and Goodman, 2017; Poeker et al., 2018; Castillo-Álvarez and Marzo-Sola, 2019).

GI-TRACT EXUDATES—BF-LPS AND FRAGILYSIN

In the human GI-tract there are 2 predominant strains of *Bacteroides fragilis* (*B. fragilis*) distinguished in part by their biosynthetic capabilities to synthesize and secrete a zinc-dependent metalloprotease toxin known as *B. fragilis* toxin (BFT) or *fragilysin*. Strains of *Bacteroides* that do not secrete BFT are called non-toxigenic *B. fragilis* while those that do secrete are called enterotoxigenic *B. fragilis* (ETBF; Allen et al., 2019). Relatively recently it has been established that enterotoxigenic strains of *B. fragilis* (ETBF) can rapidly proliferate in the mammalian GI-tract both in the absence of adequate dietary fiber and in the presence of high-fat cholesterol diets (Heinritz et al., 2016; Wexler and Goodman, 2017; Poeker et al., 2018; Zhao and Lukiw, 2018a,b). This proliferation enhances the intestinal abundance of *B. fragilis* and hence the potential of this Gram negative obligate anaerobe to secrete its formidable array of neurotoxic exudates. These primarily include: (i) the lipoglycan lipopolysaccharide (LPS), a particularly potent, pro-inflammatory LPS glycolipid subtype (BF-LPS); and (ii) the hydrolytic, extracellular zinc metalloproteinase known as ETBF-secreted *Bacteroides fragilis* toxin (BFT), also known as *fragilysin*. Recent characterization of BF-LPS and *fragilysin* have shown them to be amongst the most pro-inflammatory lipoglycans and enterotoxins known (Vines et al., 2000; Sears, 2009; Lukiw, 2016a,b; Zhao and Lukiw, 2018a,b; Batista et al., 2019; Sheppard et al., 2019). Both BF-LPS and *fragilysin* can leak through the normally protective mucosal barriers of the GI-tract intestinal endothelium to induce substantial inflammatory pathology both systemically and after BBB transit into vulnerable CNS compartments, including the neocortical parenchyma of the brain (Fathi and Wu, 2016; Lukiw, 2016a,b; Zhao and Lukiw, 2018a,b; Barton et al., 2019; Batista et al., 2019; Fox et al., 2019; Sheppard et al., 2019; Zhao et al., 2019). Indeed, while *Bacteroides fragilis* is an anaerobic, Gram-negative, rod-shaped bacillus, and part of the normal microbiota of the human colon and is generally commensal, this microbe can cause a "smoldering" systemic infection if displaced into the bloodstream or surrounding tissue following disease, trauma or surgery (Hill et al., 2014a,b; Montagne et al., 2017; Tulkens et al., 2018; Erdo and Krajcsi, 2019; Fox et al., 2019; Patrick et al., 2019; Sarkar

and Banerjee, 2019; Sweeney et al., 2019). When the highly toxic exudates of enterotoxigenic strains of *B. fragilis* escape the microbial-dense environment of the human GI-tract they can produce substantial systemic inflammatory pathology with significant mortality and morbidity. *B. fragilis* proliferation is associated with, and causative for, bacteremia, brain and intra-abdominal abscess, cellulitis, colitis, diabetic ulcer, diarrhea, necrotizing fasciitis, sepsis, peritonitis, septicemia, association with and the development of multiple pro-inflammatory bowel cancers, systemic infection and systemic inflammation, the development of neurological diseases involving inflammatory neurodegeneration, and those neurological disorders that display a significantly elevated incidence of atypical developmental programming against a background of aging (Leshchyns'ka and Sytnyk, 2016; Agrawal et al., 2017; Shivaji, 2017; Zhao et al., 2019). Very recently LPS-induced systemic inflammation has been associated with synaptic loss and cognitive decline in multiple human neurological disorders and in animal models, and a role for LPS-mediated microglial release of pro-inflammatory cytokines (such as IL-1β) based on both *in vivo* and primary culture studies *in vitro* (Sheppard et al., 2019; Zhao et al., 2019).

GASTROINTESTINAL (GI)-TRACT AND BLOOD BRAIN BARRIER (BBB) DYSFUNCTION

Two anatomical gateways, including the gastrointestinal mucosa that includes the "GI-tract barrier" and the "blood-brain barrier (BBB)," each formed essentially by vascular epithelial and/or endothelial cells and epithelial-endothelial-derived basement membranes provide both a biophysical interface and a biological compartmentalization of the GI-tract microbiome, the systemic circulation, the brain parenchyma and distinct anatomical regions of the brain such as the neocortex (**Figure 1**). These barriers are a requisite for the essential maintenance of homeostasis and the physiological environment of each compartment; microorganisms of the GI-tract microbiome and their neurotoxins that are able to transit the single layer of epithelial cells have virtually unimpeded access into the systemic circulation (Varatharaj and Galea, 2017; Logsdon et al., 2018; Sweeney et al., 2019; Tulkens et al., 2018). Probably the most important structural components of these barriers are the multiple tight junctions between adjacent cells of vascular

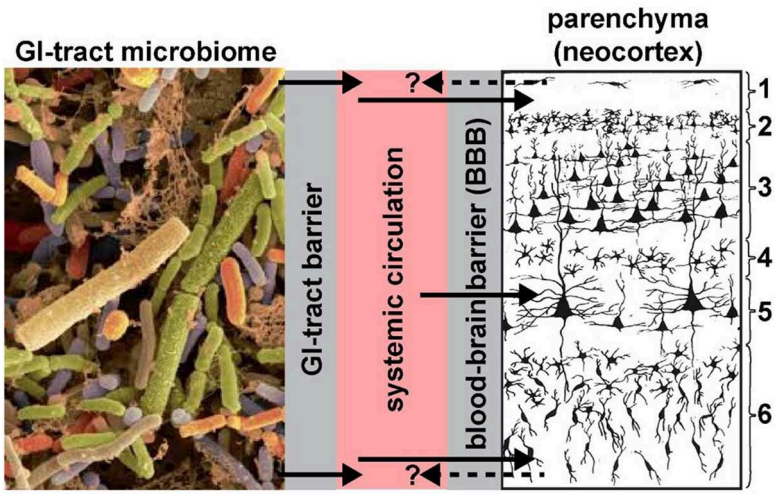

FIGURE 1 | Highly schematicized depiction of the potential transfer of GI-tract microbiome-derived pro-inflammatory neurotoxins across the GI-tract barrier into the systemic circulation, followed by translocation across the BBB into the brain parenchyma (see Hill et al., 2014a,b; Hill and Lukiw, 2015; Zhao and Lukiw, 2018a; neocortical region shown; solid black arrows). Neurotoxins identified to date include *Bacteroides fragilis* lipopolysaccharide (BF-LPS) and enterotoxins such as the *Bacteroides fragilis*-derived toxin (BFT) also known as *fragilysin*. The contribution of other pro-inflammatory neurotoxins such as GI-tract-derived amyloids and bacterial sncRNAs are not well-understood and currently very little is known concerning their neurotoxicity and CNS-effects. BF-LPS, BFT (*fragilysin*), age, dietary toxins, traumatic brain injury (TBI) and vascular disease are known to effectively disrupt endothelial cell-based biophysical barriers in part through the cleavage, disruption and/or degeneration of cell-cell adhesion proteins (Wu et al., 1998; Clement et al., 2016; Zhao and Lukiw, 2018a,b; Sweeney et al., 2019). We speculate that all of these neurotoxins together have potential to constitute a highly neurotoxic pro-inflammatory GI-tract microbiome-derived cocktail greatly detrimental to the cytoarchitecture and signaling functions of neuronal and glial cells. Gram-negative bacterial-derived LPS and related neurotoxins have been recently observed within the systemic circulation and brain parenchyma (Zhan et al., 2016, 2018; Zhao et al., 2017a,c, 2019; Zhao and Lukiw, 2018a), within and around neurons, and in the later stages of AD completely encapsulating neuronal neocortical nuclei (Hill et al., 2014b,a; Hill and Lukiw, 2015; Zhao and Lukiw, 2018a,b; unpublished observations); this later action appears to impair the exit of neuron-specific transcripts such as the neurofilament-light (NF-L) chain and synapsin-2 (SYN2) messenger RNA (mRNA) from the neuronal nuclei; both NF-L and SYN2 mRNA abundance and expression are down-regulated in LPS-treated human neuronal-glial (HNG) cells in primary culture and in AD brain (Lukiw et al., 2018; Zhao et al., 2019). Whether neurotoxins from the brain parenchyma of the neocortex can cross the BBB back into the systemic circulation (dashed black arrows with question mark) is currently not well-understood; if so, these species may be useful serum biomarkers for both the diagnosis and prognosis of AD and other types of inflammatory neurodegeneration; left panel = GI-tract microbiome magnification x ~3000 (source: http://www.lnjnbio.com; http://www.lnjnbio.com/nd.jsp?id=20; permission to reproduce granted; last accessed 26 November 2019); right panel = 6-layered structure of the human association neocortex; layers 3 and 5 are the pyramidal cell layers targeted by the AD process; other brain regions may also be affected; magnification x ~20 (source: adapted and redrawn from Martinez-Conde, 2018; last accessed 26 November 2019).

capillaries (Sweeney et al., 2018, 2019; Tulkens et al., 2018). For example, the blood-brain barrier of the CNS can selectively regulate its intracellular compartments and thereby isolate itself from rapid biochemical or biophysical changes that may occur in the systemic circulation.

The surface area of the human GI-tract barrier and the BBB is remarkably large; for example although the interior of the small intestine is only about ∼3.5 cm in diameter and ∼7 m in length (due in large part to a concentrically folded mucosa) it has a total absorptive surface area of ∼32 m^2 (Helander and Fändriks, 2014; Sweeney et al., 2019). Similarly the surface area of the 600 km of the human brain's 5 um diameter microvessels, representing the majority of the BBB, corresponds to a total surface area of ∼25 m^2 (Wong et al., 2013). Hence the maintenance of these large and formidable biophysical barriers is a very active and energy-intense biological process (Wong et al., 2013; Varatharaj and Galea, 2017; Barton et al., 2019; Erdo and Krajcsi, 2019; Sweeney et al., 2019). BF-LPS, BFT (*fragilysin*) and other GI-tract microbiome-derived exudates are remarkable in their capabilities: (i) of breaking down the intercellular junctions of these barriers via their disruptive actions on cadherins and other cell-cell adhesion molecules (Wu et al., 1998; Wong et al., 2013; Sweeney et al., 2018); (ii) of altering BBB integrity and permeability (Varatharaj and Galea, 2017; Tulkens et al., 2018; Sweeney et al., 2019); (iii) of changing BBB transport rates (Wong et al., 2013; Logsdon et al., 2018); (iv) of modulating neuroimmune signaling or the transport of immune-regulatory molecules (Erdo and Krajcsi, 2019); (v) of trafficking dietary neurotoxins and pathogens into the brain (Sweeney et al., 2019; Wong et al., 2013); and/or (vi) of inducing the release of inflammatory and neuro-immune substances from the barrier cells (Hill et al., 2014a,b; Köhler et al., 2016; Lukiw, 2016a,b; Montagne et al., 2017; Varatharaj and Galea, 2017; Tulkens et al., 2018; Sweeney et al., 2018, 2019; Erdo and Krajcsi, 2019; Fox et al., 2019; Patrick et al., 2019; Sarkar and Banerjee, 2019).

Indeed, the most recent research evidence continues to strengthen the idea that one major, and virtually unlimited, source of pro-inflammatory neurotoxic signals in inflammatory neurodegeneration such as those typified by the AD process may originate from internally derived noxious exudates such as those supplied via the diet and metabolized by the GI-tract microbiome (Sweeney et al., 2018, 2019; Erdo and Krajcsi, 2019). Because of aging, traumatic brain injury (TBI), cerebrovascular deficits (some of which may be genetic), neurovascular pathology or neuroinflammatory brain degeneration, neurotoxic molecules can "leak" into the systemic circulation, prompting some investigators to propose that progressive neurodegenerative diseases such as AD supplied via the reflect a malfunction of key biophysical barriers including those of the GI-tract and BBB, and that AD is in fact a "*defective barrier*" disease (Bhattacharjee and Lukiw, 2013; Montagne et al., 2017; Sweeney et al., 2018, 2019; Erdo and Krajcsi, 2019). The abundance of blood-borne bacterial components including LPS, for example, represents a variable component of the human blood serum that can elicit variable systemic pro-inflammatory and innate-immune-modulatory responses in the host, resulting in systemic-immune activation by pathogen-associated molecular patterns (PAMPs), a process sometimes referred to as "*microbial translocation*" (Zhao et al., 2017a,b; Tulkens et al., 2018; Di Lorenzo et al., 2019; Logsdon et al., 2018; Patrick et al., 2019). Multiple recent reports further suggest that GI-tract dysbiosis and "leaky gut syndrome" constitute a vastly under-appreciated, under-studied and critical pathophysiological passageway for transport of GI-tract microbiome-derived neurotoxins across GI-tract and blood–brain biological barriers resulting in an age-related progression from systemic inflammation to neurovascular disease to CNS inflammation and degeneration that progressively contribute to critical aspects of neuropathology associated with age-related neurodegenerative disorders. These include neuropathological disorders such as AD, anxiety, autism spectrum disorder (ASD), depression, epilepsy, multiple sclerosis, Parkinson's disease (PD), prion disease, systemic inflammatory response syndrome, and other incapacitating and/or ultimately lethal neurological diseases of the human CNS (Hill et al., 2014a,b; Köhler et al., 2016; Li and Yu, 2017; Varatharaj and Galea, 2017; Zhao et al., 2017a,b, 2019; Griffiths and Mazmanian, 2018; Di Lorenzo et al., 2019; Fox et al., 2019; Patrick et al., 2019; Sarkar and Banerjee, 2019).

BF-LPS AND THE INDUCTION OF THE PRO-INFLAMMATORY TRANSCRIPTION FACTOR NF-κB AND microRNA-146a

The extruded lipopolysaccharide shed from the human GI-tract microbiome-abundant *Bacteroides fragilis* (BF-LPS): (i) is one of the most pro-inflammatory and neurotoxic lipoglycans known (Sears, 2009; Fathi and Wu, 2016; Lukiw, 2016a,b; Allen et al., 2019); (ii) is linked to synaptic loss and cognitive decline in human patients and in animal models (Sheppard et al., 2019); and (iii) is recognized by the Toll receptors TLR2, TLR4, and/or CD14 microglial cell receptors, as are the pro-inflammatory and hydrophobic 42 amino acid amyloid-beta (Aβ42) peptides whose accumulation are a characteristic feature of AD brain (Sears, 2009; Zhao et al., 2015; Zhao and Lukiw, 2015; Lukiw, 2016a,b; Batista et al., 2019; Sheppard et al., 2019). Additional LPS-mediated pro-inflammatory actions, pathogenic mechanisms and neurodegeneration-promoting activities remain incompletely understood but remarkable progress is being made both in transgenic animal models and in human patient studies (Zhan et al., 2018; Zhao and Lukiw, 2018a,b; Barton et al., 2019; Sarkar and Banerjee, 2019; Sheppard et al., 2019; Wu et al., 2007). For example, LPS-induced synaptic loss and the impairment of cognition appear to be in part the result of a modified microglial activation, reactive oxidative species (ROS) or cytokine generation and oxidative stress damage, disruption of the intercellular adhesion proteins associated with the GI-tract or blood-brain barriers, the ROS mediated oxidation, atrophy, destruction and loss of synapse-related proteins, elevations in neuroinflammatory signaling or any combination of these events (Barton et al., 2019; Batista et al., 2019; Sheppard et al., 2019; Wu et al., 2007). One recently described BF-LPS mediated pathogenic and AD-relevant pathway is the robust activation of NF-κB (p50/p65) in human brain cells in primary culture and

induction of a pro-inflammatory signaling pathway involving an NF-kB-regulated microRNA-146a, and the subsequent chronic and pathogenic over-stimulation of innate-immune and neuro-inflammatory pathways (Zhao and Lukiw, 2018b). These include deficits in the innate-immune system modulator complement factor H (CFH), decreases in the expression of the essential presynaptic neuronal phosphoprotein synapsin-2 (SYN2) and down-regulation of the neuron-specific neurofilament light chain (NF-L) cytoskeletal protein (Lukiw et al., 2018; Zhan et al., 2018; Zhao et al., 2019). These pathological signaling pathways appear to strongly contribute to synaptic disorganization and decline, neuronal atrophy and inflammation-mediated amyloidogenic neuropathology which are all characteristic attributes of the AD-affected brain.

UNANSWERED QUESTIONS

The neurobiological signaling connections between the GI-tract microbiome and CNS disease remain incompletely understood. Eighteen years after the elucidation and characterization of the genes expressed in the human genome (Venter et al., 2001), the staggering genetic complexity of the human GI-tract and oral microbiomes have been analyzed with remarkable and unexpected results (Tierney et al., 2019). It is truly extraordinary that the potential contribution to human health and disease by the GI-tract microbiome with a total mass, complexity and diversity, and number of genes exceeding that of the liver could have been almost completely overlooked as recent as just ∼10 years ago.

Several fundamental questions remain concerning nature of the microorganisms of the GI-tract microbiome, their compartmentalization within the GI-tract and their potential effects on the neuropathology, neurobiology, and the pathogenetics of inflammatory neurodegeneration and neuropsychiatric disease. It will be further interesting to discover: (i) the evolutionary history of the GI-tract microbiome and for example, why just 2 of 52 bacterial phyla were selected and evolved to be both dominant and symbiotic within the entire human metagenome; (ii) what patterns of microbial abundance, speciation, complexity, stoichiometry, dysbiosis and GI-tract-derived mixtures of neurotoxins are the most effective in promoting pathogenic inflammatory neuro-degeneration; (iii) if the incidence of blood-borne GI-tract-derived toxic elements in the systemic inflammation could be used as a pathological biomarker or be of prognostic value for AD and other progressive, age-related, neurodegenerative diseases; (iv) what would be the contribution of combinations of the microbial constituents including archaebacteria, fungi, protozoa, viruses, and other GI-tract resident microbes of the GI-tract microbiome to enhance neurological health; (v) the intriguing possibility that the composition of the GI-tract microbiome could be altered through diet, probiotics and/or prebiotics to optimize human neurological health; (vi) if the penetration of epithelial barriers by bacterial products occurs in the oral cavity in periodontal disease with similar systemic effects; (vii) the mechanism of the duality of GI-tract abundant Gram-negative anaerobic bacteria such as *B. fragilis* in behaving in both pro- and anti-inflammatory capacities, the role of capsular polysaccharides and IL-10 secreting B and T cells in this transition, and how the role of *B. fragilis* can switch from an abundant beneficial microbe and commensal microorganism to a highly neurotoxic one (Ramakrishna et al., 2019); and (viii) perhaps most importantly, if medical researchers along with neurologists and dieticians could devise a strategy, perhaps through "*personalized medicine*," that promotes the lowering of noxious GI-tract microbes and their secretions that would optimize life-long GI-tract microbiome function and CNS health. This approach might minimize the risk of developing AD and other highly incapacitating human diseases as we age. Furthering our molecular-genetic and mechanistic understanding of how different secreted components of the GI-tract microbiome negatively affect the CNS may uncover potential and novel strategic approaches for the GI-tract microbiome-based modulation of neurological function, and the more effective clinical management of terminal, age-related neurological disorders.

CONCLUDING REMARKS

The appreciation of a potential contribution from the GI-tract microbiome to human neurological health and devastating behavioral, amnestic and cognitive disorders such as AD is a relatively recent one (Bhattacharjee and Lukiw, 2013), and gathering recent evidence continues to strengthen this association (Johnson and Foster, 2018; Patrick et al., 2019; Sarkar and Banerjee, 2019; Sheppard et al., 2019; Strandwitz et al., 2019; Sweeney et al., 2019; Ticinesi et al., 2019; Tierney et al., 2019; Zhao et al., 2019). Dietary manipulations of the GI-tract microbiome including diets enriched in biologically soluble and insoluble fiber, that seem to neutralize the potentially neurotoxic secretions from Gram-negative bacilli such as *Bacteroides fragilis* might provide a life-long resolution to defer the development of human neuro-inflammatory degenerative disease (Heinritz et al., 2016; Chen et al., 2017; Poeker et al., 2018). It is becoming increasingly established that the contribution of the GI-tract microbiome and GI-tract microbiome-derived neurotoxins to pathogenic signaling associated with inflammatory neurodegeneration is: (i) age-related and progressive; (ii) contains multiple neurotoxic components with capability to breach biophysical barriers; (iii) constitute a virtually unlimited supply of BF-LPS, BFT (*fragilysin)* and other neurotoxins; and (iv) that the GI-tract microbiome comprises a "staggering" microbial genetic complexity, and the recent finding that at least half of all the genes identified are unique to each individual further underscores the interesting parallel in the heterogeneity between GI-tract microbiome composition and AD risk, onset and development (Patrick et al., 2019; Strandwitz et al., 2019; Ticinesi et al., 2019; Tierney et al., 2019). Of further recent interest is the potential involvement of the GI-tract microbiota-brain axis with the mental status of the host in that certain "*psychotropic bacteria*" and their secreted array of "*psychobiotics*" appear to influence the mental health of the host (Beck et al., 2019; Cheng et al., 2019; Kelly et al., 2019). Given that AD was originally referred to as a progressive and dementing "*senile psychosis*," efficacious manipulation of the GI-tract microbiome might

not only attenuate inflammatory neurodegeneration, synaptic disorganization and cognitive decline but also optimize healthy neuroimmune, neuroendocrine, humoral and brain signaling pathways that also promote well-being, anti-depressive and anxiolytic behaviors in patients affected by the AD process.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Louisiana State University Institutional Review Board—only post-mortem human tissues were used in these studies. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

WL distilled the results from all laboratory experiments at the LSU laboratories and performed literature searches of recent peer-reviewed publications in this research area, compiled all data, and wrote this manuscript.

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Research on microRNAs, ethnobiology, botanical neurotoxins, pro-inflammatory and pathogenic signaling in the Lukiw laboratory involving the microbiome, the innate-immune response, amyloidogenesis, synaptogenesis, and neuro-inflammation in AD, prion and in other human neurological- and plant-viroid-based diseases was supported through an unrestricted grant to the LSU Eye Center from Research to Prevent Blindness (RPB); the Louisiana Biotechnology Research Network (LBRN) and NIH grants NEI EY006311, NIA AG18031, and NIA AG038834 (WL). The content of this manuscript was solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Aging, the National Center for Research Resources, or the National Institutes of Health.

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Case-Control Study of the Effects of Gut Microbiota Composition on Neurotransmitter Metabolic Pathways in Children With Attention Deficit Hyperactivity Disorder

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Background: Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric condition that may be related to an imbalance of neural transmitters. The gut microbiota is the largest ecosystem in the human body, and the brain-gut axis theory proposes that the gut microbiome can affect brain function in multiple ways. The purpose of this study was to explore the gut microbiota in children with ADHD and assess the possible role of the gut microbiota in disease pathogenesis to open new avenues for ADHD treatment.

Methods: A case-control design was used. We enrolled 17 children aged 6–12 years with ADHD who were treated in the Pediatric Outpatient Department of the First Medical Center of the Chinese PLA General Hospital from January to June, 2019. Seventeen children aged 6–12 years were selected as the healthy control (HC) group. Fecal samples of cases and controls were analyzed by shotgun metagenomics sequencing. Alpha diversity and the differences in the relative abundances of bacteria were compared between the two groups. Functional annotations were performed for the microbiota genes and metabolic pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Results: There was no significant difference in the alpha diversity of gut microbiota between the ADHD and HC groups. Compared with HCs, *Faecalibacterium* and *Veillonellaceae* were significantly reduced in children with ADHD ($P < 0.05$), *Odoribacter* and *Enterococcus* were significantly increased [linear discriminant analysis (LDA) > 2]. At the species level, *Faecalibacterium prausnitzii*, *Lachnospiraceae bacterium*, and *Ruminococcus gnavus* were significantly reduced in the ADHD group ($P < 0.05$), while *Bacteroides caccae*, *Odoribacter splanchnicus*, *Paraprevotella xylaniphila*, and *Veillonella parvula* were increased ($P < 0.05$). Metabolic pathway analysis revealed significant between-group differences in the metabolic pathways of neurotransmitters (e.g., serotonin and dopamine) ($P < 0.05$).

Conclusion: Composition differences of gut microbiota in subjects with ADHD may contribute to brain-gut axis alterations and affect neurotransmitter levels, which could contribute to ADHD symptoms.

Keywords: attention deficit hyperactivity disorder, child, gastrointestinal microbiome, shotgun metagenomics sequencing, neurotransmitter

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric disorder that occurs most frequently in school-age children and is characterized as inattention with or without excessive impulsivity and hyperactivity (Abramov et al., 2019). Previous studies have reported that ADHD pathogenesis may be associated with dysregulation of neurotransmitters such as dopamine, serotonin (5-hydroxytryptamine, 5-HT), and norepinephrine (Magula et al., 2019; Stewart et al., 2019; Suzuki et al., 2019). Others have shown that the incidence of ADHD may have a certain degree of heritability, and genes related to dopamine, norepinephrine, and 5-HT transmission have been found to be abnormally expressed in children with ADHD (Banerjee and Nandagopal, 2015; Karmakar et al., 2017; Kim et al., 2018). Although various theories have been proposed, the pathogenetic mechanisms underlying ADHD have not been fully clarified, which limits the development of new treatments.

Gut microbiota alterations may be associated with neurological conditions including Alzheimer's disease, epilepsy, and autism (Fan et al., 2019; Rude et al., 2019). Many researchers have proposed the existence of bidirectional regulation of the brain-gut axis, which involves gut microbiota metabolites that affect neurotransmitter levels, thereby influencing brain function (Melli et al., 2016; Khalil et al., 2019; Lacorte et al., 2019). In addition, nervous system activity can also impact gut microbiota composition. This bidirectional regulation is accomplished via complex neuroendocrine pathways (Khalil et al., 2019). The gut microbiota can adjust these pathways by regulating the levels of neurotransmitters and inflammatory factors and affecting the hypothalamic-pituitary-adrenal axis (Bermúdez-Humarán et al., 2019). Therefore, abnormal intestinal flora composition may lead to abnormal neurotransmitter secretion, which may promote the development of neuropsychiatric diseases.

We conducted a case-control study to analyze differences in intestinal flora composition between children with ADHD and healthy control (HC) children, explore ADHD pathogenesis, and investigate potential new treatments for ADHD.

MATERIALS AND METHODS

Study Subjects

Seventeen children aged 6–12 (median 8 years) with ADHD were selected from the Pediatric Outpatient Department of the First Medical Center of the PLA General Hospital between January and June, 2019. The inclusion criteria were: (1) The Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS, Present and Lifetime Version scales) was used to diagnosis ADHD, and subjects met the diagnostic criteria for ADHD in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (Ng et al., 2019) based on the opinion of an experienced child psychiatrist (GY or LW); (2) no history of respiratory or digestive tract infection within 1 month; (3) no use of probiotics within 1 month; (4) no history of digestive diseases or other chronic diseases; (5) body mass index (BMI) < 20 kg/m^2 (because obesity could cause gut microbiota abnormalities) (Salah et al., 2019); and (6) no allergic diseases such as allergic rhinitis or asthma. Seventeen children from different families aged 6–12 years (median 8 years) were selected as the HC group in the same period. The inclusion criteria were the same except that there was no diagnosis of ADHD based on DSM-5 criteria by K-SADS. All of the participating children were born full-term with normal deliveries. Subjects were excluded if they were on a special diet (e.g., vegetarian). All parents of the participating children completed the Conners Parent Rating Scales (CPRS) to assess ADHD symptom severity and exclude subjects with depressive or anxiety symptoms. Participants maintained their regular dietary patterns for a week, and a food diary was recorded for participants from both groups during this period in order to exclude the potential influence of any changes in diet on the intestinal flora. Stool samples were collected at 8:00 am in the Pediatric Outpatient Department and stored in a sterile plastic cup at −80°C prior to testing.

The study was approved by the PLA General Hospital Ethics Committee (no. 2018-278). All subjects' guardians were informed about the intentions of this study, and gave written informed consent was obtained in accordance with the Declaration of Helsinki.

Sequencing and Analysis

DNA Sequencing

A total of 34 stool samples were collected from 17 ADHD patients and 17 age-matched HCs. We applied shotgun metagenomic sequencing to the whole genome of the microorganisms for each specimen. Bead beating was performed to rupture the bacteria, DNA was extracted with HiPure Stool DNA kits (Angen Biotech Co., Ltd., Guangzhou, China), and Qubit 4.0 software (Thermo Fisher Scientific, Waltham, MA, United States) was used for quality assessment. The library was prepared with a KAPA Hyper Prep Kit (KAPA Biosystems, Wilmington, MA, United States) and paired-end sequencing was performed on an Illumina NovaSeq platform (Illumina, San Diego, CA, United States) with a reading length of 150 bp (PE150).

Species Abundance and Gene Function Annotations

All genome sequencing data were preprocessed by KneadData[1] to screen out low-quality short frame sequences and chimeric sequences among the structural primer sequences (Bolger et al., 2014). Bowtie2 (Langmead and Salzberg, 2012) was then used to align the reads with the human genome for host sequence contamination removal. This was carried out with human reference genome hg19)[2].

HUMAnN2 (version v0.11.2) was used to analyze the species abundance, gene function, and metabolic pathways related to the processed sequencing data (Franzosa et al., 2018). HUMAnN2 first used MetaPhlAn2 (version 2.7.7, Li et al., 2014) to match the sequence with the established core genes to quickly locate the species included in the microbiota. Sequences were then compared with the pan-genome of the identified species and mapped to corresponding phylogenetic levels. The abundance of genes or gene families, and metabolic pathways were analyzed at different phylogenetic levels of interest.

To determine the gene functional annotations, we employed the Bowtie2 (version 2.3.4.3) to map the sequences after removing low-quality sequences and host sequences, to Integrated Gene Catalog databases and Kyoto Encyclopedia of Genes and Genomes (KEGG).On this basis, gene abundance and alpha diversity indexes were calculated, which involves using the Shannon, Chao1, and Simpson indexes to calculate the entropy values of gene abundance. Euclidean distance was also computed as the measurement of beta diversity, followed by principal component analysis (PCA) and permutational multivariate analysis of variance (PERMANOVA). PCA was performed using ade4 package and PERMANOVA was carried out using vegan package (R version 3.5.3)[3].

Bioinformatics Analysis

Chi-square tests were performed by SPSS 21.0 to compare sex differences between the ADHD and HC groups, and independent-sample *t*-tests were used to compare age, BMI, and CPRS scores. Wilcoxon tests were used by SPSS 21.0 to assess differences in species abundance and gene function between the ADHD and HC groups. The LDA effect size (LEfSe) method was used to determine the most differentially abundant taxa at the genus and species levels between the two groups.

RESULTS

Comparison of Clinical Data Between the ADHD and HC Groups

A total of 17 ADHD children were included in this study, including 14 (82.3%) males and 3 (17.7%) females with a median age of 8 (25th and 75th percentiles: 7, 10) and a mean BMI of 16.1 ± 1.2 kg/m^2. The 17 HCs included 13 (76.5%) males and 4 (23.5%) females with a median age of 8 (7, 9.5) and a mean BMI of 15.9 ± 1.1 kg/m^2. There was no significant difference in the distributions of sex, age, or BMI between the two groups ($P > 0.05$). More children in the ADHD group (12, 70.5%) developed symptoms of constipation than in the HC group (2, 11.7%).The total CPRS scores were significantly different between the ADHD and HC groups (10.3 ± 4.2 vs. 2.2 ± 0.63, respectively; $P < 0.05$). There were no significant differences in the subscores for psychosomatic symptoms (0.56 ± 0.34 vs. 0.53 ± 0.41) or anxiety (0.42 ± 0.32 vs. 0.51 ± 0.35) ($P > 0.05$, **Table 1**).

Analysis of Intestinal Flora Diversity

The Shannon (9.67 ± 0.42 vs. 9.52 ± 0.25), Chao1 (61.5 ± 11.6 vs. 57.5 ± 9.8), and Simpson (0.89 ± 0.07 vs. 0.88 ± 0.06) indexes were calculated to assess the alpha diversity of fecal microbiota in the ADHD and HC groups. There were no significant differences in index values between the two groups (**Figure 1A**). At the genus level and the species level, PERMANOVA could not discriminate the ADHD from the HC group due to significant individual variation (**Figures 1B,C**).

Analyses of Fecal Bacterial Community Abundance

At the genus level, Wilcoxon tests showed that *Faecalibacterium* and *Veillonellaceae* were significantly reduced in the ADHD group, while *Odoribacter* was significantly higher ($P < 0.05$, **Figure 2A**). The LEfSe results also indicated that *Enterococcus* was significantly increased in the ADHD group (LDA > 2, **Figure 2B**).

At the species level, Wilcoxon tests showed that *Faecalibacterium prausnitzii*, *Lachnospiraceae bacterium*, and *Ruminococcus gnavus* were significantly decreased in the ADHD group, while *Bacteroides caccae*, *Odoribacter splanchnicus*, *Paraprevotella xylaniphila*, and *Veillonella parvula* were significantly increased ($P < 0.05$, **Figure 2C**). The results of LEfSe showed that *Odoribacteraceae* and

TABLE 1 | Descriptive data of the ADHD and HC groups.

	ADHD (*n* = 17)	HC (*n* = 17)	*P*
Sex, *n* (%)			0.671
Male	14 (82.3%)	13 (76.5%)	
Female	3 (15%)	4 (23.5%)	
Age, years; median (25th and 75th percentiles)	8 (7, 10)	8 (7, 9.5)	0.701
BMI, mean (SD)	16.1 (1.2)	15.9 (1.1)	0.652
Constipation, *n*(%)	12 (70.5%)	2 (11.7%)	<0.05
ADHD symptom severity, mean (SD)			
Total CPRS score	10.3 (4.2)	2.2 (0.63)	<0.05
Conduct problems	3.1 (1.46)	0.16 (0.27)	<0.05
Impulsive–hyperactivity	1.5 (0.59)	0.16 (0.22)	<0.05
Hyperactivity	3.4 (0.65)	0.05 (0.21)	<0.05
Learning problems	1.9 (0.57)	0.21 (0.34)	<0.05
Psychosomatic	0.56 (0.34)	0.53 (0.41)	0.452
Anxiety	0.42 (0.32)	0.51 (0.35)	0.523

ADHD, attention-deficit/hyperactivity disorder; BMI, body mass index; CPRS, Conners Parent Rating Scales; HC, healthy control.

[1]https://bitbucket.org/biobakery/kneaddata

[2]http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/hg19.fa.gz

[3]https://www.r-project.org/

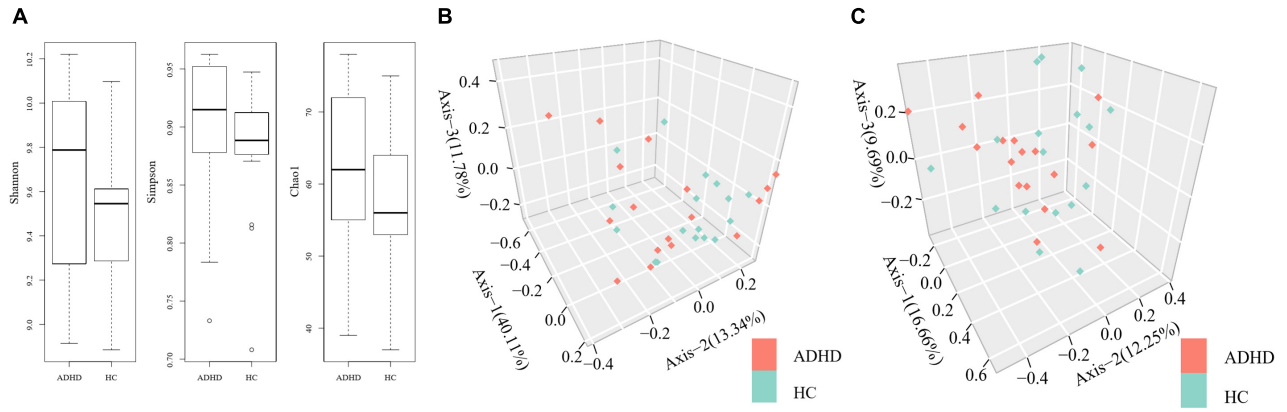

FIGURE 1 | (A) Comparison of alpha diversity indexes between the ADHD and HC groups. **(B)** Permutational multivariate analysis of variance (PERMANOVA) of microbial communities of the participants at the genus level. **(C)** PERMANOVA of microbial communities of the participants at the species level.

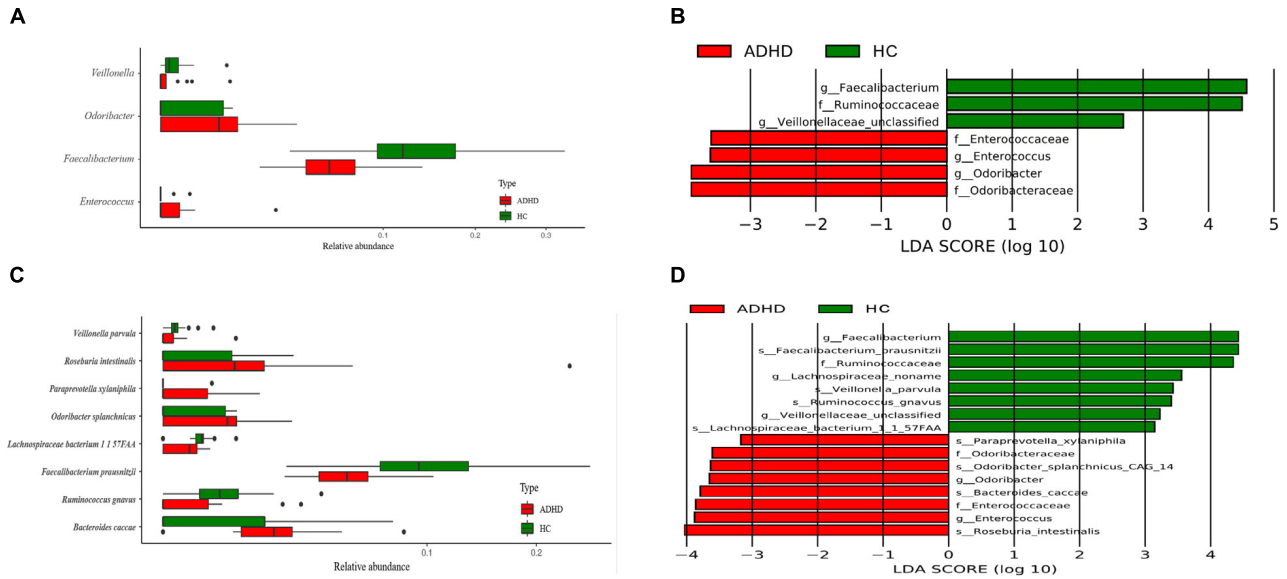

FIGURE 2 | Comparison of different bacteria at the genus and species level between the ADHD and HC groups. **(A)** Wilcoxon test result at the genus level ($P < 0.05$). **(B)** LEfSe result at the genus level (LDA > 2). **(C)** Wilcoxon test result at the species level ($P < 0.05$). **(D)** LEfSe result at the species level (LDA > 2).

Enterococcaceae were significantly increased in the ADHD group, while *Ruminococcaceae* was significantly decreased (LDA > 2, **Figure 2D**).

KEGG Analysis of Metabolism

A total of 6294 KEGG Orthology (KO) terms were used to annotate the genes. Wilcoxon tests showed 91 KOs that were significantly different between the two groups ($P < 0.01$, **Figure 3**). These included terms related to the neurotransmitter dopamine; the genes encoding the catalytic subunit of protein phosphatase-1 (PP1), threonine synthase, and 6-pyruvoyl-5,6,7,8-tetrahydropterin were significantly upregulated in the ADHD group, while the gene encoding 4-hydroxy threonine-4-phosphate dehydrogenase was significantly downregulated ($P < 0.05$, **Figure 4**).

DISCUSSION

The mammalian intestinal tract contains more than 100 trillion microorganisms; as the largest ecosystem in the body, it influences host physiological functions (Agus et al., 2018). The brain-gut axis theory proposes that there is a bidirectional regulatory mechanism between the intestinal flora and the brain. Children with ADHD may have abnormal neurotransmission, and the intestinal flora may regulate the level of neurotransmitters via complex neuroendocrine pathways (Richarte et al., 2018). A systematic review revealed two studies that assessed the correlation between ADHD and intestinal flora (Lacorte et al., 2019). Both employed 16S rRNA-sequencing technology and only analyzed the difference in gut microflora (Jiang et al., 2018; Prehn-Kristensen et al., 2018).

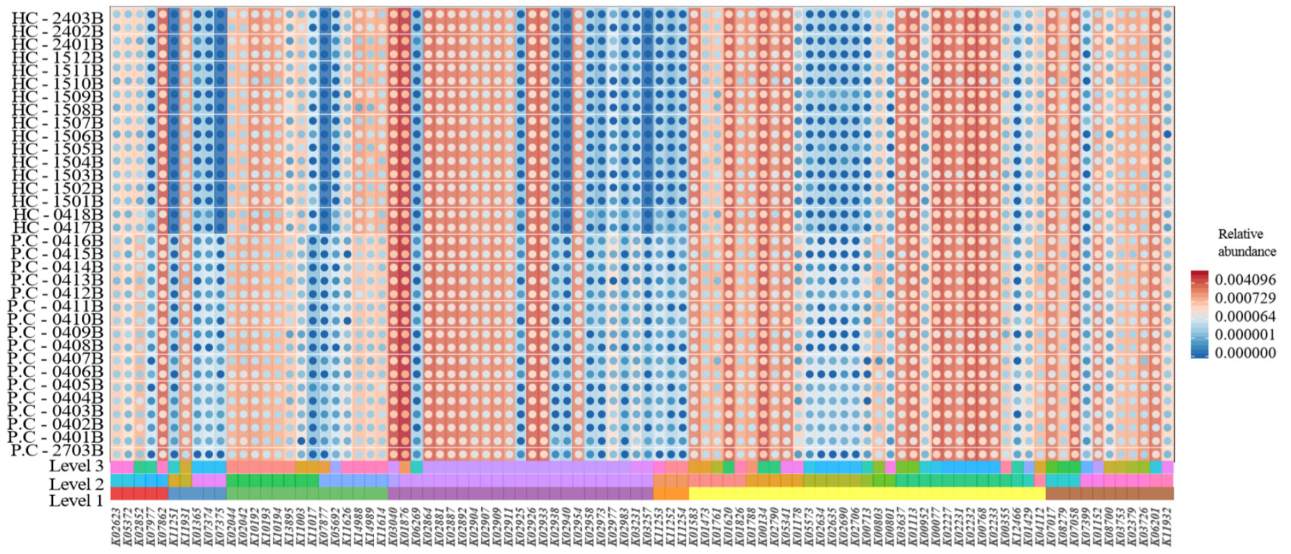

FIGURE 3 | Comparison of Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotations between the ADHD and HC groups.

We also applied shotgun metagenomic sequencing to the whole genomes of microorganisms for each specimen, and KEGG was used to analyze the metabolic pathways and identify possible pathogenetic mechanisms.

Similar to earlier reports (Jiang et al., 2018; Prehn-Kristensen et al., 2018), we found obvious differences in the gut microbiota of the ADHD and HC groups. Contrary to one study (Prehn-Kristensen et al., 2018), we found the alpha diversity of intestinal flora was not significantly different between groups, but subjects with ADHD had significantly lower levels of *Faecalibacterium*. However, unlike a previous report (Jiang et al., 2018), we also found that the ADHD group had significant decreases in *Veillonellaceae*, while *Enterococcus* and *Odoribacter* were significantly increased. At the species level, *F. prausnitzii*, *L. bacterium*, and *R. gnavus* were significantly reduced in the ADHD group, while *B. caccae*, *O. splanchnicus*, *P. xylaniphila*, and *V. parvula* were significantly increased. Additionally, we found that children with ADHD were more prone to constipation; consistent with our finding, previous studies have reported that the imbalance of intestinal flora is closely related to the occurrence of constipation (Huang et al., 2018; Wen et al., 2018; Wang L. et al., 2019).

The pathogenesis of ADHD remains unclear. One research group reported that abnormal levels of neurotransmitters are involved in the disease process (Kovács et al., 2019). Based on this theory, central nervous system (CNS) stimulants such as methylphenidate hydrochloride are widely used as first-line treatments for ADHD. The mechanism may involve inhibition of presynaptic reuptake of noradrenaline and dopamine; higher synaptic levels of these neurotransmitters may help to control symptoms, but clinical treatment effects vary among patients (Wigal et al., 2017).

Previous studies have shown that the gut microbiota could affect the brain-gut axis and contribute to the pathogenesis of neurological diseases including Parkinson's, epilepsy, autism spectrum disorders, and tic disorders (Zhao et al., 2017; Kovács et al., 2019; Rude et al., 2019). Early intestinal flora establishment can affect nervous system development, resulting in anxiety behaviors and other mental health problems after maturity, and treatment of pregnant female rats with low-dose antibiotics has been shown to lead to an imbalance of intestinal flora, with subsequent alterations in the behavior of offspring (Borre et al., 2014; Leclercq et al., 2017; Zhao et al., 2017). In another study where gut bacteria from patients with schizophrenia were transplanted into germ-free mice, the mice developed psychotic symptoms due to altered regulation of the glutamine-glutamate-gamma-aminobutyric acid pathway by the transplanted gut bacteria (Zheng et al., 2019). Similarly, transplantation of intestinal flora from patients with Parkinson's disease into a germ-free Parkinson's disease mouse model significantly increased motor symptoms of the mice, which were ameliorated by antibiotic treatment (Sampson et al., 2016).

According to the results of our experiment, we speculated that the abnormality of intestinal flora might be one of the bases of the onset of ADHD, combined with previous studies, we proposed the following conjecture about its mechanism of action. In this study, children with ADHD exhibited a reduction of *Faecalibacterium*. This has been observed in both animal and human studies and has been implicated in various allergic diseases such as asthma, eczema, and allergic rhinitis (Penders et al., 2007; Arrieta et al., 2015; Melli et al., 2016). In clinical practice, atopic children have a 30–50% increased risk of ADHD (Schans et al., 2017). We therefore speculate that the reduction of this bacterial genus may generate allergies via the brain-gut axis by affecting neurotransmitter release and inducing the pathogenesis of ADHD. One study reported that ADHD was more likely to be induced by diets high in fat, protein, and sugar, which also decrease *Faecalibacterium*

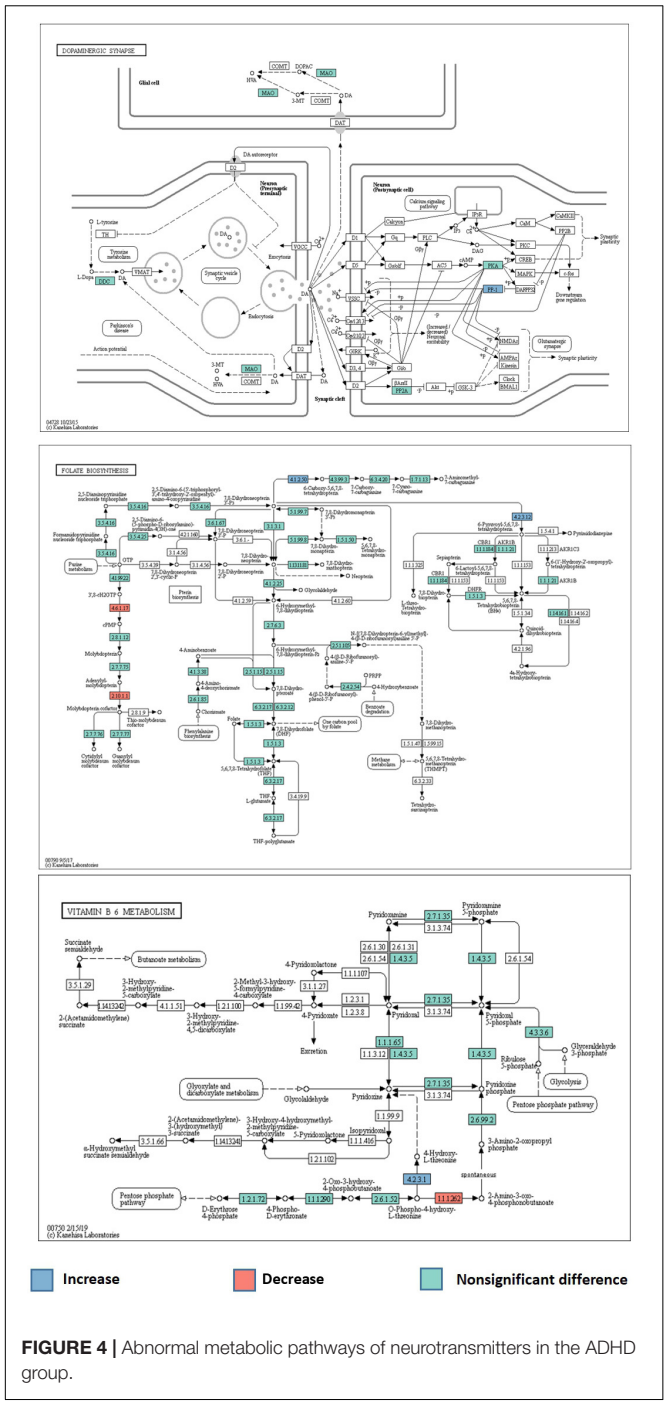

FIGURE 4 | Abnormal metabolic pathways of neurotransmitters in the ADHD group.

levels (Howard et al., 2011). *Faecalibacterium* may exert anti-inflammatory effects, and the abnormal levels may lead to higher expression of inflammatory factors (Qiu et al., 2013; Quévrain et al., 2016). Notably, children with ADHD have significantly higher levels of inflammatory cytokines than normal children (Mitchell and Goldstein, 2014). Inflammatory cytokines can cross the blood–brain barrier (BBB) and affect nervous system development and brain function (Wong et al., 2016). We therefore hypothesized that *Faecalibacterium* dysregulation may cause changes in inflammatory cytokine levels and participate in ADHD pathogenesis.

We also found that the proportion of *Enterococcus* was significantly increased in the ADHD group, and *Enterococcus* has been reported to be closely related to neurotransmitter release. One study demonstrated that *Enterococcus* abundance is significantly increased in mice lacking the 5-HT transporter (Singhal et al., 2019); deficiency of this transporter can lead to decreased 5-HT levels, which is related to ADHD onset (Wang et al., 2018). Interestingly, a study showed *Enterococcus* could lead to excessive intestinal conversion of levodopa (the first-line treatment for Parkinson's disease) into dopamine, however, peripheral dopamine cannot penetrate the BBB to enter the CNS, thus reducing the effectiveness of levodopa (Maini Rekdal et al., 2019). Furthermore, the abnormal increase in *Enterococcus* could also cause excessive activation of tyrosine decarboxylase, which increases the decarboxylation of tyrosine and phenylalanine in the gastrointestinal tract, leading to decreased levels in the CNS and subsequent low levels of levodopa (the drug precursor of dopamine) (Maini Rekdal et al., 2019). Both of these pathways can affect the concentration of dopamine in the CNS, which may aggravate Parkinson's symptoms (Maini Rekdal et al., 2019). Previous studies have shown that ADHD onset is related to decreased CNS levels of dopamine (Roncero and Álvarez, 2014; Ledonne and Mercuri, 2017). As above, we speculate that the observed increase in *Enterococcus* may lower intracranial dopamine and contribute to the development of ADHD. In addition, our observation of a higher proportion of *Odoribacter* in subjects with ADHD is similar to the results of a previous study that found higher *Odoribacter* levels in individuals with pediatric acute-onset neuropsychiatric syndrome (PAN) and pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) (Quagliariello et al., 2018). Additionally, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis of this study showed that the dopamine metabolic pathway was significantly reduced in PAN and PANDAS (Quagliariello et al., 2018). *Odoribacter* may cause abnormalities in dopamine metabolism that contribute to ADHD. Previous studies (Quagliariello et al., 2018; Maini Rekdal et al., 2019) found that abnormal *Enterococcus* and *Odoribacter* levels were associated with dysregulated neurotransmitter production. Abnormal levels of these bacteria were also found in our study, suggesting a role in the development of ADHD.

Finally, we performed KEGG analysis to determine the gene functional annotations and abnormalities in metabolic pathways, to verify the speculation of the role of gut microbiota in the pathogenesis of ADHD. Reduced dopamine levels in the CNS may contribute to ADHD pathogenesis. We identified differences in the dopaminergic synaptic pathways between the ADHD and HC groups; the gene encoding PP1 catalytic subunit was significantly upregulated, which was considered to increase synaptic sodium ion flux. Dopamine receptors are transmembrane sodium/chloride-dependent transporters that belong to the family of transporters of norepinephrine, 5-HT, and dopamine, and are referred to as neurotransmitter: sodium symporters (NSS) (Navratna and Gouaux, 2019).

Prolonged sodium-related signal transduction results in the excessive activation of NSS. Metabolic pathway alterations may cause abnormal neurotransmitter transport and reduce their concentrations in the CNS, which could contribute to ADHD. Numerous studies have reported that vitamin B6 plays a key role in nervous system development and neurotransmitter production. A randomized controlled trial of 216 children with ADHD and 216 healthy children found lower vitamin B6 levels in children with ADHD (Wang L.J. et al., 2019). In line with this finding, KEGG analysis indicated abnormalities in the metabolic pathway of vitamin B6 in the ADHD group. The genes encoding 4-hydroxy threonine-4-phosphate dehydrogenase and threonine synthase were significantly downregulated and upregulated, respectively, which could lead to abnormal levels of pyridoxal 5′-phosphate, which is an important coenzyme of aromatic amino acid decarboxylase (AADC) (Montioli et al., 2019). AADC is a key enzyme of dopamine metabolism that converts levodopa into dopamine in the CNS (Baek et al., 2018). A decrease in its activity could lead to the reduction of dopamine concentrations, which could contribute to ADHD onset. In the folate metabolic pathway, a significant upregulation of the gene encoding 6-pyruvoyl-5,6,7,8-tetrahydropterin could promote the generation of tetrahydrobiopterin (BH4). However, tryptophan hydroxylase is a rate-limiting enzyme that catalyzes 5-HT synthesis, with oxygen and BH4 as substrates (Opladen et al., 2016; Scotton et al., 2019). Upregulation of the gene encoding 6-pyruvoyl-5,6,7,8-tetrahydropterin may lead to the conversion of excessive tryptophan into 5-HT in the intestinal tract, and 5-HT has difficulty crossing the BBB, resulting in decreased CNS 5-HT concentrations, which may contribute to ADHD.

There are several limitations to this study. First, our sample size was relatively small. Second, we did not perform transplantation of intestinal flora to confirm that gut microbiota composition affects ADHD symptoms.

CONCLUSION

In summary, our results demonstrate that gut microbiota alterations occur in children with ADHD, which may contribute to abnormal metabolism of neurotransmitters. We cautiously speculated that the abnormal intestinal flora might be one of contributing factors of ADHD, the underlying mechanism may be related to changes in microbial functions that affect the function of the neuroendocrine system, leading to reduced levels of 5-HT and dopamine in the CNS, and ultimately to ADHD. Further studies should be carried out to investigate the CNS levels of dopamine and 5-HT, and animal studies are needed for functional verification.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the PLA General Hospital Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LW and W-RG contributed equally to the manuscript. LW and GY contributed to the study conception and design. W-RG, SZ, Y-LS, BW, and LW organized the database. W-RG, Y-LS, SZ, LW, and GY performed the statistical analysis. W-RG, LW, and GY wrote the first draft of the manuscript. All authors wrote sections of the manuscript, contributed to the manuscript revision, and read and approved the submitted version.

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frontiers in Cellular and Infection Microbiology

REVIEW
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The Links Between the Gut Microbiome, Aging, Modern Lifestyle and Alzheimer's Disease

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Gut microbiome is a community of microorganisms in the gastrointestinal tract. These bacteria have a tremendous impact on the human physiology in healthy individuals and during an illness. Intestinal microbiome can influence one's health either directly by secreting biologically active substances such as vitamins, essential amino acids, lipids et cetera or indirectly by modulating metabolic processes and the immune system. In recent years considerable information has been accumulated on the relationship between gut microbiome and brain functions. Moreover, significant quantitative and qualitative changes of gut microbiome have been reported in patients with Alzheimer's disease. On the other hand, gut microbiome is highly sensitive to negative external lifestyle aspects, such as diet, sleep deprivation, circadian rhythm disturbance, chronic noise, and sedentary behavior, which are also considered as important risk factors for the development of sporadic Alzheimer's disease. In this regard, this review is focused on analyzing the links between gut microbiome, modern lifestyle, aging, and Alzheimer's disease.

Keywords: Alzheimer's disease, gut microbiome, aging, lifestyle, circadian rhythm

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by memory loss, dramatic changes in character and behavior and an impossibility to carry out normal daily activities in the latter stages of the disease. AD incidence increases with age and is shown to affect ∼10% of people aged 65–75 and 32% of the elderly aged 80 and above (Alzheimer's Association, 2013; Prince et al., 2016). According to the World Health Organization (WHO) incidence of AD is worsening every year, thus it is postulated that there could be a threefold increase in the number of AD patients by 2050. Today it is believed that the pathophysiology of AD is driven by accumulation of different forms of amyloid beta peptide (Aß) in the brain leading to neuro-inflammation, oxidative stress, mitochondrial dysfunction, dysregulation of enzyme systems, and neuronal death. Yet, triggering mechanisms of Aβ deposition in the brain are still being investigated. To date, <5% of all AD cases have clear genetic evidence of increased production of Aβ. Mutations in three genes serve to transmit AD via autosomal-dominant inheritance: the presenilin gene (PS1) on chromosome 14, the presenilin 2 gene (PS2) on chromosome 1, and the amyloid precursor protein gene (APP) on chromosome 21. This form of AD is referred to as a familial Alzheimer's disease (FAD) and is characterized by earlier onset of the symptoms (EOAD, <65 years). However, most cases of AD have a late-onset of the symptoms (≥65 years) and an unclear genetic background

(Panegyres and Chen, 2013, 2014). This type of dementia is called sporadic late-onset AD (LOAD), and it is generally believed that the development of this pathology in the elderly is as a result of an interplay between genetic background and various factors including lifestyle, stress levels, chronic diseases (cardiovascular diseases, obesity, diabetes mellitus), and environment (Prince et al., 2014; Wainaina et al., 2014).

One of the important factors that is influencing human health and attracting increasing attention of scientists during the last two decades is gut microbiome. There are ∼1,000 species and 7,000 strains of bacteria that inhabit the human intestine (1,013–1,014 microorganisms in total), among which the most common are bacteria attributed to *Firmicutes* (51%) and *Bacteroidetes* (48%) (The Human Microbiome Project et al., 2012). *Firmicutes* include both gram-positive and gram-negative species such as those belonging to the genus *Lactobacillus* (gram-positive), *Eubacterium* (gram-positive), *Clostridium* (gram-positive). *Bacteroidetes* comprise of gram-negative bacteria of the genus *Bacteroides* and *Prevotella*. The remaining 1% of bacteria belong to other divisions such as *Proteobacteria* (gram-negative, genus *Escherichia* in particular), *Actinobacteria* (gram-positive, genus *Bifidobacterium* in particular), *Fusobacteria* (gram-negative), *Spirochaetes* (gram-negative), *Verrucomicrobia* (gram-negative), and *Lentispherae* (gram-negative) (Westfall et al., 2017). Until recently, intestinal microbiome was considered to be involved in processes that take place exclusively in the intestine, such as fermentation of carbohydrates, synthesis of vitamins (in particular vitamin B and K), and xenobiotic metabolism as well as acting as a barrier to pathological bacteria. However, over the last 15 years, the functions of the intestinal microbiome have been revised owing to the establishment of a direct link between density and species composition of the intestinal microbiome and a number of pathological conditions including diabetes, obesity, and cardiovascular diseases. These diseases, in turn, are the established risk factors for the development of sporadic AD, and there is data indicating that gut microbiome influences brain functions (Westfall et al., 2017; Zhu et al., 2017; Kowalski and Mulak, 2019). Moreover, recent studies have revealed the significant differences in quantity and quality of gut microbiome in AD patients compared to mentally healthy individuals of the same age (Vogt et al., 2017; Larroya-García et al., 2018; Zhuang et al., 2018).

On the other hand, negative lifestyle aspects, among people living in our modern societies, are also considered important risk factors for the development of LOAD (van Praag, 2018). The most striking result of the epidemiological study above is that radical increases in Alzheimer's disease in Japan and substantial increase in developing countries are associated with changes in national diets (Grant, 2013). Furthermore, there are many undesirable lifestyle factors in the modern society that may contribute to AD development. These factors include unhealthy diet, lack of sleep, circadian rhythm disturbance, chronic noise, sedentary behavior etc., and, in turn, gut microbiome is highly sensitive to these factors. From this point of view, studying the links between modern lifestyle, gut microbiome and Alzheimer's disease is an important task that requires special attention. Understanding the interplays between the human microbiome and the brain, as well as the factors influencing these relations may contribute to a deeper understanding of AD etiology and may serve as a basis for the development of prophylactic measures to prevent or slow down the progression of the disease.

BRAIN-GUT-MICROBIOTA AXIS AND ALZHEIMER'S DISEASE

In the past 10 years, considerable information has been accumulated on the action of microbiome on the central nervous system (CNS) and "brain-gut-microbiota axis" conception was proposed (Kowalski and Mulak, 2019). The CNS regulates the permeability, secretion, motility, and immunity of the digestive tract by exerting its effect on the enteric nervous system, muscle tissue and the mucous layer of the intestine through the efferent autonomic nervous pathways (Carabotti et al., 2015). In turn, the intestinal microbiome is able to influence brain functions through afferent signaling pathways and through the secretion of biologically active substances (Burokas et al., 2015; Petra et al., 2015). There is a number of published data showing the effects of intestinal dysbiosis, caused by changes in diet, the use of antibiotics, non-steroidal anti-inflammatory drugs as well as the presence of pathogenic microorganisms, on cognitive functions of the brain (Gareau, 2014; Jiang et al., 2017).

For example, acute stress and infection caused by conditional pathogenic bacteria *Citrobacter rodentium* have been shown to lead to memory disorders in C57BL/6 mice (Gareau et al., 2011). In sterile Swiss-Webster mice, bred in conditions precluding postnatal existence of bacteria in the intestine, a deficit of spatial and working memory was observed independent of infection and stress. This was accompanied by reduced neurotrophic brain factor (brain-derived neurotrophic factor, BDNF) expression (Gareau et al., 2011). BDNF is one of the key neurotrophins that play an important role in synaptic plasticity, and there is evidence of reduced BDNF levels in the brain and serum of patients with Alzheimer's disease (Michalski et al., 2015). On the contrary, studies conducted by Neufeld et al. revealed increased levels of BNDF in the central amygdale of sterile mice, reduced expression of mRNA encoding the serotonin receptor (5HT1A) and the NR2B subunit of the NMDA receptor (ionotropic glutamate receptor, selectively binding N-methyl-D-aspartate) in the dentate fascia of the hippocampus (Neufeld et al., 2011).

Wang et al. have demonstrated that in rats, intestinal dysbiosis caused by the usage of ampicillin for 1 month lowered the NMDA receptor and mineralocorticoid levels in the amygdala, increased the aggressiveness of the animals and caused impaired spatial memory while the presence of the *Lactobacillus fermentumNS9* strain in the intestinal microbiome normalized these parameters (Wang et al., 2015). Another study by Liang et al. showed that probiotic *Lactobacillus helveticusNS8* significantly improved cognitive impairment caused by chronic stress in Sprague-Dawley rats bred under sterile conditions (Liang et al., 2015). *L. helveticus* NS8 also reduced plasma levels of corticosterone and adrenocorticotropic hormone and increased the content of the anti-inflammatory cytokine IL-10, restored the level of serotonin and norepinifrine, and increased expression of BDNF in the

hippocampus (Liang et al., 2015). Similar data were obtained by Luo et al. (2014) and Ohsawa et al. (2015). In addition, the probiotic *Bifidobacterium Longum 1714* improved cognitive function in male BALB/c mice (Savignac et al., 2015).

Studies of stool samples obtained from transgenic mice expressing the human APP gene and PS1 (CONVR-APPPS1, an animal model of Alzheimer's disease), showed significant differences in the composition of the intestinal microbiome of these animals compared to wildtype mice (Harach et al., 2017). In 8-month-old CONVR-APPPS1 mice there was a significant decrease in the number of *Firmicutes*, *Verrucomicrobia*, *Proteobacteria*, and *Actinobacteria*, and an increase in the content of bacteria belonging to the *Bacteroidetes* and *Tenericutes* compared to wild type mice of similar age. In addition, there was a significant decrease in Aβ deposits in the brain of CONVR-APPPS1 mice bred under sterile conditions compared to animals of the same genotype bred under standard conditions. Microbiota obtained from the intestines of CONVR-APPPS1 mice bred under normal conditions when introduced into intestines of mice bred under sterile conditions led to an increase in pathological Aβ deposits in the central nervous system, while fecal transplantation from wild-type mice did not lead to a significant increase in Aβ levels in the brain.

Studies carried out on laboratory animals are confirmed by clinical data obtained in the study of the intestinal microbiome of the elderly. The association of brain amyloidosis with pro-inflammatory intestinal bacterial taxa and peripheral markers of inflammation in people of old age suffering from cognitive disorders was shown (Cattaneo et al., 2016). The results of this study demonstrated that, in dementia patients with amyloidosis, an increased level of pro-inflammatory cytokines in the blood (IL-6, CXCL2, NLRP3, and IL-1β) was accompanied by a reduced content of *E. rectale* and an increased content of *Escherichia/Shigella* in stool samples. A positive correlation was also demonstrated between pro-inflammatory cytokines and the number of pro-inflammatory intestinal bacteria belonging to the *Escherichia/Shigella* taxon in stool samples, while a negative correlation was found between pro-inflammatory cytokines and the number of anti-inflammatory intestinal bacteria belonging to the *E. rectale* taxon.

A study of the composition of the intestinal microbiome in patients at the Alzheimer's Disease Research Center (Wisconsin Alzheimer's disease Research Center, USA) revealed significant differences in the composition of the intestinal microbiome in patients with AD and healthy people at the phylum and species levels (Vogt et al., 2017). These studies demonstrated a decrease in the number of bacteria in the *Firmicutes* and *Actinobacteria phyla* (in particular, bacteria of the genus *Bifidobacterium*), and an increase in the number of bacteria belonging to the *Bacteroidetes* and *Proteobacteria* phyla in the intestinal microbiome of AD patients. In general, quantitative differences were found between 13 genera of bacteria in AD patients and healthy study participants. In addition, a differential correlation was shown between the levels of individual bacterial genera in the intestine and cerebrospinal markers of AD, such as Aβ42/Aβ40, p-tau, as well as the Aβ/p-tau ratio (Vogt et al., 2017). Studies conducted at Chongqing Medical University (China) also revealed significant differences in the composition of bacteria present in the bowels of patients with AD in taxonomic groups such as *Bacteroides*, *Actinobacteria*, *Ruminococcus*, *Lachnospiraceae*, and *Selenomonadales* (Zhuang et al., 2018). However, qualitative changes in the intestinal microbiome in Chinese patients differed somewhat from those in the United States. Zhuang et al. showed a decrease in the number of bacteria belonging to the phylum *Bacteroidetes*, while the number of bacteria in the phylum *Firmicutes* remained unchanged compared with healthy controls. These differences may be related to a number of factors, including comorbidities, ethnicity, lifestyle, and dietary preferences (Tasnim et al., 2017).

Irrespective of bacterial taxa, the functional composition of the gut microbiota may also be important (Lozupone et al., 2012). In this regards, Liu et al. conducted function analysis of microbiome in AD patients, patients with amnestic mild cognitive impairment (aMCI) and healthy controls (HC) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) functional pathway (Liu et al., 2019a). They identified 5 altered functional orthologs in AD patients using level 3 KEGG pathways. For instance, in AD there were enriched orthologs related to bacterial secretion system (membrane transport) and lipopolysaccharide biosynthesis (glycan biosynthesis and metabolism) compared to HC or aMCI subjects. In contrast, the orthologs related to N-Glycan biosynthesis and phenylalanine, tyrosine and tryptophan biosynthesis, and histidine metabolism in amino acid metabolism were reduced in AD patients, but enhanced in aMCI patients when compared to HC.

The results of these studies demonstrate that the changes in the taxonomic and functional composition of the intestinal flora are able to influence brain functions. Published data also provide an evidence of the effect of intestinal microbiome on the development of amyloid pathology and indicate the possible role of intestinal microbiome as one of the factors of AD pathogenesis. In turn, gut microbiome is a dynamic modifiable system highly sensitive to lifestyle and aging. Thus, in the subsequent chapters we discuss the modern lifestyle factors and aging-related gut microbiome influence and their relations to AD pathology.

GUT MICROBIOME AND AGING

Since advanced age is a major risk factor for AD, age-related physiological changes, including changes in the microbiome, may play a certain role in the development of dementia. In this regard, a number of studies have shown that the composition of the gut microbiome undergoes significant changes with age (Salazar et al., 2017; Nagpal et al., 2018). It was shown that general age-related changes in the composition of the intestinal microflora include an increase in the number of facultative anaerobes, changes in species dominance, while, at the same time, there is stability in the total number of anaerobes (Mariat et al., 2009; Satokari et al., 2010). Hopkins and co-authors observed that the levels of *Bifidobacterium* and *Lactobacillus* were lower in the group of elderly people compared to those of young individuals (Hopkins and Macfarlane, 2002). While the adult organism contains 4–5 species of the genus *Bifidobacterium*,

only one of the dominant species of this genus is found in old age: *Bifidobacterium adolescentis*, or phenotypically close *Bifidobacterium angulatum* and *Bifidobacterium longum* (Gavini et al., 2001; Hopkins and Macfarlane, 2002). One of the possible explanations for the reduced number of species and quantitative composition of *Bifidobacteria* in the elderly people is the decrease in their adhesion to the intestinal wall due to changes in the chemical composition and structure of the colon mucous membrane, causing restricted functionality and immunological reactivity in the intestine as well as increased susceptibility to gastrointestinal infections (He et al., 2001). In turn, the bacteria *Bifidobacterium* and *Lactobacillus* are actively involved in the production of aminobutyric acid (γ-Aminobutyric acid, GABA) (Junges et al., 2018; Strandwitz, 2018). GABA is the most important inhibitory mediator of the central nervous system of humans and other mammals involved in neurotransmitter and metabolic processes in the brain. It has been proven that the level of aminobutyric acid in the intestine correlates with its level in the CNS. Decrease in the number of *Bifidobacterium* and *Lactobacillus* leads to brain dysfunction associated with synaptogenesis disorders, depression, and cognitive impairment (Strandwitz, 2018).

Many authors have noted that Bacteroides species diversity changes with age (Bartosch et al., 2004; Layton et al., 2006). In studies conducted by a group of scientists under the leadership of Tongeren, *Bacteroides/Prevotella, Eubacteriumrectale/Clostridium coccoides*, and *Ruminococcus* prevailed in the microbiota of people aged between 70 and 100 years (van Tongeren et al., 2005). The growth of proteolytic bacteria, such as *Fusobacteria*, *Propionibacteria*, and *Clostridia*, was shown in the intestinal microbiota of elderly people leading to the development of putrefactive processes, especially in patients with post antibiotic therapy. This is confirmed by data on the increase of proteolytic activity (Hopkins and Macfarlane, 2002; Woodmansey et al., 2004). Also, an increased number of pro-inflammatory enterobacteria, streptococci, staphylococci, and yeast cells were found which may be associated with an elevated level of serum antibodies to commensal (normal) intestinal microflora, such as *Escherichia coli* and *Enterococcus faecalis*.

UNHEALTHY NUTRITION LINKED TO ALZHEIMER'S DISEASE AND GUT MICROBIOME

So-called "Western diet" (WD), which is characterized by high intake of saturated fats and added sugars (Weisburger, 1997), is one of the symbols of the modern lifestyle and it is an established risk factor for AD development (Grant, 1999, 2016; Noble et al., 2017). For example, it has been demonstrated that AD rates increased from 1% in 1985 to 7% in 2008 in Japan, and this increase is associated with nutritional transition from the traditional Japanese diet to a Western diet (Dodge et al., 2012). In fact, preclinical experiments have confirmed that high fat diet (HFD) may change the gut microbiota and contribute to development of dementia (Studzinski et al., 2009; Nam et al., 2017; Sah et al., 2017; Sanguinetti et al., 2018). These studies demonstrated that HFD promoted cognitive impairment by inducing oxidative stress and deteriorative neuronal apoptosis via inactivation of Nrf2 signaling pathway (Studzinski et al., 2009; Nam et al., 2017; Sah et al., 2017; Sanguinetti et al., 2018). Nam and coauthors showed that HFD significantly increased amyloid deposition and reduced cognition of 12-months old APP23 mice (Nam et al., 2017). In this study, RNA-seq results showed that genes related to immune response, such as Trem2 and Tyrobp in HFD mice were upregulated, but expression of the genes related to neuron projections and synaptic transmission was decreased. The authors demonstrated that levels of 24 lipid sub-species in the brain were significantly modulated by HFD.

In turn, recent study of microbiome-metabolome signatures in 3xTg-AD mice genetically predisposed to AD and fed a normal or fatty diet have demonstrated that high-fat feeding and genetic predisposition to neurodegenerative disease share common abnormalities in the gut microbiome (Sanguinetti et al., 2018). The authors showed that HFD changed bacterial composition in both colon and caecum, and also lead to reduced abundance of the microorganisms compared to normal diet-fed animals (ND). In this study, HFD mice had elevated abundances of *Firmicutes* than *Bacteroidetes* at phylum levels, *Rikenellaceae*, *Lachnospiraceae*, *Enterococcaceaeand* S24.7 at family level, as well as elevated amount of fecal ribose. High level of *Clostridium* and *Staphylococcus* were also found in the caecum. Study of serum and fecal metabolites revealed a deficiency in unsaturated fatty acids and choline, and an excess in ketone bodies, lactate, amino acids, TMA, and TMAO in 3xTg-AD mice fed a fatty diet. These metabolic changes were associated with high abundance of *Enterococcaceae*, *Staphylococcus*, *Roseburia*, *Coprobacillus,* and *Dorea*, and a low level of *Bifidobacterium*, which in turn are related to cognitive impairment and cerebral hypometabolism.

SEDENTARY BEHAVIOR LINKED TO ALZHEIMER'S DISEASE AND GUT MICROBIOME

Sedentary lifestyle is becoming a significant public health issue in many countries despite being linked to a number of chronic health conditions (Owen et al., 2010). Accumulating evidence indicates that sedentary behavior can be a risk factor for cognitive decline (Wheeler et al., 2017), while physical exercise may be an effective strategy for preventing dementia (Fenesi et al., 2016). It has been shown that the mechanisms underlying the neuroprotective influence of physical activity on Alzheimer's disease are: the production of antioxidant enzymes and growth factors and decrease in ROS and neuroinflammation, the concentration of Aβ plaques and tau protein in the brain (Chen et al., 2016b).

There is also data indicating that exercise can influence gut microbiome (Fernandez et al., 2018), and this effect is especially prominent in obese people and sedentary women (Allen et al., 2017; Bressa et al., 2017). In addition, recent data has demonstrated that physical exercise and probiotics are able to reduce the levels of Aß in the brain and slow

down progression of AD symptoms in transgenic APP/PS1TG mice (Abraham et al., 2019). The authors demonstrated that physical training was capable of increasing abundance of butyrate-producing bacteria i.e., *Butyrivibrioproteoclasticus* and *Marvinbryantiaformatexigens*, and reducing pro-inflammatory bacteria, such as Clostridium, *Eubacterium*, and *Roseburia*. Moreover, exercise decreased the levels of H_2O_2 generating bacteria *L. johnsonii*. It has been suggested that butyrate is a key regulator of inflammatory processes that induces mucin production and reduces the penetration of LPS from intestine into the bloodstream. The authors also concluded that physical activity or proper nutrition alone has a weak effect on dementia, whereas together their effects become significant.

SLEEP DEPRIVATION LINKED TO ALZHEIMER'S DISEASE AND INTESTINAL MICROBIOME

Insufficient sleep is another important public health issue of the twenty-first century (Chattu et al., 2018), and it has been suggested that disrupted sleep may promote the development of Alzheimer's disease (Ju et al., 2013a,b; Shokri-Kojori et al., 2018). For example, animal studies have demonstrated that acute sleep deprivation significantly increases Aβ levels in brain interstitial fluid (Kang et al., 2009), and, in contrast, natural, and anesthetic sleep increases the interstitial space and subsequently enhances convective exchange of cerebrospinal fluid with interstitial fluid resulting in increased rate of Aβ clearance (Xie et al., 2013). Clinical studies have also shown that healthy individuals have morning decrease in Aβ42 in cerebrospinal fluid, but 24 h of total sleep deprivation undoes this decrease (Ooms et al., 2014). Similarly, it has been found that slow wave sleep disruption correlates with an increase in Aβ40 level in cerebrospinal fluid (Ju et al., 2017) and that one night of sleep deprivation induces Aβ accumulation in the brains of healthy individuals (Shokri-Kojori et al., 2018).

It is of our interest that chronic sleep disruption impacts gut microbiome. A study in animals has revealed that chronic sleep fragmentation alters taxonomic profiles of fecal microbiota and induces systemic and adipose tissue inflammation and insulin resistance (Poroyko et al., 2016). Similarly, randomized within-subject crossover study conducted by Benedict et al. demonstrated that partial sleep deprivation (PSD) in normal-weight young individuals affects the human gut microbiota. In particular, PSD increased *Firmicutes:Bacteroidetes* ratio with a higher abundance of the families *Coriobacteriaceae* and *Erysipelotrichaceae*, and lower abundance of *Tenericutes* (Benedict et al., 2016). Contrary to this, Zhang at al. reported that major microbial populations were not altered in sleep-restricted rats and healthy human subjects (Zhang et al., 2017). The authors concluded that the microbiome is largely resistant to changes during sleep restriction and that sleep disruption and microbial dysbiosis are independent health risk factors (Zhang et al., 2017). However, in another research, better sleep quality in healthy older adults was associated with better neuropsychological test performance and higher abundance of microbial phyla *Verrucomicrobia* and *Lentisphaerae* in the stool samples (Anderson et al., 2017). Collectively, these studies suggest that a lack of sleep combined with obesity, diabetes and high-fat diet can be a risk factor for Alzheimer's disease and is associated with changes in the gut microbiome.

CIRCADIAN RHYTHMS, INTESTINAL MICROBIOME AND ALZHEIMER'S DISEASE

A phenomena known as "social jetlag," or the mismatch between social and biological clocks, is common in the modern society and causes circadian rhythm disruption (CRD) (Farhud and Aryan, 2018). One of the causes of CRD is light pollution, which is a typical hallmark of the big cities (Chepesiuk, 2009). It is a matter of fact that sleep deprivation and CRD is one of the common and earliest signs of AD, and there is increasing evidence that CRD might be a contributing factor in AD pathogenesis (Wu et al., 2003; Musiek, 2015; Musiek and Holtzman, 2016; Phan and Malkani, 2019). In support of this notion, there is a study demonstrating that Aβ production is regulated by circadian rhythms with peak concentrations of Aβ occurring during wakefulness (Kang et al., 2009), which is in agreement with the data discussed in the previous chapter. A recent study has shown that targeted deletion of the core clock gene *Bmal1* in *APPPS1-21* transgenic mice resulted in disruption of daily hippocampal interstitial fluid Aβ oscillations, increased expression of *Apoe* and promoted amyloid plaque accumulation (Kress et al., 2018). In addition, it has been demonstrated that the level of melatonin, one of the important regulators of circadian system, is reduced in AD patients (Wu et al., 2003). There is data indicating that melatonin and the circadian rhythms regulate the intestinal microbial flora (Zhu et al., 2018; Parkar et al., 2019), and that circadian rhythm disruption by abnormal light–dark (LD) cycles results in the dysfunction of the intestinal barrier and increases the number *Ruminococcus torques* but reduces that of *Lactobacillus johnsonii* (Deaver et al., 2018). In addition, circadian disruption affects functional gene composition of gut microbiome leading to downregulation of the genes involved in promoting host beneficial immune responses and upregulation of the genes involved in the synthesis and transportation of lipopolysaccharides (LPS) (Deaver et al., 2018), which is similar to the changes of the functional composition of the gut microbiome in AD patients (Liu et al., 2019a).

CHRONIC NOISE STRESS, INTESTINAL MICROBIOME AND ALZHEIMER'S DISEASE

A large number of sources of noise pollution has appeared in the human environment since the onset of post-industrial era (Passchier-Vermeer and Passchier, 2000), thus making chronic noise another hallmark of the modern lifestyle (Seidman and Standring, 2010). Epidemiological and experimental studies showed that chronic noise has been associated with

cardiovascular diseases, hearing impairment, changes in the immune system and birth defects (Passchier-Vermeer and Passchier, 2000; Ising and Kruppa, 2004). Recently, an etiological association between chronic noise exposure and Alzheimer disease was proposed (Cui and Li, 2013). In 2018, a retrospective cohort study conducted by Carey and coauthors demonstrated positive association between residential levels of noise and air pollution across London and incidence of dementia (Carey et al., 2018). A number of animal studies have shown that chronic noise induces tau pathology in the hippocampus and the prefrontal cortex (Manikandan et al., 2006; Cui et al., 2009, 2012a,b). Also, there is published data demonstrating upregulated expression of amyloid precursor protein (APP) and its cleavage enzymes, β- and γ-secretases upon chronic noise exposure (Cui et al., 2015), and the involvement of the corticotropin-releasing factor system in noise-induced alteration in Aβ production (Gai et al., 2017). Furthermore, Cui and et al. reported that cognitive impairment and Aβ accumulation in exposed-to-chronic-noise young SAMP8 mice was associated with the dysregulation of intestinal microbiota (Cui et al., 2018). The authors also found downregulation of endothelial tight junction proteins both in the intestine and brain and upregulation of serum neurotransmitter and inflammatory mediator levels in the mice. Further analysis of gut microbiota has revealed increased abundance of *Firmicutes* and reduced quantity of *Bacteroidetes* on the phylum level, whereas *Candidatus Jettenia, Denitratisoma,* and *SM1A02* levels were increased in noise exposed SAMP8 mice (the genus level) (Cui et al., 2018). Taken together, the studies suggest that chronic noise affects gut microbiome and can be one of the AD triggers.

POSSIBLE MECHANISMS UNDERLYING THE EFFECT OF GUT MICROBIOME ON THE PATHOGENESIS OF ALZHEIMER'S DISEASE

Reducing the number and species diversity of many beneficial anaerobes such as *Bifidobacterium* and *Lactobacillus*, as well as a shift in the diversity of the intestinal microbiota toward conditional pathogenic and pathogenic microorganisms, results in changes in local intestinal chemical and immunological parameters and induces the translocation of the gut bacteria into focal lymphoid tissue (Nagpal et al., 2018). These factors contribute to an increase in permeability of the intestinal and blood-brain barriers and the penetration of pathological microflora and their metabolites into the brain (Tran and Greenwood-Van Meerveld, 2013; Elahy et al., 2015).

On the other hand, intestinal bacteria are able to excrete functional amyloid peptides and lipopolysaccharides (LPS) in large quantities. Amyloid peptide in bacteria contributes to various physiological processes on the surface of bacterial cells, such as biofilm formation, adhesion, interaction with other bacterial and eukaryotic cells, etc. Its structure and biophysical properties are similar to human pathological amyloid (Evans et al., 2018). For example, pro-inflammatory conditional pathogenic bacteria of the intestine such as *Escherichia coli, Baccilus subtilis, Salmonella tyrhimurium*, and *Salmonella enterica* are able to secrete large amounts of the bacterial amyloid peptide *curli* (Hufnagel et al., 2013; Schwartz and Boles, 2013). The *curli* peptide, like Aβ, forms a secondary structure of β-folded sheets and stains with thioflavin and Congo red (dyes used to stain the brain's amyloid plaques). It was shown that the main structural subunit of the *curli* peptide, the precursor of amyloid gA (gA amyloid precursor), has in its structure sections similar to Aβ42, and that sections can be recognized by the human TLR2 receptor (tall-like receptor 2) (Rapsinski et al., 2015). Interaction of TLR2 with *curli* peptide or human Aβ42 leads to the activation of bone marrow macrophages and their production of pro-inflammatory cytokines, such as IL-6 and IL-1β (Rapsinski et al., 2015). In a similar study, microbial amyloid was shown to be able to activate T-lymphocytes and induce the production of pro-inflammatory interleukins IL-17A and IL-22(Nishimori et al., 2012). These cytokines are able to penetrate the blood-brain barrier and cause the production of reactive oxygen species, activation of the TLR2/1 and NFkB signaling pathways in microglia and astrocytes, which is directly related to neuroinflammation and neurodegeneration (Perriard et al., 2015; Sun et al., 2015; Zhan et al., 2018). Besides Chen et al. demonstrated that oral contamination of old rats (previously subjected to antibiotic treatment) with wild *E. coli* strain, capable of producing the functional curli peptide, led to an increase in brain tissue microgliosis and astrogliosis and increased expression of TLR2, IL6, and TNF (Chen et al., 2016a).

In addition to the amyloid peptide, many intestinal bacteria secrete LPS. LPS are the main components of the outer cell wall of gram-negative bacteria and, in the case of penetration from the intestinal cavity into the bloodstream, can cause neuro-inflammatory reactions. Published data indicates that the LPS level in the blood plasma of patients suffering from sporadic lateral sclerosis and AD is three times higher than the physiological age norm (Zhang et al., 2009). Post-mortem studies revealed that the level of LPS in the neocortex and hippocampus in patients with AD was two to three times (and in some cases 26 times) higher than in older people of the same age who did not suffer from cognitive disorders (Zhao et al., 2017). Studies on laboratory animals showed that intraventricular administration of LPS for 4 weeks can cause chronic neuroinflammation, nerve cell death of II and III layers of the entorhinal cortex and impairment of the long-term synaptic plasticity of the neurons of the dentate gyrus of the hippocampus, which is one of the characteristic signs of damage to the temporal lobes of cerebral hemispheres inAD (Hauss-Wegrzyniak et al., 2002).

There is also evidence that LPS secreted by bacteria *Bacteroidesfragilis* can activate the pro-inflammatory transcription factor NFkB involved in the pathogenesis of AD in human primary microglial cells (Zhao and Lukiw, 2018). NFkB induces transcription of pro-inflammatory miRNAs, such as miRNA-9, miRNA-34a, miRNA-125b, miRNA-146a, and miRNA-155, activating neuro-inflammatory mediators and inhibiting phagocytosis (Zhao and Lukiw, 2018). For example, micro-RNA-34a has been shown

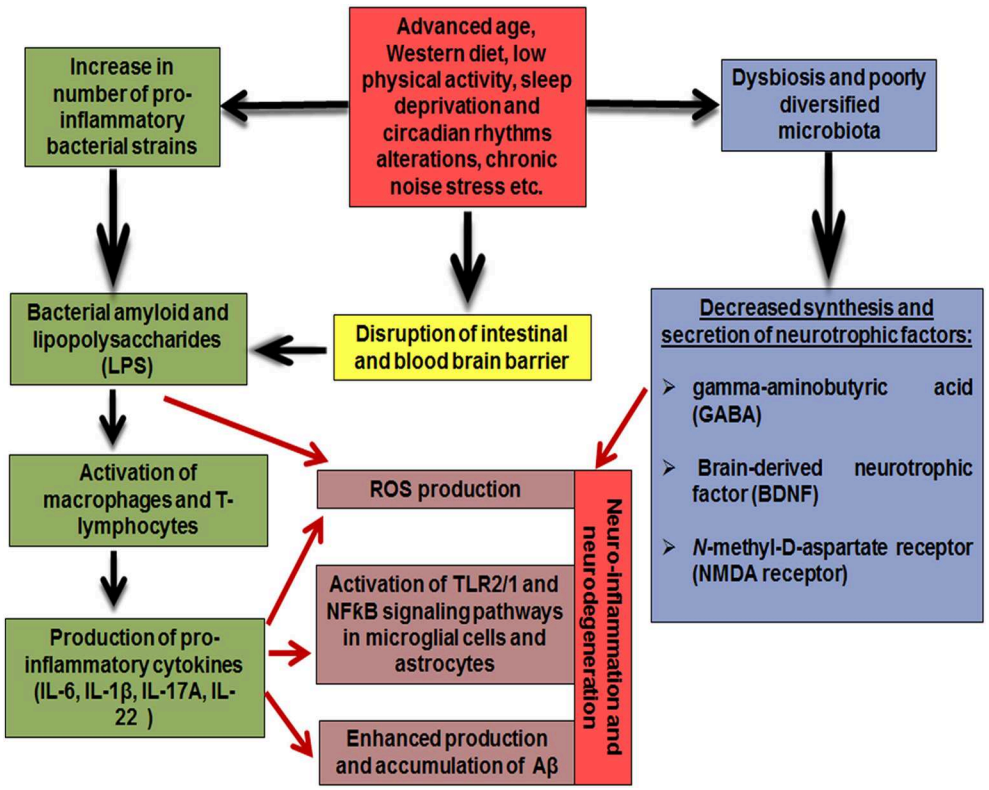

FIGURE 1 | Mechanisms underlying the effect of gut microbiome on the pathogenesis of Alzheimer's disease.

to inhibit TREM2 expression (the triggering receptor expressed on microglia/myeloid cells-2), thereby disrupting the phagocytic ability of microglia and increasing Aβ42 accumulation (Bhattacharjee et al., 2016). In support of this concept, the intraperitoneal administration of LPS to mice line C57BL/6J led to an increase in Aβ42 level in the brain and induced cognitive deficits (Kahn et al., 2012). *In vitro*, endotoxins secreted by *Escherichia coli* strains (*E. coli*) accelerated Aβ aggregation and fibril formation (Asti and Gioglio, 2014). Jaeger and colleagues showed that intraperitoneal administration of LPS disrupts Aβ transport through the blood-brain barrier, increasing its influx and decreasing efflux (Jaeger et al., 2009). It was also shown that intraventricular infusion of LPS in combination with ascorbic acid increased the immunoreactivity of intra-neuronal beta-amyloid (Hauss-Wegrzyniak and Wenk, 2002).

Thus, the composition of gut microbiome changes significantly with aging: the diversity of beneficial bacteria, such as *Lactobacillus and Bifidobacteria,* decreases, and, in a contrast, a number of "unhealthy" pro-inflammatory bacteria, such as *Propionibacteria, Fusobacteria, Shigella*, and *Clostridia* increases. Furthermore, unhealthy modern lifestyle factors including high fat diet, sedentary behavior, lack of sleep, circadian rhythm disturbance and chronic noise alter the composition of gut microbiome in the same way, thus, exacerbating negative impact of aging. In turn, lack of probiotic strains affects synthesis and secretion of the neurotrophic factors, such as BDNF, NMDA receptor and GABA, while pro-inflammatory gut microbiota taxa are capable of secreting bacterial amyloid and lipopolysaccharides, which are considered to be neurotoxic. Since elderly people experience impaired barrier functions of the intestinal wall and the blood-brain barrier, these endotoxins are able to penetrate from the intestinal cavity into the bloodstream, and further into the brain tissue, and have a direct and/or systemic negative effect on the structure and functions of the CNS and promote the development of neuro-inflammation and neurodegeneration (**Figure 1**).

ORAL MICROBIOME AND ALZHEIMER'S DISEASE

There is new scientific evidence published recently that aside the gut microbiome, oral microflora is also able to influence brain functions (Orr et al., 2020). Numerous studies have shown that periodontal disease is associated with neurodegeneration and cognitive decline (Kamer et al., 2008; Cerajewska et al., 2016; Wang et al., 2019). Chang and coauthors reported that chronic periodontitis of 10 years duration was associated with a 1.707-fold increase in the risk of developing AD (Chen et al., 2017). A nationwide, retrospective, matched-cohort study

in Taiwan showed that patients with chronic periodontitis and gingivitis have a higher risk of developing dementia compared to those with healthy gums (Tzeng et al., 2016). Moreover, recent accumulating evidence has demonstrated a causal relationship between oral microbiome and AD (Paganini-Hill et al., 2012; Harding et al., 2017; Liu et al., 2019b; Panza et al., 2019; Olsen and Singhrao, 2020). For example, *P. gingivalis,* the most common periodontal bacteria causing periodontal disease, was capable of inducing accumulation of amyloid-beta plaques and neurofibrillary tangles following experimental oral infection in mice (Dominy et al., 2019). In turn, serum antibodies for *P. gingivalis* have been found to be elevated in AD patients (Kamer et al., 2009), and protein-degrading enzyme gingipain produced by *P. gingivalis,* was found in the brain of Alzheimer's patients (Singhrao and Olsen, 2019). Dominy et al. have demonstrated that oral administration of small-molecule inhibitors of gingipain block gingipain-induced neurodegeneration, decreased *P. gingivalis* load in the mouse brain and the host Aβ42 response *to P. gingivalis* brain infection (Dominy et al., 2019). It was also found that chronic systemic *P. gingivalis* infection causes Aβ accumulation in inflammatory monocytes/macrophages via the activation of CatB/NF-κB signaling (Kunkle et al., 2019).

In addition, there is data indicating the significant differences in quantity and quality of oral microbiome in AD patients compared to mentally healthy individuals of the same age. For example, Liu et al. have demonstrated lower richness and diversity of salivary microbiome in patients with Alzheimer's disease compared to healthy controls (Liu et al., 2019b). The authors reported a relatively high level of *Moraxella*, *Leptotrichia*, and *Sphaerochaeta* and significantly decreased number of *Rothia* in the saliva of AD patients (Liu et al., 2019b). However, these authors emphasize the limitations of the study due to the absence of many periodontal bacteria in saliva which exist within the subgingival niche or dental plaques (Filoche et al., 2009). Therefore, a comprehensive picture of the full composition of oral microbiome in patients with AD requires further research.

CONCLUSION

Recent studies strongly suggest that gut and oral microbiome is capable of modulating the neurochemical and neuro-metabolic signaling pathways of the brain through the formation of a two-way communication axis involving the endocrine and immune systems, and contribute to the development of neuro-inflammation and neurodegeneration. In turn, there is a strong correlation that exists between AD and modern lifestyle factors. It is a fact that unhealthy diet, lack of sleep, circadian rhythm disturbance, chronic noise, and sedentary behavior are linked to neurodegeneration. By focusing on the mechanism for interaction between lifestyle factors and AD, we can evaluate the contribution of changing modern society to the increase in prevalence of AD. However, tackling this issue is impossible without understanding its intertwined relationship with other aspects, and gut microbiome is crucial for this interaction. From this point of view, the study of the composition of the intestinal microbiome in patients with AD and healthy aging people is of considerable interest. This could unearth novel associations between intestinal microbiome, lifestyle, and dementia, and help to develop practical recommendations for the prevention and treatment of this severe pathology.

AUTHOR CONTRIBUTIONS

SA conceptualized the structure, wrote the first draft, and edited the final version of the manuscript. BU co-wrote the first draft. A-RM, AKa, YS, AT, FO, and AKu made substantial contribution to the content. All authors approved the final version of the manuscript.

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Altered Caecal Neuroimmune Interactions in the Neuroligin-3^{R451C} Mouse Model of Autism

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The intrinsic nervous system of the gut interacts with the gut-associated lymphoid tissue (GALT) *via* bidirectional neuroimmune interactions. The caecum is an understudied region of the gastrointestinal (GI) tract that houses a large supply of microbes and is involved in generating immune responses. The caecal patch is a lymphoid aggregate located within the caecum that regulates microbial content and immune responses. People with Autism Spectrum Disorder (ASD; autism) experience serious GI dysfunction, including inflammatory disorders, more frequently than the general population. Autism is a highly prevalent neurodevelopmental disorder defined by the presence of repetitive behavior or restricted interests, language impairment, and social deficits. Mutations in genes encoding synaptic adhesion proteins such as the R451C missense mutation in neuroligin-3 (NL3) are associated with autism and impair synaptic transmission. We previously reported that NL3^{R451C} mice, a well-established model of autism, have altered enteric neurons and GI dysfunction; however, whether the autism-associated R451C mutation alters the caecal enteric nervous system and immune function is unknown. We assessed for gross anatomical changes in the caecum and quantified the proportions of caecal submucosal and myenteric neurons in wild-type and NL3^{R451C} mice using immunofluorescence. In the caecal patch, we assessed total cellular density as well as the density and morphology of Iba-1 labeled macrophages to identify whether the R451C mutation affects neuro-immune interactions. NL3^{R451C} mice have significantly reduced caecal weight compared to wild-type mice, irrespective of background strain. Caecal weight is also reduced in mice lacking Neuroligin-3. NL3^{R451C} caecal ganglia contain more neurons overall and increased numbers of Nitric Oxide (NO) producing neurons (labeled by Nitric Oxide Synthase; NOS) per ganglion in both the submucosal and myenteric plexus. Overall caecal patch cell density was unchanged however NL3^{R451C} mice have an increased density of Iba-1 labeled enteric macrophages. Macrophages in NL3^{R451C} were smaller and more spherical in morphology. Here, we identify changes in both the nervous system and immune system caused by an autism-associated mutation in Nlgn3 encoding the postsynaptic cell adhesion protein, Neuroligin-3. These findings provide further insights into the potential modulation of neural and immune pathways.

Keywords: caecum, mice, autism, neuroimmune, gut-associated lymphoid tissue

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INTRODUCTION

Emerging evidence suggests that altered communication between the nervous system and inflammatory pathways is associated with multiple diseases including autism. Both altered inflammatory activity (Wei et al., 2011) and a maternal history of autoimmune diseases, such as rheumatoid arthritis and celiac disease, is associated with an increased risk of autism (Atladóttir et al., 2010). The gut-associated lymphoid tissue (GALT) plays a crucial role in mucosal immunity and microbial populations. Caecal patches are lymphoid aggregates located at the blind end of the caecum and contain various immune cells such as macrophages and dendritic cells.

The precise role of the caecum is unclear, but it has been suggested that the appendix in humans houses a "reserve population" of commensal microbes (Randal Bollinger et al., 2007). The caecal patch contributes to gut homeostasis and is a major site for the generation of IgA-secreting cells that subsequently migrate to the large intestine (Masahata et al., 2014). Secretory IgA plays an important role in regulating the activities and compositions of commensal bacteria populations in animal models (Fagarasan et al., 2002; Suzuki et al., 2004; Peterson et al., 2007; Strugnell and Wijburg, 2010). However, whether caecal innervation and immune function are altered in preclinical models of neural disorders is unknown.

Autism is a neurodevelopmental disorder affecting 1 in 59 children (Loomes et al., 2017; Baio et al., 2018). In many autism patients, core features (impairments in social interaction, communication, and repetitive and/or restrictive behaviors) are present along with immunological dysfunction (Marchezan et al., 2018) and gastrointestinal (GI) disorders (Valicenti-McDermott et al., 2006; Buie et al., 2010; Coury et al., 2012). Individuals with autism are four times more likely to experience frequent GI symptoms including alternating diarrhea and constipation, and abdominal pain compared to children with typical development (McElhanon et al., 2014). Interestingly, Inflammatory bowel disease (IBD) is present at significantly higher rates in people with autism than the general public (Kohane et al., 2012). Autism-associated GI dysfunction includes increased GI permeability along with altered motility (Horvath and Perman, 2002; Parracho et al., 2005; Kohane et al., 2012; Neuhaus et al., 2018). Mice expressing the Neuroligin-3 R451C mutation exhibit autism-relevant behaviors including impaired social interaction (Tabuchi et al., 2007; Etherton et al., 2011), a heightened aggression phenotype (Burrows et al., 2015; Hosie et al., 2019), impaired communication (Chadman et al., 2008) and increased repetitive behaviors (Rothwell et al., 2014). Furthermore, the robust aggression phenotype in these mice is rescued by a clinically relevant antipsychotic, risperidone (Burrows et al., 2015), highlighting that this model is useful for preclinical studies. These mice also show altered GI motility, in line with the notion that alterations in the nervous system may also affect the ENS to result in GI dysfunction (Gershon and Ratcliffe, 2004; Hosie et al., 2019).

Most research to date in animal models of autism has focused on replicating the core traits of ASD, in addition to using invasive techniques to highlight changes in neural network activity in the brain (Tabuchi et al., 2007; Halladay et al., 2009; Lonetti et al., 2010; Etherton et al., 2011; Patterson, 2011; Schmeisser et al., 2012; Varghese et al., 2017; Hosie et al., 2018). Using these approaches, it is well established that many gene mutations identified in autism patients affect neuronal function. Here we assessed whether the autism-associated R451C mutation in Neuroligin-3 affects gross caecal morphology, enteric neuronal populations or immune cells within the caecal patch.

METHODOLOGY

Animals

Adult male $NL3^{R451C}$ mice (8–14 weeks old) and wild type (WT) littermate controls from two different colonies were used in this study. Neuroligin 3 knockout mice ($NL3^{-/-}$; 12 weeks old) were also examined. $NL3^{R451C}$ mutant mice (B6;129-$Nlgn3^{tm1Sud}$/J) were originally obtained from Jackson Laboratories (Bar Harbour, MI, USA) and maintained on a mixed background ($mbNL3^{R451C}$) strain at the Biomedical Sciences Animal Facility, The University of Melbourne (Hosie et al., 2019). These mice were then backcrossed onto a C57BL/6 background for more than 10 generations (i.e., $B6NL3^{R451C}$ mice) and maintained at the animal facility at RMIT University, Bundoora, Australia. In contrast, $NL3^{-/-}$ mice (Radyushkin et al., 2009; Leembruggen et al., 2019) were bred on a C57BL/6NCrl background at the Florey Institute of Neurosciences and Mental Health. All $NL3^{R451C}$ mice were culled by cervical dislocation following RMIT University and The University of Melbourne animal ethics guidelines (AEC# 1727, AEC# 1513519). $NL3^{-/-}$ mice were cervically dislocated and fresh tissue was collected for other applications (AEC# 14095). All data from mutant mice were compared with matched WT littermate controls from the respective cohorts to remove environmental and additional genetic factors (i.e., data from $mbNL3^{R451C}$ animals were compared with mbWT mice; $B6NL3^{R451C}$ vs. B6WT mice and C57BL/6NCrl $NL3^{-/-}$ mice vs. C57BL/6NCrl WT littermates).

All mice from each cohort were housed in mixed genotype groups of up to six per cage to minimize the impact of environmental factors. This study was carried out following the Basel Declaration and all experiments conducted at RMIT University were approved by the RMIT University Animal Ethics Committee and experiments conducted at The University of Melbourne were approved by The University of Melbourne Animal Ethics Committee.

Caecal Collection

The caecum was collected and weighed from $B6NL3^{R451C}$, $mbNL3^{R451C}$ mice and $NL3^{-/-}$mice. The caecum from each mouse was opened and pinned with the mucosa facing upwards and submerged in 0.1 M PBS on a petri dish lined with sylgard (Sylgard Silicone Elastomer, Krayden Inc., Denver, CO, USA), enabling visualization of the lymphoid patch (i.e., the caecal patch). Images of caecal tissue with a measuring scale were captured and caecal area measured using ImageJ software (ImageJ 1.52a, NIH, Bethesda, MD, USA).

Wholemount Tissue Preparation

Caecal myenteric and submucosal plexus neurons were revealed by microdissection using fine forceps and dissecting spring scissors. The submucosal plexus was revealed by removing the mucosal layer and carefully exposing neurons adjacent to the circular muscle within the caecal tissue. To obtain the myenteric plexus, the circular muscle was then peeled away from the remaining caecal tissue. A small area of tissue (approximately 0.5 cm^2) containing myenteric and submucosal plexuses was transferred into a small Petri dish, submerged in 0.1 M PBS for labeling by immunofluorescence.

Wholemount Immunofluorescence for Neuronal Populations

Immunofluorescence staining was performed on wholemount caecal tissue samples to assess for potential differences in neuronal cell numbers between $NL3^{R451C}$ and WT mice. Wholemount samples of myenteric and submucosal plexus were incubated at room temperature (RT) for 30 min in 0.01% Triton (to permeabilize the tissue for improved access by primary and secondary antibodies) with 10% CAS-block (Invitrogen Australia, Mt-Waverley, Australia; to reduce non-specific binding of antibodies). Then, tissues were incubated with 30 μl primary antisera; human anti-Hu (1:5,000, a pan-neuronal marker; a gift from Dr. V. Lennon, Mayo Clinic, Rochester, MN, USA) and sheep anti-neuronal Nitric Oxide Synthase (NOS; 1:400; Abcam, Eugene, OR, USA) and kept at 4°C overnight in a sealed container. After incubation, caecal tissues were washed with 0.1 M PBS (three washes of 10 min duration). Secondary antisera (30 μl) were applied to the samples and left for 2.5 h at RT on a shaker incubator (Digital Shaking Incubator OM11, Ratek, Australia). Caecal tissues were mounted using fluorescence mounting medium (DAKO Australia Private Limited; Botany, NSW, Australia).

Imaging of Caecal Neuronal Populations

Images of caecal tissue containing the submucosal, myenteric plexus were analyzed using ImageJ (ImageJ 1.52a, NIH, Bethesda, MD, USA) and Imaris software (Imaris 64X 9.1.0; Bitplane AG, UK). 10 myenteric ganglia and 10 submucosal ganglia were selected from each wholemount caecal tissue sample (n = 5 $NL3^{R451C}$ and n = 5 WT samples). From each ganglion, the number of Hu and NOS stained cells were counted.

Caecal Patch Tissue Collection

Caecal tissues including caecal patch samples were fixed in 4% formaldehyde solution at 4°C overnight. The next day, tissue samples were washed three times (10 min per wash) with filtered 0.1 M PBS. The caecal patch was excised from the caecal tissue using spring scissors. Caecal patch samples were subsequently placed into a 30% sucrose solution in distilled water overnight at 4°C for cryoprotection. Caecal patches were placed in a cryomold (Tissue-Tek Cryomold, Sakura, Finetek, USA) filled with optimal cutting temperature compound (Tissue-Tek, OCT compound, Sakura, Finetek, USA). Cryomolds containing caecal patch samples were then snap frozen using liquid nitrogen and tissue blocks stored at −80°C. Frozen caecal patch samples were sectioned at 6-micron thickness using a cryostat (Leica CM1950 Clinical Cryostat, Leica Biosystems Nussloch GmbH, Germany) and collected on positively charged slides (Thermo Fisher Scientific, Waltham, MA, USA Menzel-Glaser, Superfrost^R plus, New Hampshire, USA and stained for Haematoxylin & Eosin (H&E) to assess for overall cell density.

Caecal Patch Image Analysis

Images were obtained using an Olympus slide scanner microscope (VS120-S5; Olympus Australia Private Limited; Melbourne, VIC, Australia) and the cell density within the caecal patch was analyzed using ImageJ software (ImageJ v1.52a, NIH, Bethesda, MD, USA). The entire area of each caecal patch was selected to calculate the area of the caecal patch and cell numbers within that area. The total number of cells was then divided by the area of interest to calculate the number of cells per 100 μm^2.

Caecal Patch Immunofluorescence

Immunofluorescence was also performed on cross-sections of caecal patch tissue samples to assess for altered density and morphology of macrophages. To observe a subpopulation of immune cells within the caecal patch, immunofluorescence for the immune cell marker Iba-1 (1:3,000, Abcam, USA) was conducted. The sections were incubated for 30 min with 0.1% triton and 10% CAS-block at RT. Thirty microliters of primary antibody was subsequently applied to each section and kept at 4°C overnight in a moisture sealed container. After incubation, caecal patch sections were washed with 0.1 M PBS (3 × 10 min washes). Secondary antiserum was applied to the samples and left for 2.5 h at RT on a shaker incubator. Caecal sections were mounted using fluorescence mounting medium (DAKO Australia Private Limited; Botany, Australia) containing DAPI (4′,6-diamidino-2-phenylindole) and stored at 4°C overnight. Tissue samples were imaged using a confocal electron microscope (Nikon Confocal Microscope: A1; Version 4.10). A Z-series of images of caecal patch sections (30 μm thickness) were captured and saved in the ND2 file format. Imaris software (Imaris 64X 9.1.0; Bitplane AG, UK) was used for 3D cellular reconstruction of Iba-1 labeled macrophages.

Statistical Analysis

Potential statistical differences between groups were identified using Student's t-tests.

RESULTS

Mouse body weight, caecal weight, and caecal tissue area were assessed to determine if anatomical changes occur in the presence of the autism-associated R451C mutation in mice. To address whether the R451C mutation and the *Nlgn3* gene itself plays a broader role in caecal weight, ceacae from $NL3^{R451C}$ mice bred on two different background strains were weighed, and caecal weights from mice lacking Nlgn3 compared to WT littermates were also compared.

The average body weight of WT (n = 39) and $NL3^{R451C}$ (n = 34) mice was similar (26.38 ± 0.4 g and 26.46 ± 0.4 g, WT

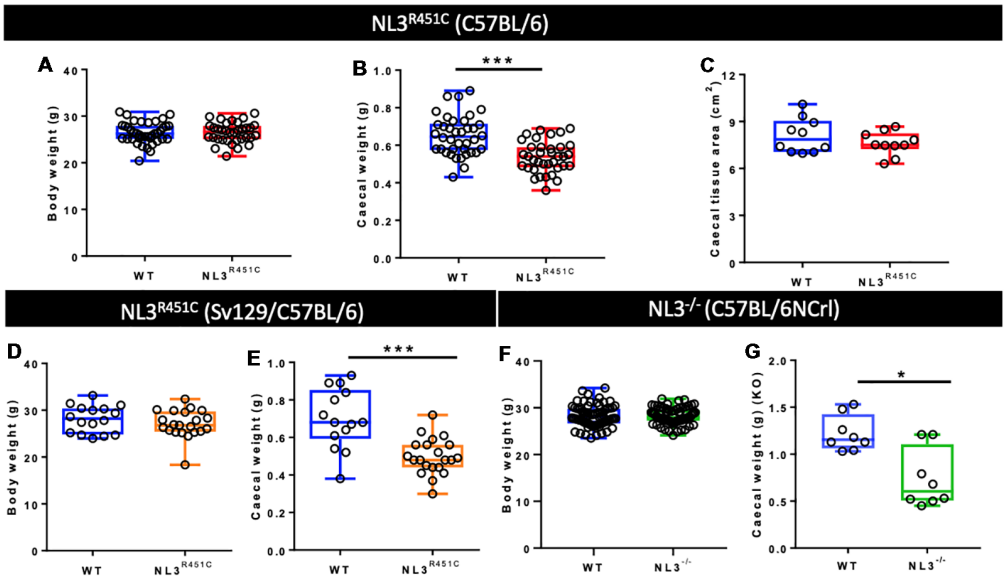

FIGURE 1 | Bodyweight, caecal weight, and caecal tissue area. **(A)** Pure C57BL/6 background wild type (WT) and NL3^{R451C} mice (red) show similar body weights. WT (n = 39) and NL3^{R451C} (n = 34) mice; p = 0.88. **(B)** Caecal weight is reduced in NL3^{R451C} C57BL/6 background mice; WT (n = 38) and NL3^{R451C} (n = 36) mice. **(C)** Similar caecal tissue area for WT (n = 15) and NL3^{R451C} (n = 16) mice. **(D)** In mixed background mice (orange), no differences in body weight were found. WT (n = 16) and NL3^{R451C} (n = 21) mice; p = 0.30. **(E)** Caecal weight is also reduced in NL3^{R451C} (orange) mixed background mice. WT (n = 14) and NL3^{R451C} (n = 21) mice). **(F)** Body weight is unchanged in a large cohort of WT (n = 70) and NL3$^{-/-}$ (n = 71) mice; p = 0.95. **(G)** Reduced caecal weight in NL3$^{-/-}$ (green) mice. WT (n = 8) and NL3$^{-/-}$ (n = 8) mice. Student's t-test *$p < 0.05$; ***$p < 0.001$. Each symbol indicates an individual mouse. Mixed background mice were bred on a mixed Sv129/C57BL/6 genetic background; KO: NL3$^{-/-}$ mice (bred on C57BL/6NCrl mice).

and NL3^{R451C} respectively; p = 0.88; **Figure 1A**). To determine if the reduction in NL3^{R451C} caecal weight was due to a reduction in the size of the caecum itself, total caecal tissue area was measured. No difference between the caecal area of WT (n = 15) and NL3^{R451C} (n = 16) mice was observed (7.99 ± 0.36 and 7.75 ± 0.5 cm^2, respectively; p = 0.51; **Figure 1B**). To determine if the R451C mutation affects caecal structure in mice, the fresh caecal weight from 38 WT and 36 NL3^{R451C} mice was recorded. NL3^{R451C} caecae were significantly lighter than WT (0.65 ± 0.02 g and 0.54 ± 0.01 g, WT and NL3^{R451C} respectively; p = 0.0001; **Figure 1C**). A role for the *Nlgn3* gene in influencing caecal weight is supported by similar observations in NL3^{R451C} mice bred on a mixed background strain and in Nlgn3$^{-/-}$ (NL3$^{-/-}$) mice in which the *Nlgn3* gene is deleted. In mice expressing the R451C mutation bred on a mixed background (mb) strain, the average body weight was similar (28.11 ± 1.01 g and 27.1 ± 0.9 g, WT and mbNL3^{R451C} n = 16 and n = 21, respectively; p = 0.30; **Figure 1D**). Caecal weight was also reduced in mb strain mutant littermates (0.69 ± 0.11 g, 0.49 ± 0.28 g; WT (n = 14) and mbNL3^{R451C} (n = 21), respectively; $p < 0.0001$; **Figure 1E**). Bodyweight is unchanged in a large cohort of WT (n = 70) and NL3$^{-/-}$ (n = 71) mice aged 10–12 weeks; p = 0.95; **Figure 1F**. Similar to data from both the C57BL/6 and mb strains of NL3^{R451C} mice, KO (NL3$^{-/-}$) mice also revealed a reduction in caecal weight (1.16 ± 0.5 g and 0.61 ± 0.53 g; WT and NL3$^{-/-}$, respectively, n = 8 in each group; p = 0.02; **Figure 1G**). These findings suggest a role for the *Nlgn3* gene in regulating caecal weight in mice.

To investigate whether the NL3^{R451C} mutation alters neural populations in the caecal submucosal and myenteric plexus, immunofluorescence for the pan-neuronal marker Hu and NOS (which labels approximately 20–40% of myenteric neurons capable of synthesizing NO (Sang and Young, 1996), the major inhibitory enteric neurotransmitter of the ENS) was conducted. Wholemount preparations of WT (**Figures 2A–C**) and NL3^{R451C} (**Figures 2D–F**) submucosal plexus were labeled with Hu and NOS to quantify neuronal subpopulations. The total number of neurons (i.e., labeled by Hu) per submucosal ganglion was increased in NL3^{R451C} mice (5 ± 0.2 and 6 ± 0.2 neurons, WT and NL3^{R451C}, respectively, n = 5 in each group; p = 0.04; **Figure 2G**). Similarly, NL3^{R451C} mice showed increased numbers of NOS immunoreactive neurons per ganglion (2 ± 0.2 and 3 ± 0.2 cells; WT and NL3^{R451C} respectively, n = 5 in each group; p = 0.003; **Figure 2H**). In submucosal neurons, there was also an increased percentage of NOS neurons per ganglion in WT and NL3^{R451C} mice (43 ± 3% and 55 ± 3%; WT and NL3^{R451C} respectively; p = 0.02; **Figure 2I**).

Wholemount preparations of WT (**Figures 3A–C**) and NL3^{R451C} (**Figures 3D–F**) myenteric plexus were labeled with Hu and NOS. Similar to findings in the submucosal plexus, more myenteric neurons (labeled for Hu) were seen in NL3^{R451C} mice (11 ± 0.3 and 15 ± 1 neurons/ganglion, WT and NL3^{R451C} respectively, n = 5 in each group; p = 0.002; **Figure 3G**). The number of NOS stained caecal myenteric neurons per ganglion was also increased in NL3^{R451C} mice (5 ± 0.3 and 9 ± 0.2 neurons/ganglion, WT and NL3^{R451C}, respectively,

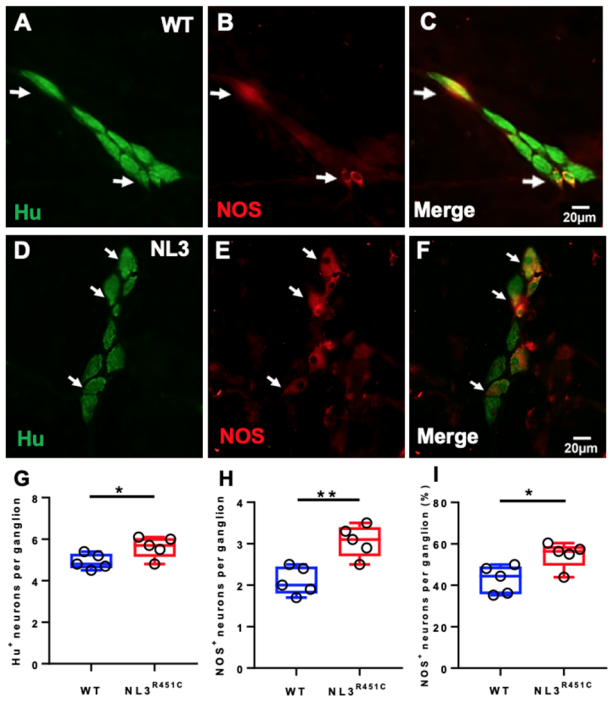

FIGURE 2 | Caecal submucosal neuronal numbers and proportions. WT caecal submucosal plexus ganglia labeled for Hu **(A)** Nitric Oxide Synthase (NOS; **B**); and overlap illustrated in the merged image **(C)**. $NL3^{R451C}$ caecal submucosal plexus ganglia labeled for Hu **(D)** NOS **(E)**; merge **(F)**. Scale bar = 20 μm. **(G)** The total number of Hu labeled neurons per ganglion. **(H)** The total number of NOS stained cells per ganglion. **(I)** Proportions of NOS stained neurons/ganglion; $n = 5$ in each group. Each symbol indicates an individual mouse. Bars in boxplots indicate the mean and range of the data. White arrows indicate neurons immunoreactive to both Hu and NOS. Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

$n = 5$ in each group; $p < 0.0001$; **Figure 3H**). The percentage of NOS stained neurons per myenteric ganglion was also increased in $NL3^{R451C}$ mice (41 ± 1.3% and 58 ± 2.0%; WT and $NL3^{R451C}$ respectively; $p = 0.0001$; **Figure 3I**). These data show that the R451C mutation results in increased numbers of caecal submucosal and myenteric neurons in mice.

To assess whether the R451C mutation alters the GALT structure, we measured total cell density in H&E stained cross-sections of the caecal patch of WT (**Figures 4A,B**) and $NL3^{R451C}$ (**Figures 4C,D**) mice. Caecal patch cellular density was similar in both genotypes (1276 ± 48 and 1428 ± 22 cells/100 μm^2, WT and $NL3^{R451C}$ mice respectively; $n = 8$ in each group; $p = 0.28$; **Figure 4E**).

In healthy intestinal mucosa, mononuclear phagocytes comprising both macrophages and dendritic cells are the most abundant leukocyte population and play an important role in maintaining homeostasis (Kühl et al., 2015). However, little is known about the morphology and role of macrophages associated with GALT in the intestine (den Haan and Martinez-Pomares, 2013) such as the caecal patch. Caecal patch samples were labeled with the pan-nuclear marker, DAPI, and a pan-macrophage antiserum targeting the ionized calcium-binding adaptor molecule 1 (Iba-1) to determine whether the R451C mutation affects these immune cells in WT (**Figures 5A–D**) and $NL3^{R451C}$ (**Figures 5E–H**) within the caecal patch. $NL3^{R451C}$ caecal patch tissue had a higher density of Iba-1 stained cells (14 ± 0.7 cells/100 μm^2, $n = 5$) compared to WT mice (10.5 ± 1 cells/100 μm^2, $n = 4$; $p = 0.02$; **Figure 5I**). The volume of Iba-1 stained cells in WT was larger than in $NL3^{R451C}$ mice (928.5 ± 97 μm^3 and 559.7 ± 58 μm^3; WT ($n = 4$) and $NL3^{R451C}$ ($n = 5$), respectively; $p = 0.01$; **Figure 5J**). Iba-1 stained cells in $NL3^{R451C}$ mice showed increased sphericity (0.6 ± 0.04 and 0.7 ± 0.02 arbitrary units; WT ($n = 4$) and $NL3^{R451C}$ ($n = 5$) respectively; $p = 0.007$; **Figure 5K**). These results suggest that the autism-associated R451C mutation in *Nlgn3* alters macrophage density and morphology within the caecal GALT.

DISCUSSION

The nervous system and the immune system are in constant bidirectional communication (reviewed in Margolis et al., 2016). Altered immune responses and gut dysfunction commonly occur in individuals genetically susceptible to autism (Coury et al., 2012). Altered neuronal communication in autism (Betancur et al., 2009; Grabrucker et al., 2011; Huguet et al., 2016), likely contributes to changes in the peripheral nervous system, and therefore GI function (Hosie et al., 2019; Leembruggen et al., 2019).

A main finding from this study is the clear reduction of caecal weight in mice expressing the Neuroligin-3 R451C mutation. Importantly, in addition to our findings on a pure C57BL/6 genetic background, caecal weight was also reduced in mice bred on a mixed background in a different animal facility. These findings, therefore, confirm a persistent effect of the gene mutation and rule out genetic susceptibility due to background strain or environment. Furthermore, mice lacking Neuroligin-3 expression ($NL3^{-/-}$ mice) that were bred in a third animal facility, and therefore experienced a different environment to the two $NL3^{R451C}$ strains, also had reduced caecal weight. Together, these findings suggest that the Nlgn gene plays a role in caecal neuroimmune physiology and that the reduction in weight is unlikely solely due to diet, microbial populations, and other environmental factors. A reduction in caecal weight has also been reported in a mouse model of obesity. For example, obese mice fed a high-fat diet (diet-induced obese mice) had caecal weights approximately 50% reduced compared to controls, and this reduction was restored by antibiotic treatment (Soto et al., 2018). Since obesity is associated with increased inflammation, our observations in $NL3^{R451C}$ mice might also indicate elevated inflammatory cytokine levels, which remain to be assessed.

The reduced caecal weight in $NL3^{R451C}$ mice may indicate changes in caecal mucus thickness. The hydrophilic mucus layer that coats the GI tract plays an important role in innate host defense (Mowat, 2003). Changes in the mucus thickness could contribute to an altered immune response in the host organism (Liévin-Le Moal and Servin, 2006; McGuckin et al., 2009). Accordingly, altered mucus thickness along the GI tract

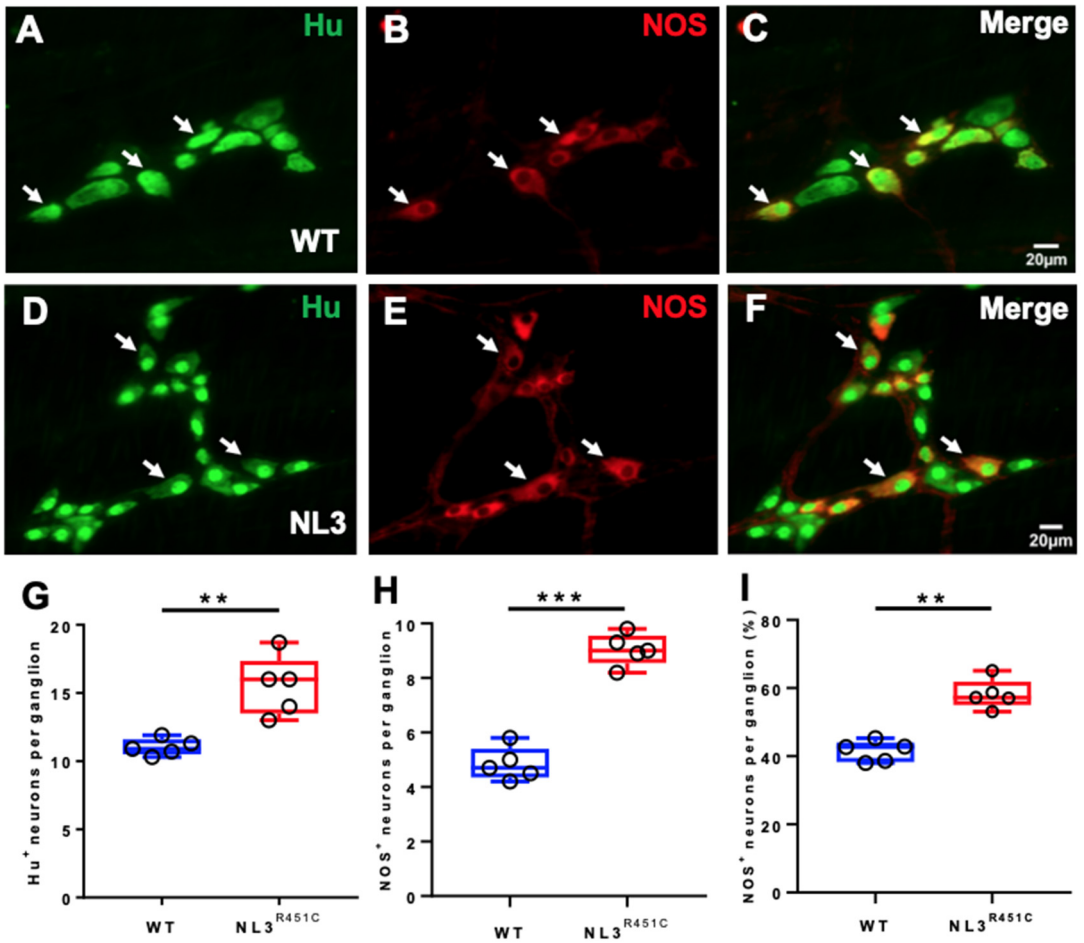

FIGURE 3 | Caecal myenteric neuronal numbers and proportions. WT caecal submucosal plexus ganglia labeled for **(A)** Hu (green), **(B)** NOS (red) and **(C)** merge. $NL3^{R451C}$ caecal submucosal plexus ganglia labeled for **(D)** Hu (green) **(E)** NOS (red), **(F)** merge. Scale bar = 20 μm. **(G)** The number of Hu^+ neurons/ganglion. **(H)** The number of NOS immunoreactive neurons/ganglion. **(I)** The percentage of NOS neurons/ganglion; $n = 5$ in each group. Each symbol indicates an individual mouse. Bars in boxplots indicate the means and range of the data. White arrows indicate neurons immunoreactive to both Hu and NOS. Student's *t*-test $^{**}p < 0.01$; $^{***}p < 0.001$.

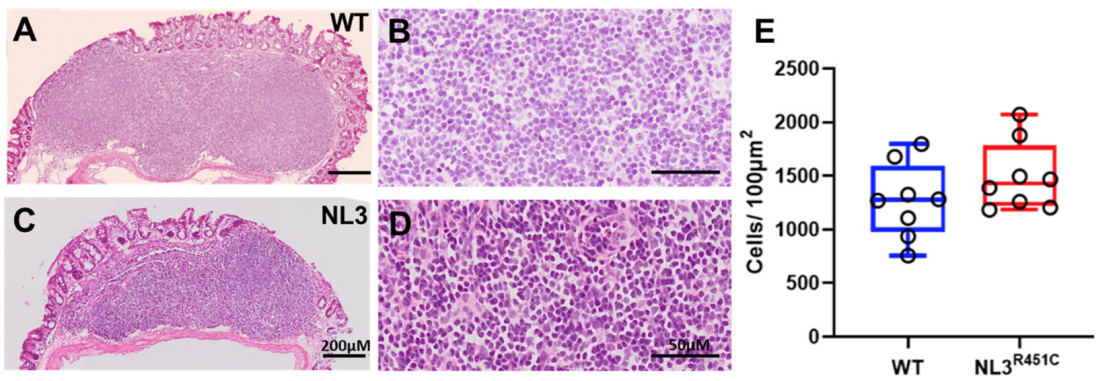

FIGURE 4 | Caecal patch cell density. Haematoxylin and Eosin (H&E) stained transverse sections of caecal patches from WT **(A,B)** and $NL3^{R451C}$ **(C,D)** mice. **(E)** There was no difference in overall caecal patch cell density in WT ($n = 8$) and $NL3^{R451C}$ mice ($n = 8$). Each symbol indicates an individual mouse. Bars in boxplots indicate the mean and range of the data.

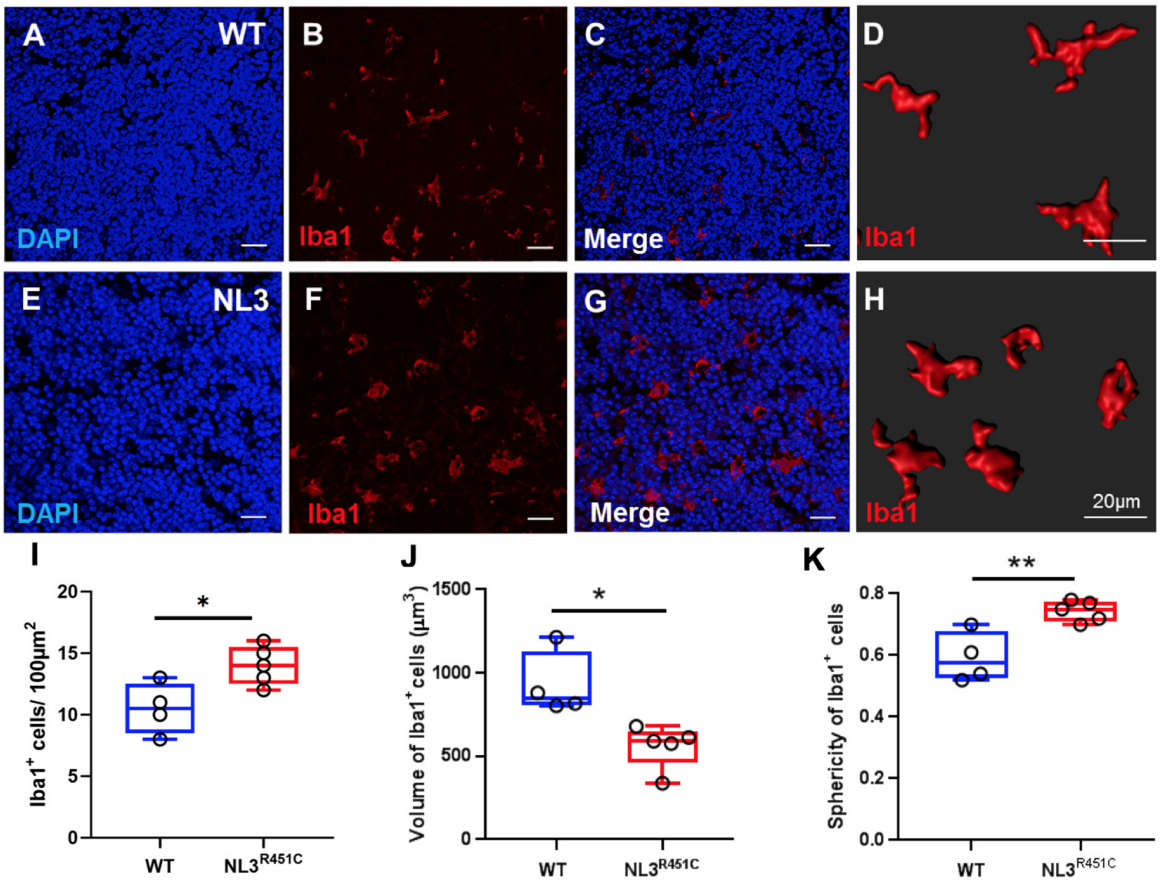

FIGURE 5 | Caecal patch macrophage density and morphology. WT caecal patch tissue labeled for **(A)** DAPI and **(B)** Iba-1, **(C)** merge; **(D)** 3-D reconstruction of Iba-1 labeled cell morphology. $NL3^{R451C}$ caecal patch tissue labeled for **(E)** DAPI **(F)** Iba-1, **(G)** merge, **(H)** 3-D reconstruction of Iba-1 labeled cell morphology. **(I)** Density of Iba-1 stained cells in WT and $NL3^{R451C}$ caecal patch tissue. **(J)** Volume of Iba-1 stained cells in WT and $NL3^{R451C}$ caecal patch tissue. **(K)** Sphericity of Iba-1 stained cells in WT and $NL3^{R451C}$ mice. Each symbol indicates an individual mouse (n = 4 WT and n = 5 $NL3^{R451C}$). Bars in boxplots indicate the mean and range of the data. Student's t-test $^{*}p < 0.05$; $^{**}p < 0.01$. Scale bars = 20 µm.

may contribute to GI dysfunction which is commonly observed in children with autism. Based on studies in preclinical models of other disorders, aberrant mucus production may be present alongside other phenotypic traits. For example, caecal tissue sampled from a mouse model of stroke (72 h after brain injury) showed decreased numbers of mucus-producing goblet cells compared to sham-treated mice (Houlden et al., 2016). Reductions in goblet cell number and size were also reported in mice during the development of ulcerative colitis (Van der Sluis et al., 2006; Johansson et al., 2008). Although potential changes in caecal weight were not correlated with these observations, a thinning of the adherent mucus layer and reduced total mucus volume within the caecum may contribute to the significant reduction in caecal weight in $NL3^{R451C}$ mutant mouse strains identified here.

The enteric nervous system (ENS) regulates GI motility and secretion, as well as nutrient uptake and gut immune and inflammatory processes (Goyal and Hirano, 1996). The two main cell populations of the ENS are neurons and enteric glial cells (EGCs; Jessen, 2004). Many studies have identified enteric neuron pathologies in the context of inflammatory disease (Marlow and Blennerhassett, 2006; Boyer et al., 2007; Winston et al., 2013; Talapka et al., 2014; Rahman et al., 2015; Li et al., 2016), but how alterations in the ENS might affect inflammatory pathways remains largely unknown. Nevertheless, altered neuronal activity has previously been implicated in altering immune function, where reports investigating NO levels in human colonic and rectal mucosal biopsies in active ulcerative and Crohn's disease showed elevated expression of Nitric oxide synthase (NOS; Rachmilewitz et al., 1995; Ljung et al., 2006).

Changes in enteric neuronal numbers are reported in animal models demonstrating GI dysfunction (Schneider et al., 2001; de Fontgalland et al., 2014; Hosie et al., 2019). Our findings that both submucosal and myenteric neuronal numbers are increased in $NL3^{R451C}$ mice caecal tissue indicate that the R451C mutation likely alters neuronal populations during development. These results are in agreement with our previous report showing increased jejunal neuronal numbers in adult $NL3^{R451C}$ mice bred on a mixed genetic background (Hosie et al., 2019). In addition to a potential developmental effect, these findings suggest that the

$NL3^{R451C}$ mutation may influence caecal function. Specifically, we speculate that the R451C mutation could alter the rhythmic caecal "churning" of waste that occurs post digestion and before expulsion *via* the colon, however, this hypothesis remains to be investigated. The contractile activity of the GI tract is neurally regulated so given that the R451C mutation is expressed in the gut in these models (Hosie et al., 2019), it would indeed be of interest to assess whether $NL3^{R451C}$ mice show altered caecal motility.

In addition to characterizing changes in enteric neuronal populations in $NL3^{R451C}$ mice, we investigated the effects of the autism-associated R451C mutation on macrophages in caecal tissue using the pan-macrophage marker, Iba-1. $NL3^{R451C}$ mice showed increased numbers of Iba1 stained cells in caecal patch tissue compared to WT mice. Also, the volume of Iba-1 immunoreactive cells was decreased and are more spherical in $NL3^{R451C}$ mutant mice compared to WT littermates. These findings could indicate that macrophages within $NL3^{R451C}$ caecal patch tissue are present in a more reactive state compared to WT mice, with potential implications for immune pathways in this model. Similar observations were reported in disease conditions such as IBD, where both the number and morphology of intestinal macrophages are altered (Mowat and Bain, 2011; Bain and Mowat, 2014). Moreover, macrophages are integral to the pathogenesis of Crohn's disease (Smith et al., 2011).

In summary, to further assess the impact of the neuroligin-3 R451C mutation on both the enteric nervous system and the immune responses within the caecum, experiments should investigate changes in mucus properties, potential alterations in the mucus-producing goblet cells of the epithelium, inflammatory pathways, and caecal function; including permeability and motility, in this model. Each of these areas of investigation will yield valuable findings about the fundamental role of the caecum in mice as well as the pathophysiology resulting from this mutation.

CONCLUSION

This is the first study to assess the impact of the Neuroligin-3 R451C mutation on caecal structure at both an anatomical and cellular level in mice. The observation that the Neuroligin-3 gene plays a role in regulating caecal weight across multiple genetic backgrounds and environments identifies a new role for the *Nlgn3* gene in mice. This work also highlights the caecum as a region of interest within the GI tract that may play a central role in modulating neuro-immune interactions. In the context of neurodevelopmental disorders, our findings that an autism-associated mutation that affects nervous system function also impacts GALT have implications for identifying novel interactions between the enteric nervous system, microbes located within the gut lumen, immune pathways and potential therapeutic targets for GI dysfunction.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by RMIT University and The University of Melbourne animal ethics committees (AEC# 1727, AEC#1513519).

AUTHOR CONTRIBUTIONS

GB, SH, AF, and EH-Y conceptualized, supervised and designed the research. SS, EH-Y, GB, and JN harvested the tissue and SS and GB undertook the research. SS, SH, and EH-Y drafted the initial version of the manuscript. All authors read and contributed to the final manuscript.

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REVIEW
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Overview of Brain-to-Gut Axis Exposed to Chronic CNS Bacterial Infection(s) and a Predictive Urinary Metabolic Profile of a Brain Infected by *Mycobacterium tuberculosis*

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A new paradigm in neuroscience has recently emerged – the brain–gut axis (BGA). The contemporary focus in this paradigm has been gut → brain ("bottom-up"), in which the gut-microbiome, and its perturbations, affects one's psychological state-of-mind and behavior, and is pivotal in neurodegenerative disorders. The emerging brain → gut ("top-down") concept, the subject of this review, proposes that dysfunctional brain health can alter the gut-microbiome. Feedback of this alternative bidirectional highway subsequently aggravates the neurological pathology. This paradigm shift, however, focuses upon non-communicable neurological diseases (progressive neuroinflammation). What of infectious diseases, in which pathogenic bacteria penetrate the blood–brain barrier and interact with the brain, and what is this effect on the BGA in bacterial infection(s) that cause chronic neuroinflammation? Persistent immune activity in the CNS due to chronic neuroinflammation can lead to irreversible neurodegeneration and neuronal death. The properties of cerebrospinal fluid (CSF), such as immunological markers, are used to diagnose brain disorders. But what of metabolic markers for such purposes? If a BGA exists, then chronic CNS bacterial infection(s) should theoretically be reflected in the urine. The premise here is that chronic CNS bacterial infection(s) will affect the gut-microbiome and that perturbed metabolism in both the CNS and gut will release metabolites into the blood that are filtered (kidneys) and excreted in the urine. Here we assess the literature on the effects of chronic neuroinflammatory diseases on the gut-microbiome caused by bacterial infection(s) of the CNS, in the context of information attained via metabolomics-based studies of urine. Furthermore, we take a severe chronic neuroinflammatory infectious disease – tuberculous meningitis (TBM), caused by *Mycobacterium tuberculosis*, and examine three previously validated CSF immunological biomarkers – vascular endothelial growth factor, interferon-gamma and myeloperoxidase – in terms of the expected changes in normal brain metabolism. We then model the downstream metabolic

effects expected, predicting pivotal altered metabolic pathways that would be reflected in the urinary profiles of TBM subjects. Our cascading metabolic model should be adjustable to account for other types of CNS bacterial infection(s) associated with chronic neuroinflammation, typically prevalent, and difficult to distinguish from TBM, in the resource-constrained settings of poor communities.

Keywords: gut-brain axis, tuberculous meningitis, immunological biomarker, metabolism, urinary profiling, chronic neuroinflammation, bacterial infectious diseases

INTRODUCTION

A new paradigm in neuroscience has emerged in recent years – the brain–gut axis (BGA) – involving bidirectional communication between the brain and gut. This implicates a variety of pathways, including the enteric nervous system (ENS), central nervous system (CNS), gastrointestinal tract (GIT), endocrine system/GI hormones, and immune response, all integrated to orchestrate the bidirectional feedback loop of the BGA. As averred by Hippocrates, the Greek physician acknowledged by many as the father of modern medicine, "*All disease starts in the gut.*" The gut-microbiome is made up of innumerable microbes, which function in a mutualistic relationship with the human host (Collins et al., 2012; Zhu et al., 2017). Currently, scientific evidence supports the notion that homeostatic imbalance is initiated in the gut-microbiome, mediated by several microbe-derived molecules, in the gut–brain ("bottom-up") direction of communication (Foster and Neufeld, 2013; Martin et al., 2018). Stable gut microbiota are essential for normal gut physiology and contribute to appropriate signaling along the BGA (Forsythe et al., 2010; Cryan and Dinan, 2012; Schroeder and Bäckhed, 2016). Over the past decade, however, neuroscience research on the BGA has focused on how perturbations in the gut-microbiome affect the brain in a feedback loop, centered on the premise of "*you are what you eat*" and "*gut feelings*" (Moos et al., 2016; Sherwin et al., 2016; Zmora et al., 2019). Considering the bottom-up motif, particularly its perturbations in the gut-microbiome, can have a clear and direct effect on the host's psychological state-of-mind (depression, anxiety, bipolar disorder), behavior (autism) and also in the pathogenesis and/or progression of various neurodegenerative diseases (Alzheimer's, Parkinson's, and multiple sclerosis). These disorders associated with the bottom-up direction of communication have been succinctly and meticulously detailed in many topical research reviews (Mayer et al., 2014; Konturek et al., 2015; Powell et al., 2017; Zhu et al., 2017; Martin et al., 2018; Ambrosini et al., 2019). Perturbations of the BGA associated with non-communicable neurological diseases – to what degree, the precise mechanism involved, and their appropriate therapy – are not yet well understood. Many studies on the role of microbiota in the pathogenesis of neurodegenerative/psychiatric diseases exist, however, and their main findings are summarized in **Table 1**.

The focus of this review is on the brain–gut ("top-down") direction of the BGA. In particular, perturbations of brain metabolism induced by invading bacteria and, as a consequence, gut dysbiosis. Within the contemporary paradigm of a perturbed BGA, most of the relevant research centers on non-communicable neurological diseases, synonymous with a slow, gradual progression of neuroinflammation. However, the link between the brain–gut concept and CNS bacterial infection(s) is less prevalent in the literature, and hence the focus of this review. The most recent and comprehensive review of the BGA was by Cryan et al. (2019). However, only a very small section, amounting to half a page, discusses infections and the brain, even though bacterial penetration of the blood–brain barrier (BBB), and subsequent infection, leads to a cascade of events within the brain, modulating a feedback effect on the host gut-microbiome (Dando et al., 2014; Bauer et al., 2016; Martin et al., 2018). Bacterial infection(s) of the CNS induce an inflammatory response via glia mediators, pivotal to establishing communication between the host's immune system and the brain (DiSabato et al., 2016) and, ultimately, generating sustained feedback on the BGA (Geyer et al., 2019).

As a proof of a novel concept for the BGA, we use three previously validated immunological CSF markers of tuberculous meningitis (TBM) – vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ), and myeloperoxidase (MPO) – to model/predict the metabolic changes, and are the basis for postulating a metabolic cascade, expected within the brain of a TBM patient. It is well known that important diagnostic and prognostic information related to alterations in metabolic cascades and disruption of homeostasis can be characterized through metabolite profiling of urine (An and Gao, 2015; Emwas et al., 2015). Hence, logic dictates that if the BGA exists then the impact of chronic CNS bacterial infection(s) (such as TBM) should be reflected in the host's urine.

BRAIN–GUT CONCEPT

According to the brain–gut ("top-down") concept, the brain can alter the community structure and function of the gut-microbiome in a bidirectional interaction feedback loop, characterized by continuous communication between the CNS and the GIT (Zhu et al., 2017; Karol and Agata, 2019). The GIT is a highly complex organ involved in multiple dynamic physiological processes, while interacting with the gut-microbiome – an extensive and diverse community of bacteria (Parker et al., 2018). The brain nerves (e.g., vagus nerve), which control unconscious tasks, run from the brainstem to the gut, maintaining the physical bidirectional communication between the CNS and intestinal wall. The brain-to-gut signaling pathway affects host–bacteria interactions in the GIT by influencing

TABLE 1 | Main findings from studies describing the role of microbiota in the pathogenesis of neurodegenerative/psychiatric diseases.

Disorders	Main findings	References
Neurodegenerative		
Parkinson's disease (PD)	(i) Gut microbiota influence the activity of enteric neurons, affecting cellular α-synuclein (α-syn) secretion, characterized by the accumulation and aggregation of α-syn in the substantia nigra (SN).	Braak et al., 2003
	(ii) Gastrointestinal dysfunction is present in ∼80% of PD patients.	Mulak and Bonaz, 2015
	(iii) α-Synucleinopathy is suggested to be an early indicator of PD pathology.	Nair et al., 2018
	(iv) The vagal nerve, which serves as channel for α-syn from the ENS to the CNS, is crucial for the communication between gut microbiota and the brain.	Ulusoy et al., 2013; Scheperjans et al., 2015; Fitzgerald et al., 2019
	(v) Pathological hallmarks of PD are a loss of dopaminergic neurons in the SN and the presence of cytoplasmic eosinophilic inclusions termed Lewy bodies (LBs).	Lebouvier et al., 2009
	(vi) Immunolabeling with α-syn antibodies have become the reference standard in the assessment of LBs and Lewy neurites in both the CNS and peripheral nervous system. Hence, α-synucleinopathy affects all levels of the BGA.	Lebouvier et al., 2009
Alzheimer's disease (AD)	(i) AD is characterized by a deposition of amyloid beta (Aβ) followed by the formation of plaques, characterized by a progressive decline in cognitive function.	Wang et al., 2014; Jouanne et al., 2017
	(ii) Gut microbiota produce amyloids which aid bacterial cell binding, and form part of the biofilm protecting these from destruction by host immune factors.	Friedland and Chapman, 2017
	(iii) Bacterial amyloid proteins exposure to the host, from the gut, may be detrimental since they prime of the host's immune system against endogenous production of neuronal amyloids in the brain.	Kowalski and Mulak, 2019
	(iv) Bacterial lipopolysaccharides are increased in the neocortex and the hippocampus in AD.	Zhao et al., 2017
	(v) Calprotectin is indicative of inflammation and has be detected in elevated amounts in the CSF, brain and fecal matter of AD patients.	Kowalski and Mulak, 2019
Multiple sclerosis (MS)	(i) MS is a demyelinating disease, clinically associated with autoimmune disease. Progressive degradation of the integrity of the epithelia that comprise cellular barriers essential to maintaining the integrity of both intestine and CNS, have been associated in MS patients suffering from autoimmunity, resulting in paralysis and other related symptoms of MS.	Ochoa-Repáraz and Kasper, 2014; Dendrou et al., 2015; Ochoa-Repáraz et al., 2018
	(ii) Clinical signs of MS are relapse of sensory, motor and cerebellar complications; while an acute disease stage is a characteristic feature of the relapsing-remitting MS (the latter of which are often diagnosed with neuronal dysfunction).	Johnston and Joy, 2001; D'Amico et al., 2016; Connick et al., 2018
	(iii) Secondary-progressive MS develops and transcends into progressive neurological impairment.	D'Amico et al., 2016
	(iv) Dysbiosis affects the immunological responses of the host to the microbiota, as described in an experiment where germ-free mice with an immune dysfunction, were characterized by an imbalance between pro- and anti-inflammatory immune cells in the gut, where after colonization of the gut with commensal microbes restored immune function.	Mazmanian et al., 2005; Kirby and Ochoa-Repáraz, 2018; Ochoa-Repáraz et al., 2018
Neuropsychiatric		
Autism spectrum disorders (ASD)	(i) Dysbiosis in children with ASD has been show to contribute to both gastrointestinal and CNS abnormalities.	Wang et al., 2011; Santocchi et al., 2016
	(ii) Short-chain fatty acid producing bacteria, and their metabolites, especially propionic acid, has been indicated to adversely affect the CNS and contribute to autism behavior by modulating the BGA.	De Angelis et al., 2015
	(iii) Behavioral abnormalities are accompanied by imaging abnormalities in the sensory and emotion regulation regions of the brain.	Green et al., 2013
	(iv) Abnormally elevated levels of lipopolysaccharides have also been associated with the pathogenesis of autism.	Fattorusso et al., 2019
	(v) 40% of ASD patients complain of GI symptoms; abnormalities such as chronic diarrhea, constipation, vomiting, feeding problems, reflux and abdominal pain, as well as anxiety.	Mayer et al., 2014; Fattorusso et al., 2019
	(vi) Patients with ASD also have high fecal and urinary levels of bacterially derived p-cresol, and further exposure to p-cresol has been shown to contribute to the severity of behavioral symptoms and cognitive impairment in ASD.	Altieri et al., 2011; Persico and Napolioni, 2013; Gabriele et al., 2014
	(vii) Optimized remedies that are practiced include rehabilitation, educational therapy and psycho-pharmacological approaches.	Fattorusso et al., 2019
Depression, anxiety, and major depressive disorder (MDD)	(i) Pre-clinical studies of depression, anxiety and MDD indicate that the altered brain function associated with these, can partly be attributed to disturbances in the gut microbiota composition.	Bercik et al., 2011; Park et al., 2013; Jiang et al., 2015; Kelly et al., 2016
	(ii) Studies have shown that the microbiome has the capacity to influence on emotional behavior, and is associated with various parameters relating to depression pathogenesis and severity.	Bercik et al., 2011; Clemente et al., 2012; Cryan and Dinan, 2012
	(iii) Hippurate, dimethylamine and dimethylglycine, all by-products of gut microbiota, have been detected in abnormal concentrations in MDD patients which further substantiates the aforementioned observations.	Zheng et al., 2013, 2016
	(iv) Increased severity in depression and anxiety have been noted following bacterial infection in patients.	Naseribafrouei et al., 2014

the enteric microbiota indirectly via an altered intestinal permeability, or directly via signaling molecules released into the gut lumen from immune and enterochromaffin cells, thereby increasing motor, sensory and secretory modalities of the GIT (Rhee et al., 2009; Grenham et al., 2011; Eisenstein, 2016). Those signaling systems that allow the brain, in this crosstalk communication, to influence gut-microbiome functions in the GIT, are: (1) the endocrine-immune system, (2) the hypothalamus–pituitary–adrenal (HPA) axis, (3) the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), and (4) enteric nervous system (ENS) (Rhee et al., 2009; Grenham et al., 2011; Cong et al., 2015). These signaling systems are interlinked systematically to form a complex reflex network, with afferent and efferent fibers (O'Mahony et al., 2011). Hence, activation of any of these signaling systems, either alone or in combination, might influence the composition and functionality of enteric microbiota (Rhee et al., 2009). For instance, under conditions of chronic stress the brain recruits these same mechanisms, by activation of the HPA axis in the brain, to regulate cortisol secretion. Cortisol in turn affects various immune cells (including cytokine secretion) locally in the gut, subsequently inducing changes to microbiota composition, and increasing the gastrointestinal permeability (de Punder and Pruimboom, 2015; Kelly et al., 2015; Farzi et al., 2018). Hence, an exceedingly complex array of signaling systems, all interlinked, lies between the brain and gut in the "top-down" concept (Aziz and Thompson, 1998; Collins and Bercik, 2009; O'Mahony et al., 2009; Forsythe et al., 2014; Khlevner et al., 2018; Weltens et al., 2018; Zhao et al., 2018).

The CNS is well shielded by the BBB, the major site of blood–CNS exchange. The barrier comprises microvascular endothelial cells, astrocytes and pericytes, and is tasked with the regulated passage of molecules into and out of the brain (Abbott et al., 2010; Sochocka et al., 2017b). Neurotropic bacteria are capable of evading host defenses, gaining access to the CNS (Dando et al., 2014), with >95% of brain abscesses caused by bacterial infection(s) (Sonneville et al., 2017). Furthermore, the brain may become particularly susceptible to bacterial infection(s), if the BBB is chronically compromised by an initial infection (Mendes et al., 1980; Cantiera et al., 2019). Various brain cells – microglia (resident macrophages), endothelial, ependymal, neuronal and glial (astrocytes and oligodendrocytes) – convey innate immune molecules that prompt the recruitment of leukocytes into the infected CNS compartments, in order to combat invading neurotropic bacteria (Klein et al., 2017). This process results in a series of initial neuroinflammatory events within the brain, as well as phagocytosis of the infecting bacteria, in an attempt to control disease progression. Neuroinflammation in the CNS is mediated by the production of cytokines and chemokines, that are pivotal in the coordinated communication between the immune system and the brain (DiSabato et al., 2016). The host's inflammatory reaction in the CNS is initiated by the recognition of the invading pathogens, which in turn leads to the local production of mediators by the glial cells comprising microglia and astrocytes (Grandgirard et al., 2013). Thus, acute inflammatory feedback is triggered by rapid and early activation of mediators released by activated glial cells in the CNS due to the infectious agent. However, when the presence of an infectious agent persists, a chronic state of inflammation within the brain results (Sochocka et al., 2017a) and the activated glial cells are altered beyond "normal" proportions, which results in progressive neurodegeneration (Kempuraj et al., 2017; Sochocka et al., 2017a). Pattern recognition receptor (Newton and Dixit, 2012; Suresh and Mosser, 2013) activation initiates the release of pro-inflammatory cytokines and chemokines, in order to modulate the immune response, leading to pleocytosis of white blood cells (Janowski and Newland, 2017). This in turn triggers an increased BBB permeability and the influx of leukocytes from the blood into the CNS at the site(s) of infection (Waisman et al., 2015; Kempuraj et al., 2017). Although this is the mechanism by which the brain attempts to restore homeostasis and protect itself against the invading pathogen (More et al., 2013), the chronic production of immune cells induces neurodegeneration. Since activated microglia have both neuroprotective and neurotoxic functions (Kim, 2003; Nimmerjahn et al., 2005; Dando et al., 2014; Liechti et al., 2015; Doran et al., 2016), various toxic molecules released by the microglia during the immune response may also inflict neuronal injury.

BACTERIAL INFECTIONS OF THE CNS AND THEIR EFFECT ON THE BRAIN–GUT AXIS

Most bacterial CNS infections present acutely, including subacute and chronic forms. Common acute bacterial CNS infections involve *Streptococcus agalactiae,* Gram-negative bacilli including *Escherichia coli, Klebsiella pneumoniae*, *Listeria monocytogenes, Neisseria meningitidis,* and *Streptococcus pneumoniae* (Durand et al., 1993; Gray, 1997; Grandgirard et al., 2013; Zhou, 2019), while subacute and chronic bacterial CNS infections, besides *Mycobacterium tuberculosis*, involve *Borrelia burgdorferi*, *Leptospira interrogans*, *Treponema pallidum, Mycobacterium leprae*. Microbial pathogens can gain entry into CNS by penetrating the BBB or via the olfactory (Kristensson, 2011). The nasopharynx is the usual portal of entry for major meningeal pathogens. Pathogens penetrate the olfactory epithelium, and could potentially cross epithelial barriers into the subarachnoid space; compromising the epithelial tissue by exposure to bacterial virulence factors, directly infecting the olfactory sensory neurons (Dando et al., 2014; Rey et al., 2018). Meningeal invasion subsequently follows via penetration of the cellular barriers of the CNS. The putative cascade of events caused by bacterial infection(s) of the brain that alter permeability of the gut – discussed in detail below, ultimately leads to dysbiosis.

(1) Within the cascade, the first step of bacterial invasion involves transitioning across the compromised BBB into the subarachnoid space. Pathogens can cause disruption of the BBB, which enables their passage into the brain. The various host defenses are usually inadequate to control the infection. Leukocytes traverse the BBB and patrol the brain parenchyma under normal conditions.

During inflammation, as result of infection, the BBB junctions (adherens and tight) that regulate the flux of ions, polar molecules, and macromolecules from the systemic circulation can be compromised, thus traffic is greatly increased at these junctions. Bacteria may cross the BBB by transcellular penetration after bacterial adhesion to endothelial cells or via infected leukocytes. Pinocytosis, increased by leukocytes combating bacteria that might have invaded following disruption of tight junctions or via the "Trojan horse" mechanism – phagocytes infected with the pathogen transverse the BBB (Kim, 2003; Pulzova et al., 2009). Leukocytes, activated by inflammatory molecules released during infection, cross the BBB by a multistep process that involves attachment to, and invasion through, the post-capillary venule wall and the surrounding endothelial and parenchymal basement membranes which differ in their laminin composition and permeability (Owens et al., 2008; Kristensson, 2011; Dando et al., 2014). During infection of the CNS various acute pathological events may occur which further compromise the CNS. The brain parenchyma is populated by resident immune cells, the microglia, which are highly specialized tissue macrophages.

(2) Microglia cells, the primary immune effector cells in the brain, continuously survey the brain parenchyma and respond to very subtle alterations in their microenvironment and in the brain's structural integrity (Nimmerjahn et al., 2005). Microglia are highly motile immune effector cells in the brain that respond to neuronal infection and damage. The role of microglia in a healthy brain, along with immediate reaction to brain damage, is paramount in response to the prevention of any kind of major brain damage. Microglia are considered essential for communication in the intrinsic immune system of the CNS, as well for intercellular crosstalk between astrocytes and neurons (Kreutzberg, 1996; Stollg and Jander, 1999; Streit, 2002; Streit et al., 2004; Akiyoshi et al., 2018). Microglia maintain CNS health via mediators involved in the function of neurogenesis, modeling of synapses, excitotoxicity prevention and regulation of neuroinflammation. Short-chain fatty acids derived from the gut-microbiome play a pivotal role in the function and maturation of microglia. Hence, microglia are crucial mediators in the interaction between the CNS and the gut microbiota (Wang et al., 2018; Abdel-Haq et al., 2019).

(3) Bacterial cell wall material, enzymes, and toxins cause direct injury to neurons and indirect damage by increasing vascular permeability that causes edema and further injury. Microglial cells respond to bacterial pathogens and neuronal injury by the production of reactive oxygen species (ROS), nitrous oxide, and peroxynitrite. Immune response also contribute to neurotoxicity via release of proteases and excitatory amino acids. Several signaling molecules, such as catecholamines, serotonin, dynorphin and cytokines, used by the host for neuronal and neuroendocrine signaling, are also likely to be secreted into the gut lumen (Rhee et al., 2009).

(4) Bacterial pathogens may target neurons and glial cells, inducing inflammation and exerting direct cytopathic effect due to the release of their products. Thereafter, brain cell apoptosis begins to occur. For example, Pneumolysin and hydrogen peroxide (H_2O_2) are direct triggers of *Streptococcus pneumoniae*. H_2O_2 rapidly diffuses through eukaryotic cell membranes to damage intracellular targets thus increasing intracellular Ca^{2+}, damaging mitochondria, and causing the release and translocation of mitochondrial apoptosis-inducing factor. Increased intracellular ROS and Ca^{2+} precedes morphologic changes that lead to brain cell apoptosis (Mitchell and Andrew, 1997; Lipton and Nicotera, 1998; Braun et al., 2002; Janowski and Newland, 2017). Brain cell apoptosis leads to neuronal injury in the form of brain manifestations, such as: basal ganglia and thalami communication that become obstructive, cranial nerve dysfunction, minor focal neurological signs, infiltrates of inflammatory cells, exudation of protein-rich fluid, and edema (Gray, 1997; Hussein and Shafran, 2000; Van de Beek et al., 2004; Østergaard et al., 2005; Al Khorasani and Banajeh, 2006; Hähnel and Bendszus, 2009; Abdulrab et al., 2010).

(5) Pathogenic bacteria that causes meningitis exhibit antiphagocytic capsular polysaccharide ability which enables survival within the blood. Hence, changes in the gut involves hematogenous dissemination of bacteria, initiating meningitis via mucosal adhesion of the organism and subsequent systemic invasion (Seib et al., 2009; Harvey et al., 2011; Dando et al., 2014). The intestinal immune system is tasked to maintain homeostasis within the gut-microbiome via the processes of minimizing direct contact between intestinal bacteria and the epithelial cell surface (stratification), and confining penetrant bacteria to intestinal sites and limiting their exposure to the systemic immune compartment (compartmentalization) (Hooper et al., 2012; Macpherson and McCoy, 2013). Mucosal surfaces represent the major interface and constitute the point of entry of most infectious pathogens, and are in contact with potentially injurious antigens (Janeway et al., 2001; Kaetzel, 2005).

(6) Stratification of intestinal bacteria on the luminal side of the epithelial barrier also depend on secreted immunoglobulin A (IgA). IgA specific for intestinal bacteria is produced with the help of intestinal dendritic cells that sample the small numbers of bacteria penetrating the overlying epithelium. Some meningeal pathogens produce proteases that cleave to human immunoglobulin subclasses (e.g., IgA1), allowing adherence of bacterial strains to mucosal surfaces and crossing the mucosal barrier (Lorenzen et al., 1999; Hooper et al., 2012; Brooks and Mias, 2018). IgA1 proteases separate the pathogen-recognition (Fab) and host signaling (Fc) components of the antibody, thereby severing communication with host defense cells. This also leaves pathogens coated with cleaved Fab fragments and camouflaged from the immune system. IgA1 proteases disable this important defense immune molecule allowing for direct escape of the

invading pathogen from host immunity (Woof and Russell, 2011; Marshall et al., 2017). This communication/crosstalk involving the gut microbiota from the CNS encompasses several channels along various neural, enteric and immune systems. Sensory and motor fibers from the vagus nerve connect the gut and the brainstem, and serve as a conduit for neural signals involving the microglia. Increased CNS inflammation signals vagal efferent nerves to relay information about the immune status of the brain to the gut and the gut microbes. In the same manner, vagal afferents transduce and relay information from the GIT to the CNS, signaling microglia via increased production of various pro-inflammatory cytokines that modulate neuroinflammation (Goehler et al., 1999, 2005; Borovikova et al., 2000; Forsythe et al., 2014; Abdel-Haq et al., 2019).

URINE REFLECTS DYSBIOSIS WITHIN BACTERIAL CNS INFECTION(S)

The CNS can communicate with the gut via signaling molecules carried by the CSF and blood, which in turn may alter gut composition and physiology. Evidence for this communication between the gut and the brain includes the following: (1) it is well known that toxins or abnormal metabolites that enter the bloodstream are ultimately removed from the blood, in an attempt to maintain a state of cellular homeostasis, and excreted via the urine (Li, 2015; Wu and Gao, 2015); (2) biomarkers for various neurological diseases are detected using body fluids including CSF, blood and urine (An and Gao, 2015). The CSF transfers waste products to the blood, which is filtered by the kidneys, whereby blood-borne waste products accumulate in the urine and are then excreted (Wu and Gao, 2015). It is also well known that various perturbations or other physiological changes in the human body – such as an altered microbiome, for instance – may change what is considered a normal urinary metabolome fingerprint into a new disease-specific fingerprint (Want et al., 2010; Emwas et al., 2015; Wu and Gao, 2015). There exists well-described examples in the literature of metabolites found in urine that are associated with microbial metabolism or microbial–host co-metabolism and found to change in response to diseases where gut dysbiosis is the predominant perturbation (Holmes et al., 2011; Vernocchi et al., 2016; Dumas et al., 2017; Malatji et al., 2019). Furthermore, urine is considered the preferred sample matrix for the detection of certain metabolites, which are otherwise difficult to detect from a blood sample due to their low concentrations. Moreover, urine collection is considered relatively non-invasive (Bouatra et al., 2013; Li, 2015). For these reasons, the metabolomics of urine has been successfully exploited for new biomarker discovery in various diseases, including neuropsychiatric disorders, such as schizophrenia, major depressive disorder, bipolar disorder, and autism spectrum disorder (Yap et al., 2010; Cai et al., 2012; Zheng et al., 2013; Chen et al., 2014), and various neurodegenerative diseases, such as PD, AD, and MS (Luan et al., 2014). Based on the premise that the urine contains the accumulation of all end-product metabolites of the body, logic dictates that chronic bacterial infection(s) of the CNS should, in principle, result in persistent feedback on the gut via the BGA, communicated via the CSF and blood, leading to dysbiosis and an altered urinary metabolome.

In research on infectious diseases, urinary profiling has received much attention, in particular regarding pulmonary tuberculosis (TB) – a disease caused by *Mycobacterium tuberculosis* (Mtb) – about which several studies have been conducted using urine for the detection of clinically relevant biomarkers (Banday et al., 2011; Bonkat, 2012; Das et al., 2015; Luies and Loots, 2016; Luies et al., 2017; Preez et al., 2017; Isa et al., 2018). The detection of lipoarabinomannan (LAM), for instance, a *Mycobacterium*-specific liposaccharide from the Mtb cell wall, is an example of the basis of a well-studied commercial ELISA assay that shows promise for its diagnostic use in urine with a reported sensitivity of 74% and specificity of 86.9% in a study performed on 148 confirmed TB patients (Tessema et al., 2001); a sensitivity of 80.3% and specificity of 99% in a study conducted on 132 confirmed TB patients (Boehme et al., 2005); and a sensitivity of 44% and specificity of 89% in a study conducted on 195 TB-positive patients in a high-HIV prevalence setting (Mutetwa et al., 2009). Within TBM cases (see **Box 1**), the direct LAM-ELISA assay of CSF has similarly shown a sensitivity of 64% and specificity of 86.9% in a study including 50 TBM cases in a high-HIV-prevalence setting (Patel et al., 2009); and a sensitivity of 43% and specificity of 91% for definite TBM cases in a study performed on CSF collected from the 4th ventricle, post-mortem (Cox et al., 2015). However, Bahr et al. (2015) determined that this LAM-based TB antigen test yielded negative results for all the CSF samples (∼100) analyzed in their study, of whom 18 had a confirmed diagnosis of TBM. In a short communication the following year, Bahr et al. (2016) voiced their concern about the reliability of the LAM assay for

BOX 1 | Tuberculous meningitis (TBM).

TBM, a severe infectious disease caused by Mtb, is a chronic form of bacterial meningitis (BM), resulting in chronic neuroinflammation often associated with irreversible neurological damage/dysfunction. TBM develops in severity in progressive stages (TBM stages I, II and III), and a uniform case definition (definite, probable and possible TBM) for diagnosis has been standardized (Marais et al., 2010). TBM is the most common form of CNS-tuberculosis (TB) (Van Well et al., 2009) and is considered severe due to its high associated prevalence of mortality and morbidity (Rohlwink et al., 2019). Transmitted via infectious aerosols into the lung, Mtb may enter the circulatory system, traverse the BBB and then enter the brain meninges (Rock et al., 2008; Nicholas et al., 2012). Microglia, the resident macrophages of the brain, are the cells preferentially infected by the Mtb bacilli (Rock et al., 2005). The Rich foci (Rich and McCordock, 1933), lesions that form in the meninges, eventually rupture, spilling the Mtb microbes, cytokines and chemokines into the subarachnoid space, resulting in infection and extensive inflammation of the meninges (Dastur et al., 1995; Donald et al., 2005; Rock et al., 2008). The pathogenesis of TBM is dynamic and Mtb bacteria exhibit a resilience that allows them to survive hostile environments, which results in a persistent neuroinflammatory response if not treated correctly and swiftly (de Carvalho et al., 2010; Beste et al., 2011, 2013; Warner, 2015). Despite all efforts toward improved solutions to curbing TB since the discovery of Mtb as the causative agent in 1882, there is still a very limited understanding of Mtb infection within the host, especially so for TBM, and hence the need for new biomarkers better describing this.

use on CSF for diagnosis of TBM, and also discussed the study by Cox et al. (2015). Ultimately, the LAM-ELISA, like many other TB diagnostic tests, is not sufficient as a stand-alone assay for a definitive diagnosis of TB.

Of particular interest, as it pertains to our review, is that bacterial antigen-specific assays perform particularly poorly when used for diagnosing bacterial CNS infection from urine collected from patients, even in documented septicemia cases (Barnes et al., 1998). Barnes et al. postulated that the reason for this is that these complex polysaccharide antigens break down before excretion in urine. Using the well-tested LAM-ELISA assay, Blok et al. (2014) analyzed urine collected from 21 TBM cases and obtained a sensitivity of only 4.8% and specificity of 93.1%, and hence concluded that urinary LAM detection offers little value for the diagnosis of TBM. Although LAM is detectable in the urine of TB cases and the CSF of TBM patients, it is almost undetectable in urine collected from patients with TBM. A postulated reason for this inconsistency is the inability of LAM to transgress the BBB. This hypothesis can likely be extended to complex bacterial antigens in general, as supported by the results of Barnes et al. (1998). We therefore conclude from these Mtb-antigen-specific assay studies that the diagnosis of bacterial infection(s) of the CNS, based on the detection of bacterial antigens in urine, is not a viable option.

For this reason, we believe that the detection of the catabolic components (metabolites) of complex signaling pathways is a better option for the accurate and sensitive differential diagnosis of bacterial CNS infection(s), using urine collected from patients. Mason et al. (2016) provided proof-of-concept by using an untargeted gas chromatography–mass spectrometry (GC-MS) metabolomics approach to analyze the urine of 12 confirmed TBM cases, 19 non-TBM cases (sick controls proven negative for both TB and meningitis) and 29 controls. This explorative study identified urinary metabolite markers that showed two important changes in the TBM cases: (1) a dysfunctional host metabolism, and (2) indicators of an altered host–microbe response in TBM (Mason et al., 2016). The indicators of dysfunctional host metabolism included: lipolysis and ketosis (elevated 2-hydroxybutyric acid, 3-hydroxybutyric acid, 2-methyl-3-hydroxybutyric acid, and acetoacetic acid); perturbed energy metabolism (elevated branched-chain amino acid derivatives, citric acid cycle intermediates and vanillylmandelic acid); liver damage (from the presence of 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid, and highly elevated 4-hydroxyphenylpyruvic acid). Of greater importance to this review was the discovery of those markers serving as indicators of an altered host–microbe response in TBM, as is discussed in greater detail below.

First, Mtb-induced changes to tryptophan metabolism was evident, due to the presence of elevated urinary concentrations of indole-3-acetic acid, 5-hydroxyindole acetic acid, tryptophan, kynurenic acid and quinolinic acid, accompanied by significantly elevated levels of N-acetylanthranilic acid (the N-acetylated product of anthranilic acid; Paul and Ratledge, 1970, 1971, 1973), the latter of which is a novel microbial metabolite indicative of gut microbiota involved in the perturbed host's tryptophan metabolism (Mason et al., 2016). Using a similar but more sensitive metabolomics analytical platform (GC × GC–TOFMS), Luies and Loots (2016) independently compared urine collected from 46 confirmed TB adults to 30 TB-negative healthy controls, and identified similar urinary markers indicative of the same alterations for the host's tryptophan metabolism. They attributed these to the result of an inflammatory response due to releases of cytokines, specifically IFN-γ. Hence, an inflammatory response induced by Mtb-infection, whether in the lungs or brain, results in the release of IFN-γ, which stimulates the upregulation of tryptophan catabolism (Yoshida et al., 1981; Taylor and Feng, 1991; Blumenthal et al., 2012; Hashioka et al., 2017; Lu et al., 2017). The presence of increased urinary tryptophan catabolites therefore contributes to a differential diagnosis of Mtb-based infection, but they do not serve as uniquely distinctive biomarkers.

Second, Mtb–host related metabolites were identified. In particular, significantly elevated concentrations of methylcitric acid were speculated to be likely to have originated from the well-characterized methylcitrate cycle of Mtb (Muñoz-Elías et al., 2006; Savvi et al., 2008). Interestingly, a positive correlation between urinary quinolinic acid and methylcitric acid concentrations was observed by Mason et al. (2016) in all the TBM patients' urine samples collected both before and after Mtb-specific treatment commenced. Hence, the roles of quinolinic acid and methylcitric acid in the host are intertwined during Mtb infection, and its treatment.

Lastly, urinary metabolite markers associated with alterations to the gut-microbiome were identified as a major consequence of perturbed metabolism associated with TBM. Of the significant urinary metabolites, those that are linked to gut microbiota were identified as uracil, hippuric acid, 4-hydroxyhippuric acid, phenylacetylglutamine and 4-cresol (Mason et al., 2016). Luies and Loots (2016) also identified elevated urinary concentrations of oxalic acid and rhamnulose, as evidence for an altered gut-microbiome in pulmonary TB. In a follow-up study by Luies et al. (2017), the failure of treatment of TB via standard anti-TB combination therapy was characterized by an imbalanced gut-microbiome, with the two largest predictors for a poor treatment outcome being two altered micobiome urinary markers [3,5 dihydroxybenzoic acid and 3-(4-hydroxy-3-methoxyphenyl)propionic acid]. Additionally, another independent GC-MS metabolomics longitudinal treatment study conducted on TB patient urine (Das et al., 2015) showed a treatment-dependent trend of a deregulated tyrosine–phenylalanine axis, also associated with an abnormal microbiome. Considering these urinary TB metabolomics studies, although not yet fully understood, strong evidence exists for the association of TB disease and an altered microbiome, detectable via altered metabolite markers present in urine collected from TB patients.

Independent urinary metabolomics studies on pulmonary TB, therefore, although not related to the CNS but still involving an infectious disease distinguished by chronic inflammatory response(s), support the findings of Mason et al. (2016) in characterizing chronic neuroinflammation from TBM through urinary profiling. Herein lies the strength of untargeted metabolomics studies – the complementary evidence of three

independent, open-minded analyses of metabolomics data obtained from urine on a similar analytical platform with a common, general hypothesis of the importance of the gut microbiota. For the remainder of this review, we focus on TBM and take a validated 3-marker CSF immunological signature of TBM and discuss it in conjunction with previously identified, altered urinary metabolomics markers of TBM.

VALIDATED 3-MARKER CSF IMMUNOLOGICAL SIGNATURE OF TBM

Bacteriological confirmation of TBM from CSF is not always possible, especially in children, so that diagnosis is mostly based on a combination of clinical findings, CSF analysis and radiological results (Marais et al., 2010). Since various biomarker-based tests of the host have shown promise in extrapulmonary pleural-TB diagnostics, it has been thought that these same tests could also be used to diagnose TBM (Chegou et al., 2008). Recent technology has allowed for the screening for many such biomarkers, using as little as 3 μL of CSF via Luminex multiplex cytokine-beaded arrays. With clinical application, host biomarkers could potentially be added to the current TBM diagnostic armamentarium, in order to provide an earlier and more efficient diagnosis.

A preliminary 3-marker CSF biosignature, comprising VEGF, IL-13 and cathelicidin LL-37 (cut-off values 42.92, 37.26, and 3221.01 pg/mL, respectively), correctly diagnosed childhood TBM with a sensitivity and specificity of 52 and 95%, respectively (Visser et al., 2015). The same 3-marker CSF biosignature, tested on a different cohort of 23 children, however, revealed lower sensitivity (30.4%), yet a similar specificity (91.7%), with different cut-off values. In this same cohort of 23 children with TBM and 24 controls, VEGF, IFN-γ, and MPO provided good accuracy with an AUC of 0.97, up to 91.3% sensitivity and up to 100% specificity, with cut-off values of >9.4, >99.5, and >25,823 pg/mL, respectively (Manyelo et al., 2019). Hence, VEGF, IFN-γ, and MPO in combinaton was validated by Manyelo et al. (2019) as a 3-marker CSF immunological signature of TBM. The background behind these three markers is now described, in order to provide insights into how they led to our predictive metabolic model.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF, a 46 kDa glycosylated homodimeric cytokine protein, is expressed intracellularly in several cell types, including microglia (Cohen et al., 1996). It is a potent growth factor inducer of vascular endothelial cell proliferation, vascular permeability (Soker et al., 1997) and angiogenesis (Connolly, 1991; Yancopoulos et al., 2000). Endothelial changes associated with VEGF include: (1) separation of intercellular tight junction, (2) increased vesicle transport, and (3) formation of vesico-vacuolar organelles, all of which results in increased macromolecular transport over the endothelial barrier (Feng et al., 1996; Wang et al., 2001). Classically associated with chronic inflammatory diseases, such as rheumatoid arthritis (Fava et al., 1994), VEGF is also associated with the increased permeability, and subsequent dysfunction, of the BBB (Dobrogowska et al., 1998; Proescholdt et al., 1999; Harrigan et al., 2002) and in the pathogenesis of brain edema related to ischemia, trauma, vasculitis and tumors (Van Bruggen et al., 1999; Viac et al., 1999). VEGF exhibits direct neuroprotective effects during *in vitro* ischemia (Jin et al., 2000). Another study showed that topical application of VEGF on the cerebral cortex induces a reduction of infarct size in a rat model of transient cerebral ischemia (Hayashi et al., 1998).

In 2001, Van der Flier et al. showed no detectable CSF VEGF concentrations in patients with viral meningitis (VM), whereas 30% (11/37) of those patients with bacterial meningitis (BM) displayed detectably elevated concentrations of CSF VEGF (ranging from <25 to 633 pg/mL). Furthermore, elevated VEGF has been associated with an upregulation of MMP-9 (Wang and Keiser, 1998) – see **Box 2** – which additionally contributes to BBB disruption in BM (Paul et al., 1998). Van der Flier et al. (2001) also indicated the VEGF index in BM (calculated as $[VEGF_{CSF}/VEGF_{plasma}]/[albumin_{CSF}/albumin_{plasma}]$) to be 6.2 [0.6–42], which indicates that CSF VEGF is a result of intrathecal production. This increase in CSF VEGF could be associated with: (1) a change in mental status, (2) seizures, (3) an elevated CSF WBC count (with neutrophils being the main source of VEGF), (4) elevated CSF protein and higher CSF:serum albumin ratios (marker of BBB breakdown), (5) severe BBB disruption, and, eventually, (6) death.

Within TBM, VEGF is localized in the microvessels and perivascular cells (Matsuyama et al., 2001). Tumor necrosis-alpha (TNF-a), associated with pathogenesis of TBM (Tsenova et al., 1999), is a known inducer of VEGF (Ryuto et al., 1996). In a follow-up investigation conducted by Van der Flier et al. (2004), the prevalence of elevated CSF VEGF concentrations in TBM patients was 58% (15/26) (at 98 ± 31 pg/mL) with a calculated VEGF index of 486 ± 976, the latter once again indicative of

BOX 2 | Matrix metalloproteinases (Kolb et al., 1998; Leib et al., 2000; Shapiro et al., 2003; Lee et al., 2004).

MMPs are a large family of zinc-dependent proteolytic enzymes. Their main function involves remodeling of the connective tissues by degrading extracellular matrix molecules and are regulated by tissue inhibitors of metalloproteinases. These many compounds are subdivided according to their main substrates:

- Gelatinases: MMP-2, MMP-9.
- Collagenases: MMP-1, MMP-8, MMP-13.
- Stromelysins: MMP-3, MMP-10, MMP-11.

MMP-2 and MMP-9 digest type IV collagen and are subsequently implicated in the breakdown of the BBB via dissolution of the basement membrane underlying the endothelial cells. MMP-2 and MMP-9 production is strongly correlated with the development of neurological sequelae and induced by pro-inflammatory cytokines (IFN-γ) and other mediators (such as MPO). The amount of MMP present in CSF varies, depending on the severity of inflammation. MMP-2 and MMP-9 are detected in elevated amounts in the CSF of meningitis cases (TBM, VM and BM), with MMP- 9 correlating strongly with the number of neutrophils in VM.

TABLE 2 | Summary of CSF VEGF concentrations in different types of meningitis.

	TBM	BM	VM
CSF VEGF	142.8 pg/mL [28.1–225.7][a] 144.4 ± 75.1 pg/mL[d] 106 ± 50 pg/mL [44.9–336][e]	14.5 pg/mL [8.7–86.5][a] 47 ± 9 pg/mL [<10–174][b] 37.5 pg/mL [<20–160][c] 80.1 ± 49.5 pg/mL[d]	27.9 pg/mL [7.9–48.7][a] 27.6 ± 26.3 pg/mL[d]

[a]*Visser et al. (2015).* [b]*Van der Flier et al. (2005).* [c]*Coenjaerts et al. (2004).* [d]*Matsuyama et al. (2001).* [e]*Husain et al. (2008).*

intrathecal production. Van der Flier et al. furthermore associated the elevated concentrations of CSF VEGF in TBM with: (1) significantly greater mononuclear cell counts; (2) elevated CSF protein and higher CSF:serum albumin ratios; (3) not being significantly correlated with the elevated ICP, decreased CSF glucose nor with cerebral infarct on a CT scan; and (4) the inhibition explained the clinical effect of adjuvant corticosteroid therapy. In 2008, Hussain et al. similarly indicated significantly increased CSF VEGF levels (106 ± 50 pg/mL [44.9–336 pg/mL]) in TBM, accompanied by a strongly positive correlation between microvessel density and VEGF expression. Additionally, the investigation revealed that in excised tuberculomas: (1) VEGF expression was highest in regions of the granulomatous reaction; (2) no VEGF was present in the areas of caseous necrosis; (3) areas of caseation were devoid of angiogenesis; and (4) inflammatory mononuclear cells were positive for VEGF antigen (these included epitheloid cells, histiocytes and macrophages). Furthermore, immunohistochemical staining of excised tuberculoma demonstrated an elevated expression of VEGF in the granulomatous areas, with positivity in inflammatory mononuclear cells, Langhan's giant cells, as well as reactive astrocytes and fibrocytes.

Matsuyama et al. (2001) and Visser et al. (2015) both indicated CSF VEGF to be significantly increased in TBM compared with other types of meningitis (**Table 2**). Among the TBM cases, CSF VEGF was additionally significantly higher in those patients with hydrocephalus (196.3 ± 60.2 pg/mL vs. 119.8 ± 69.6 pg/mL) and there was a significant correlation with increased CSF protein and CSF total cell counts (Matsuyama et al., 2001). Visser et al. (2015) associated elevated CSF VEGF with raised hydrocephalus and CSF protein (>1 g/L), along with basal meningeal enhancement and hyperdensity in the basal cisterns on non-contrast CT scans. Lastly, Matsuyama et al. (2001) indicated that CSF VEGF localizes to microvessels and perivascular cells in TBM.

MYELOPEROXIDASE

Myeloperoxidase (MPO), a heme enzyme (EC 1.11.1.7) and pro-inflammatory mediator present in the primary granules of polymorphonuclear leukocytes (PMNs), participates in oxygen-dependent microbiocidal activity of PMNs and triggers oxidative stress during acute and chronic inflammatory processes, resulting in the production of ROS. MPO can be measured in CSF as an index of inflammation (Liechti et al., 2014) and leukocyte influx (Grandgirard et al., 2012). In a review by Ray and Katyal (2016), MPO was clearly associated with the etiology of neurodegenerative disorders.

MPO is synthesized in reaction to infection (Pohanka, 2013), resulting in elevated ROS. The occurrence of oxidative stress in meningitis patients is well-described in the literature (Koedel and Pfister, 1999; Ray et al., 2000; Tsukahara et al., 2000; Christen et al., 2001; Kastenbauer et al., 2002; Klein et al., 2006; Hamed et al., 2009; Loro, 2009; Koedel et al., 2010; Mirić et al., 2010; Barichello et al., 2011). Furthermore, significant increases in MPO activity have been shown in BM-induced rats (Giridharan et al., 2017), particularly within the hippocampus and frontal cortex (Barichello et al., 2011, 2014). In a study of 59 pediatric BM cases, Mirić et al. (2010) showed no significant correlation between MPO and neutrophil count in CSF; however, CSF MPO activity did correlate with various lipid peroxidation products. Additionally, H_2O_2 levels in CSF were associated with elevated BBB permeability, CSF albumin concentrations, and serum H_2O_2 concentrations. Lastly, it is important to note that MPO reacts with cell matrix metalloproteinases (MMPs – see **Box 2**), or their tissue inhibitors, and this is thought to contribute to the BBB dysfunction seen in such cases.

Borelli et al. (1999) proved that purified MPO, in the presence of H_2O_2, exerts a consistent killing effect on Mtb, and that the MPO activity is both time and dose dependent; it also requires chloride ions for efficacy. This MPO–H_2O_2–Cl_2 system produces hypochlorous acid (HOCl) via activated leukocytes (Klebanoff, 2005), which in turn serves as a strong, non-radical oxidant of a wide range of biological compounds, although it is more selective than hydroxyl radicals (Hampton et al., 1998), with the following characteristics: (1) it has a preferred substrate selectivity toward thiols and thioethers, (2) an ability to convert amines to chloramines, (3) promotes chlorination of phenols and unsaturated bonds, (4) oxidizes iron centers, (5) crosslinks proteins, and (6) is membrane permeable. HOCl has also been characterized as covalently modifying lipids and/or proteins, resulting in local tissue damage and amplification of the inflammatory cascade. Furthermore, HOCl, in the presence of nitrite (NO^{2-}) formed by stimulated PMNs, forms 3-chlorotyrosine (3Cl-Tyr), and to a lesser degree, 3-nitrotyrosine ($3NO_2$-Tyr) and N-chlorotaurine (Eiserich et al., 1998). The 3Cl-Tyr is considered a specific marker of MPO-catalyzed oxidation (Hazen and Heinecke, 1997), with GC-MS being the preferred method for quantifying it (Hazen et al., 1997; Winterbourn and Kettle, 2000). Other biomarkers of MPO-derived HOCl include: chlorohydrins, protein carbonyls, anti-HOP (hypochlorous acid-oxidized protein), antibodies, 5-chlorocytosine, and glutathione sulfonamide. Each with their advantages and disadvantages is described by Winterbourn and Kettle (2000). Based on the analyses of CSF collected from 79 confirmed pediatric BM cases, Rugemalira et al. (2019) indicated that elevated ratios of 3Cl-Tyr:para-tyrosine serves as a marker for MPO activation in CSF in pediatric BM cases, and potentially also for grading the severity of neuroinflammation. Furthermore, Rugemalira et al. (2019)

also proved that $3NO_2$-Tyr can be used as a biomarker for peroxynitrite formation and is associated with an unfavorable outcome of BM. In a study of 59 children with confirmed BM (Mirić et al., 2010), CSF MPO activity, although relatively low, was significantly increased at baseline compared to controls (n = 23), increasing even further by day 5 of treatment. It was concluded that MPO may be involved in the oxidative stress associated with BM, as well as potentially contributing to BBB disruption. Marais et al. (2016) indicated a significant increase in neutrophil-dependent inflammatory response biomarkers, including MPO, in adult TBM and HIV co-infection patients with paradoxical immune reconstitution inflammatory syndrome. Lastly, Üllen et al. (2013) indicated that BBB dysfunction associated with neuroinflammation caused by MPO can be partially reversed by using para-aminobenzoic acid (PABA) hydrazide, first shown by Forghani et al. (2012) to effectively treat multiple sclerosis in mice. PABA (or vitamin Bx) is non-essential for humans, but exhibits anti-fibrotic properties. Fibrosis in the brain occurs via the proliferation or hypertrophy of glial cells, such as microglia – microgliosis, during neurotrauma caused by infection. Subsequently, PABA may later be considered for its use as a possible adjunctive therapeutic agent in TBM, since the inhibition of MPO has been posited to be a valuable therapeutic approach to reduce oxidative-stress-mediated damage in neurodegenerative diseases (Green et al., 2004).

INTERFERON-γ

Interferon-γ (IFN-γ) is predominantly produced by $CD4^+$ T cells and functions by activating microglia, thereby stimulating lymphocyte Th1 differentiation (Farrar and Schreiber, 1993) and antimicrobial activity of the microglia (Mastroianni et al., 1997), after infection. A plethora of literature studies report the performance of IFN-γ release assays (IGRAs) for diagnosing TB under different conditions. These studies are comprehensively covered by systematic reviews and meta-analyses and include applications to diagnosing: (1) latent Mtb infection (53 studies: Diel et al., 2011); (2) latent Mtb infection in rheumatic patients (11 studies: Ruan et al., 2016); (3) latent TB in patients with autoimmune diseases under immunosuppressive therapy (17 studies: Wong et al., 2016); (4) active TB (27 studies: Sester et al., 2011); (5) active TB among HIV-seropositive individuals (11 studies: Huo and Peng, 2016); (6) active TB in immunocompetent children (15 studies: Laurenti et al., 2016), immunodiagnosis of TB (75 studies: Pai et al., 2004); (7) active and latent TB in HIV-positive populations (32 studies; Overton et al., 2018); and (8) extra-pulmonary TB (22 studies: Zhou et al., 2015). Similarly, several studies (**Table 3**) using IGRAs have also been performed using CSF as a possible sample matrix for diagnosing TBM, with the two main commercially used IGRAs tested being T-SPOT.TB and QuantiFERON-TB. IGRAs function by measuring the release of IFN-γ from T cells, after *in vitro* stimulation with Mtb antigens, such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10); they are influenced by (1) the antigenic load, (2) host responsiveness to antigens, and (3) host–pathogen interactions (Lu et al., 2017).

Consolidating from the literature, the CSF studies on IGRAs as a diagnostic tool for TBM (**Table 3**), a weighted average of the diagnostic performance of IGRAs (pooled from 326 TBM cases) was calculated to give an overall average sensitivity and specificity of 65 and 87%, respectively – insufficient for application as a stand-alone diagnostic tool. On similar data, a meta-analysis of 6 studies from the literature, all using IGRAs conducted on CSF, showed a pooled (156 cases) sensitivity of 77% (69–84%) and specificity of 88% (74–95%) for TBM diagnostic applications (Yu et al., 2016). Furthermore, IGRAs require 3–7 mL of CSF, a volume often unobtainable, especially from children and infants. Moreover, the measure of sensitivity and specificity is dependent upon a pre-defined cut-off point which is currently not yet standardized.

The use of IGRAs for the differential diagnosis of meningitis has, however, yielded a practical outcome. Chonmaitree and Baron (1991) analyzed CSF from 16 VM and 41 BM cases and determined that elevated concentrations of IFN-γ were present in 75 and 24% of these patient groups, respectively. A review of the literature (1964–1991) by Chonmaitree and Baron (1991) revealed a similar trend, showing elevated concentrations of IFN-γ in 68% (133/196) of all VM patients (based on 11 studies), whereas in patients with BM, only 28% (59/189) showed elevated IFN-γ in the pooled population (8 studies used). Hence, patients with VM exhibit higher IFN-γ levels than those with BM. Based upon quantified data in 50 patients with VM, using a radioimmunoassay, Minamishima et al. (1991) determined CSF IFN- γ to be on average 9.8 ± 7.5 UI/mL. Minamishima et al. additionally suggested that IFN-γ produced in the inflamed intrathecal space may be associated with the pathogenesis of the disease, and associated the elevated CSF IFN-γ levels with (1) CSF protein concentrations, (2) total cell counts, and (3) number of febrile episodes. San Juan et al. (2006), also using a radioimmunoassay on CSF collected from patients, calculated a mean IFN-γ for definite (n = 12) and probable (n = 8) TBM patients to be 28.7 ± 8.2 and 10.6 ± 2.8 UI/L, respectively. However, Ohga et al. (1994) showed only 3 out of the 13 BM patients investigated, and Kornelisse et al. (1997) only 20 of 35 BM patients investigated, to have CSF IFN-γ elevated to concentrations above the detection limit of 10 pg/mL. In an analysis of 30 TBM patients, Lu et al. (2016) determined, via ELISA, a mean CSF IFN-γ value for patients with TBM to be 350.97 ± 372.94 pg/mL. Lu et al. also determined that in 10 of these TBM patients the average CSF IFN-γ levels were 500.48 pg/mL before treatment and 103.62 pg/mL following 4 weeks of treatment, indicating that while IFN-γ decreased significantly (5-fold), it still remained elevated compared to the norm, after 4 weeks of treatment (that is, inflammation in the brain persisted). Mansour et al. (2005) reported a highly elevated mean concentration of CSF IFN-γ (794 ± 530 pg/mL) in 39 patients with TBM (all of whom were HIV negative) prior to receiving medication, which was correlated with markers of neuroinflamation in these individuals. Mansour et al. (2005) also showed that the CSF IFN-γ remained elevated for many weeks after treatment was begun in patients

TABLE 3 | Performance of IGRAs on CSF from TBM cases as a stand-alone diagnostic tool.

References	IGRA	TBM cases (n)	Sensitivity % (range)	Specificity % (range)
Pan et al. (2017)	T–SPOT.TB	53	61 (40–92)	97 (75–100)
Lu et al. (2017)	T–SPOT.TB	61	62 (49–74)	73 (62–82)
Qin et al. (2015)	T–SPOT.TB	12	92 (62–100)	93 (76–99)
Park et al. (2012)	T–SPOT.TB	25	72 (51–88)	79 (66–89)
Kim et al. (2010)	T–SPOT.TB	31	71 (51–86)	89 (72–98)
Patel et al. (2010)	T–SPOT.TB	38	58 (41–74)	94 (83–99)
Thomas et al. (2008)	T–SPOT.TB	10	90 (56–100)	100 (59–100)
Caliman-Sturdza et al. (2015)	QuantiFERON-TB	63	84	98
Vidhate et al. (2011)	QuantiFERON-TB	36	13 (2–40)	63 (35–85)
Weighted average diagnostic performance of IGRAs		329	65	87

with TBM, whereas in those cases diagnosed with VM and BM the CSF IFN-γ returned to undetectable concentrations within a couple of days post-treatment. Considering all of the above, patients with VM and TBM exhibit a similar increase in CSF IFN-γ levels, both far greater than in patients with BM. This suggests that CSF IFN-γ could potentially be used as a differential diagnostic marker for the exclusion of BM. Furthermore, CSF IFN-γ levels in TBM cases remain elevated for weeks following treatment, differentiating TBM from VM. However, as described previously, in order to acquire a definitive TBM diagnosis additional measures of CSF parameters are needed.

In summary, the overall trend across all CSF VEGF studies is a significantly higher concentration of VEGF in TBM patients than in other cases of meningitis. Of further note, Van der Flier et al. (2004) reports significantly increased CSF VEGF (178 ± 52 pg/mL) in TBM patients with nausea and vomiting, indicating that elevated CSF VEGF has a potential direct impact on the BGA, leading to a perturbed gut. CSF IFN-γ levels show a similar increase in TBM and VM but less so in BM. Hence, CSF IFN-γ levels could potentially be used for the exclusion of the diagnosis of BM. The HOCl produced by the MPO–H_2O_2–Cl_2 system yields similar oxidative markers in both TBM and BM.

The addition of VEGF and MPO with IFN-γ, as part of a 3-marker immunological biosignature of TBM in CSF (Manyelo et al., 2019), has yielded a diagnostic measure with an AUC of 0.97, and a sensitivity and specificity of up to 91.3% and up to 100%, respectively. Hence, this 3-marker biosignature yields excellent results for diagnosis of TBM from a CSF sample. But, what of the urinary metabolomics profile? If these three immunological markers are present in the CSF of a TBM patient, then a downstream metabolic effect, based upon the BGA, should be reflected in the urine. This concept is explored in our proposed predictive metabolic model that follows.

PROPOSED PREDICTIVE METABOLIC MODEL OF TBM IN THE BRAIN BASED UPON IFN-γ, MPO AND VEGF

Given the background of IFN-γ, MPO, and VEGF described above, and the associated metabolic pathways of these signaling compounds, we propose a predictive metabolic model for TBM in the brain based upon previously published biochemistry fundamentals. This model, illustrated in **Figure 1**, shows the interaction of the overlapping metabolic cascades initiated by TBM, and its associated 3-marker CSF immunological signature.

Our predictive metabolic model shows how increased levels of VEGF result in a persistent metabolic burst caused by the induction of angiogenesis (Stapor et al., 2014; Treps et al., 2016), whereby glycolysis, and the release of glycogen from astrocyte stores to fuel glycolysis, is increased significantly. Secondary pathways that are subsequently upregulated include: (1) the pentose phosphate pathway, that contributes to an elevated synthesis of glutathione (Ben-Yoseph et al., 1996), elevated xylulose-5-phosphate (also via phosphoenolpyruvate in the glycolysis pathway) to fuel tryptophan catabolism (Stephanopoulos and Simpson, 1997; Simpson et al., 1999; Maria et al., 2018), and elevated purine and pyrimidine synthesis (Zimmer, 1988, 1996); (2) the hexosamine pathway, which contributes to increased O- and N-protein glycosylation, imperative for the host's immune response since glycosylation controls cell migration, host defense, and antigenicity (Varki, 1993); (3) increased β-oxidation providing substrate in the form of diacylglycerol from downstream catabolism of dihydroxyacetone phosphate and activation of protein kinase C from VEGF (Takahashi et al., 1999; Harhaj et al., 2006), ultimately yielding increased acetyl-CoA; and (4) the boosted mitochondrial citric acid (TCA) cycle, due to the increased acetyl-CoA. The elevated TCA intermediate α-ketoglutarate (α-KG), previously indicated to be a urinary marker of TBM (Mason et al., 2016), contributes to glutamate synthesis and downstream glutathione (GSH) production, the latter being a needed antioxidant, synthesized in response to the elevated MPO.

Increased levels of IFN-γ, stimulated by Mtb-induced antigens (Blumenthal et al., 2012; Lu et al., 2017), specifically upregulate indoleamine dioxygenase (Yoshida et al., 1981; Taylor and Feng, 1991; Hashioka et al., 2017), the initial enzyme in the tryptophan catabolic pathway. A massive burst in tryptophan catabolism results in astrocyte-based kynurenic acid and microglia-based quinolinic acid synthesis – also previously identified urinary markers of TBM (Mason et al., 2016). Several enzymes within the tryptophan metabolic pathway require pyridoxal-5-phosphate (P-5-P), an active form of vitamin B_6, as a cofactor.

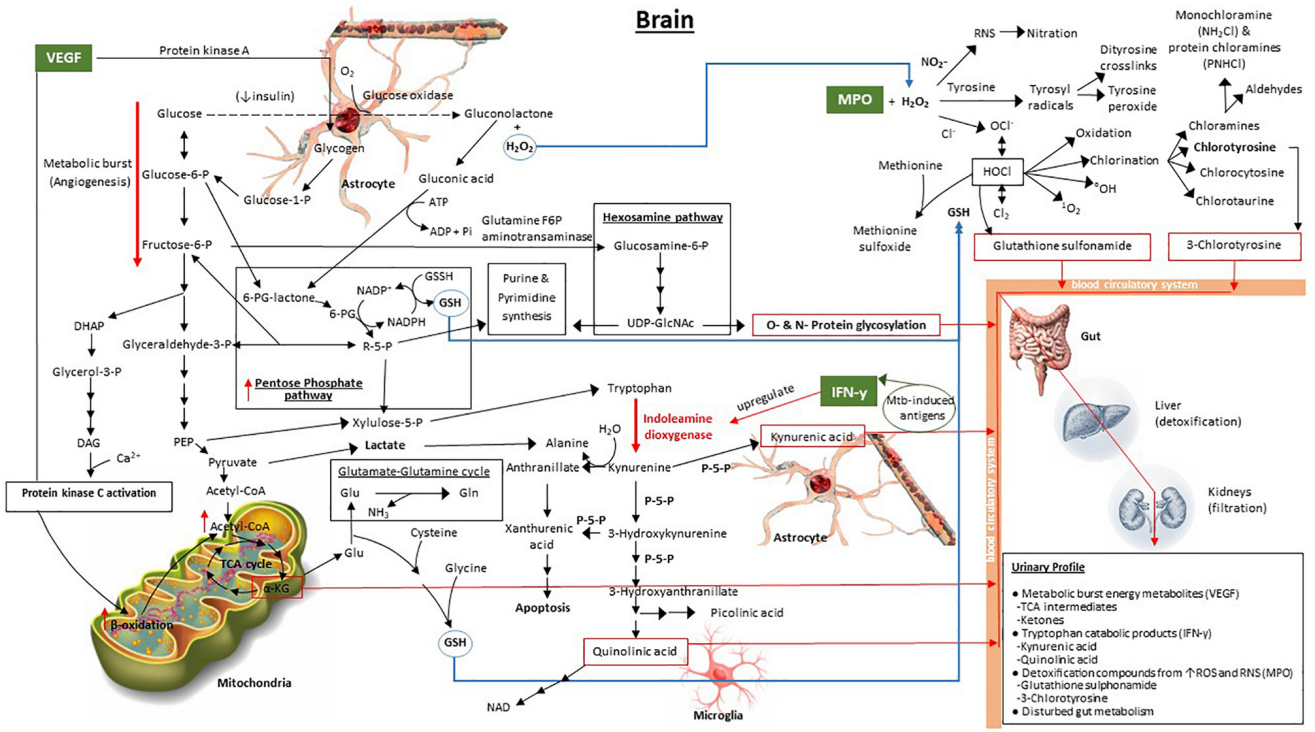

FIGURE 1 | Model predicting the downstream metabolic effects of VEGF, IFN-γ, and MPO in the brain, and the expected attendant urinary profile, in a TBM patient. The bold red arrows indicate the major upregulated metabolic pathways, namely tryptophan catabolism, induced by upregulated indoleamine dioxygenase due to IFN-γ, and increased metabolic burst (angiogenesis) via increased glycolysis. The red boxes enclose metabolic end-products within the Mtb-infected brain – protein glycosylation, α-ketoglutarate, quinolinic acid, kynurenic acid, 3-chlorotyrosine and glutathione sulfonamide – which are the predicted metabolic brain markers of TBM. The blue arrows indicate the transport of important metabolic components. The dashed black line, indicating oxidation of glucose to gluconolactone, represents a transient pathway that occurs when insulin is depleted, as indicated. 6-PG, 6-phosphogluconate; R5P, ribulose-5-phosphate; UDP-GlcNAc, UDP-N-acetylglucosamine; PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; DAG, diacylglycerol; TCA cycle, tricarboxylic acid (citric acid) cycle; α-KG, α-ketoglutarate; Glu, glutamate; Gln, glutamine; GSH, glutathione (reduced); P-5-P, pyroxidal-5-phosphate (vitamin B_6).

Deficiency of P-5-P diverts tryptophan metabolism from production of NAD to the excessive formation of xanthurenic acid (Oxenkrug, 2013), and subsequently apoptosis. One of the mechanisms of insulin resistance is inflammation-induced upregulation of tryptophan metabolism in combination with P-5-P-deficiency-induced diversion of tryptophan metabolism leading to formation of xanthurenic acid and other kynurenine derivatives that affect insulin activity (Oxenkrug, 2013). It has been shown that reduced insulin levels may lead to uncontrolled glucose metabolism – previously described in individuals with diabetes (American Diabetes Association, 2013), pulmonary TB (Preez et al., 2017) and, recently, in runners after a marathon (Stander et al., 2018), all of which are associated with a severe inflammatory response. As depicted in our model, uncontrolled glucose metabolism can result in glucose being oxidized via glucose oxidase to produce gluconolactone, in addition to hydrogen peroxide, previously reported to occur in both diabetes and pulmonary TB (Preez et al., 2017). The gluconolactone subsequently becomes siphoned into the pentose phosphate pathway via hydrolysis to form gluconic acid and phosphorylation with ATP to produce 6-phosphogluconate lactone (Dickens and Glock, 1951; Rohatgi et al., 2014). The consequential elevated H_2O_2, and its interaction with raised MPO, leads to the activation of various oxidative stress pathways (Hampton et al., 1998; Podrez et al., 2000; Klebanoff, 2005), as depicted in **Figure 1**, and described above. The two final urinary markers of elevated MPO and of HOCl, via the MPO–H_2O_2–Cl_2 system, are predicted to be glutathione sulfonamide and 3-chlorotyrosine (Winterbourn and Kettle, 2000).

Furthermore, the burst from both the glycolysis (via pyruvate) and tryptophan catabolism (via hydration of kynurenine to form alanine; Kotake and Nakayama, 1941) pathways yields increasingly elevated levels of extracellular lactate. This lactate pool is essential in the lately proposed astrocyte–microglia lactate shuttle (AMLS, Mason et al., 2015), since lactate plays a dual role of being a preferred source of energy in the brain during TBM as well as also being neuroprotective (Mason, 2017), diverting lactate away from neurons (thereby deactivating neurons to protect them) toward activated microglia in an attempt to eradicate the immediate insult/infection. This increase in lactate in TBM is predicted to be localized in the brain for immediate use and is not expected to be present in elevated amounts in the urine of TBM patients.

The predictive metabolic model presented here, although speculative, is based upon three validated immunological markers of TBM. The subsequent activated metabolic pathways in the brain are based upon biochemistry fundamentals and supported by the literature, as discussed. The end-product metabolites that act as metabolic markers of TBM are expected to cross the BBB and travel in the blood circulation and interact with the gut. The principal limitation of our model is that, in its current form, it can predict only the end-product metabolites from the TBM-infected brain, with the assumption that no other systemic co-infection is present. The complex interactions with the gut microbiota are poorly understood and require further research. However, based upon previous urinary metabolomics studies reported in this review, experimental evidence is emerging that points toward an altered gut metabolism. Hence, changes in gut metabolism became the fourth component of our proposed urinary metabolic profile of a TBM patient. The specifics of the complex relationship between host and gut-microbiome and the details of the altered metabolic profile of the gut under pathophysiological states remain a hot topic.

CONCLUSION

The significance of this review is that it takes a newly established paradigm within the neurosciences – the BGA – and critically examines the literature from a relatively unexplored niche perspective – chronic neuroinflammation caused by CNS bacterial infection(s) of the brain, using TBM as an example. We posit that if the BGA exists then chronic neuroinflammation within the brain caused by pathogenic bacteria (Mtb) will influence the gut microbiota, and the ideal biofluid to analyze this, reflecting the associated systemic changes, is urine. We support our postulate with data from published studies on urinary metabolomics, as follows.

First, the strength of untargeted urinary metabolomics is clearly demonstrated in the literature. A previous untargeted urinary metabolomics study conducted on TBM cases, by Mason et al. (2016), yielded data that, when analyzed in a non-biased, holisitic manner, resulted in a putative urinary metabolic signature characterizing TBM that was interpreted in a hypothesis-generating perspective. Independently, using similar analytical platforms in metabolomics, Das et al. (2015) and Luies and Loots (2016) examined urinary metabolomics profiles of pulmonary TB patients, and came to similar conclusions – the most significant of which, in the context of this review, were that infection by Mtb results in an altered gut-microbiome and this is substantiated by altered microbiome markers in the urine of these patients.

Second, we take an independent, and initially unrelated, study that closely examined the immunological profile of TBM, in which three specific immunological markers in the CSF associated with neuroinflammation – VEGF, IFN-γ, and MPO – were validated as diagnostic markers of TBM. We explored the background behind this 3-marker CSF immunological signature of TBM, in the context of its influence on the gut-microbiome and the subsequently altered urinary metabolome, using previously discovered urinary metabolites in TBM patients as proof (such as α-KG, and the tryptophan catabolites 3-hydroxykynurenic acid and quinolinic acid) (Mason et al., 2016). By extension, we also predict other metabolic pathways that would be expected to be changed within our model.

Third, we combined the sciences of immunology and metabolomics to create a novel integrated predictive metabolic model of TBM in the brain. By integrating relevant information from systems biology, our predictive cascading metabolic model should be adjustable to account for other types of bacterial infection(s) of the CNS that cause chronic neuroinflammation, such as neurosyphilis, bacterial brain abscesses and Lyme disease, as well as chronic non-bacterial CNS infections that are common in resource-constrained settings of poor communities, and sometimes difficult to distinguish from TBM, such as cerebral malaria and cryptococcal meningitis. Being so identified, based upon the literature, patients with VM and TBM exhibit a similar increase in CSF IFN-γ levels, both far greater than in patients with BM. Hence, a predictive metabolic model of cerebral malaria and cryptococcal meningitis would likely exclude CSF-based IFN-γ and its subsequent downstream cascading metabolic influence – that is, no downstream tryptophan metabolic catabolites. What remains to be done is to identify the unique immunological markers associated with these other bacterial infection(s) of the CNS and predict and confirm their associated downstream metabolic markers that should be reflected in urine, which could be used diagnostically or to characterize these diseases better.

In short, analysis of urinary metabolic profiles offers a wealth of metabolic information that can be traced back to an altered gut-microbiome, and to an inherently changed BGA, induced by chronic neuroinflammation from bacterial infection(s) of the CNS. This metabolic information from urine holds within it the potential to contribute to improved and early differential diagnosis of bacterial infection(s) in the CNS – a quicker and less invasive method of diagnosis than currently available. The review presented here provides support that, by taking existing validated immunological markers of infectious diseases in conjunction with metabolomics data and biochemistry fundamentals, it is possible to predict downstream metabolic products, most likely detectable via urinary metabolic profiling methods.

AUTHOR CONTRIBUTIONS

SM conceptualized the manuscript. SI, SM, and DL planned the outline of the manuscript. SI wrote the manuscript. SM and DL supervised SI in the writing of the manuscript by providing critical feedback. RS provided clinical input and critically read the manuscript. All co-authors read and approved the final draft for submission.

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REVIEW
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The Role of the Gastrointestinal Mucus System in Intestinal Homeostasis: Implications for Neurological Disorders

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Mucus is integral to gut health and its properties may be affected in neurological disease. Mucus comprises a hydrated network of polymers including glycosylated mucin proteins. We propose that factors that influence the nervous system may also affect the volume, viscosity, porosity of mucus composition and subsequently, gastrointestinal (GI) microbial populations. The gut has its own intrinsic neuronal network, the enteric nervous system, which extends the length of the GI tract and innervates the mucosal epithelium. The ENS regulates gut function including mucus secretion and renewal. Both dysbiosis and gut dysfunction are commonly reported in several neurological disorders such as Parkinson's and Alzheimer's disease as well in patients with neurodevelopmental disorders including autism. Since some microbes use mucus as a prominent energy source, changes in mucus properties could alter, and even exacerbate, dysbiosis-related gut symptoms in neurological disorders. This review summarizes existing knowledge of the structure and function of the mucus of the GI tract and highlights areas to be addressed in future research to better understand how intestinal homeostasis is impacted in neurological disorders.

Keywords: mucus, MUC-2, goblet cells, intestine, microbes, neurological disorders

PROPERTIES OF THE GASTROINTESTINAL MUCUS LAYER

The mucus layer is the first line of defense against infiltration of microorganisms, digestive enzymes and acids, digested food particles, microbial by-products, and food-associated toxins. This layer coats the interior surface of the GI tract, lubricates luminal contents and acts as a physical barrier to bacteria and other antigenic substances present in the lumen. The moist, nutrient-rich mucus layer adjacent to the epithelial barrier of the GI tract is also essential in the maintenance of intestinal homeostasis and contains a thriving biofilm including beneficial and pathogenic microbial populations.

Emerging evidence demonstrates changes in the gut-brain axis in neurological disease involving the enteric nervous system located within the wall of the GI tract. Interestingly, mucus production is regulated by molecular pathways involved in developmental processes and nervous system activity. Multiple neurological disorders present with gastrointestinal dysfunction and microbial dysbiosis but whether alterations in mucus structure and function are driving these changes is unknown.

Therefore, we propose that alterations in enteric nervous system function and mucus production may occur in neurological disease and contribute to GI symptoms and dysbiosis.

Regional Mucus Variations

Although mucus located throughout the gut contains the same biological components, mucus properties vary with regional differences in function along the gastrointestinal tract (Ermund et al., 2013, **Figure 1**).

Small Intestine

The majority of nutrient uptake from digested food occurs in the small intestine and therefore there is a single, discontinuous and more penetrable mucus layer in this region (Johansson et al., 2011). The discontinuity of the small intestinal mucus layer is important not only for the absorptive function of this region but also for the release of digestive enzymes localized in the brush border membrane of epithelial cells. Experiments assessing passage of fluorescent beads across small intestinal mucosal samples showed that small intestinal mucus in mice is penetrable by beads equivalent to the size of bacteria (i.e., 0.5–2 μ^3) and hence contains pores as large as 2 μ^2 (Ermund et al., 2013). These large mucus pores ensure efficient nutrient absorption by the host epithelium.

The bacterial content of the mucosal barrier in the small intestine is also regulated by a cocktail of antibacterial mediators such as defensins, lysozymes, and other peptides released by Paneth cells (Peterson et al., 2007). Together, these mediators repel bacteria by generating an antibacterial gradient toward the lumen (Johansson and Hansson, 2011; Vaishnava et al., 2011). Specific mediators include the abundant Regenerating islet-derived 3 (REG3) peptides, IgA, Toll-like receptor 5 (TLR5 regulates levels of anti-flagellin antibody in the gut) (Cullender et al., 2013) and phospholipase A2-IIA (Meyer-Hoffert et al., 2008; Bevins and Salzman, 2011). Overall, antibacterial peptides kill bacteria via a range of mechanisms including by the formation of aggregates, recognition, and binding to bacterial cell wall peptidoglycans, and permeabilization of bacterial cell membranes (Chairatana and Nolan, 2017). This serves to neutralize invasion by foreign particles and maintain epithelial crypts. This antimicrobial defense mechanism is critical in the small intestine due to the discontinuous and penetrable nature of the mucus in this region and is reflected by a higher density of Paneth cells and corresponding peptides (Ouellette, 2010).

Colon

The organization of the mucus layer varies along the length of the colon. In the distal colon, there are two layers of mucus, however, whether these layers adhere to the epithelium or the colonic content is under debate. In the proximal colon, the presence of two mucus layers has been queried based on histological studies in animal models.

Johansson and colleagues reported that the mouse distal colon contains two continuous mucus layers; an inner mucus layer that is ∼50 μm thick and anchored to the mucus-producing goblet cells of the epithelial membrane, and an outer mucus layer that is loosely adherent and harbors bacteria (Johansson et al., 2008). These researchers also reported that the thickness

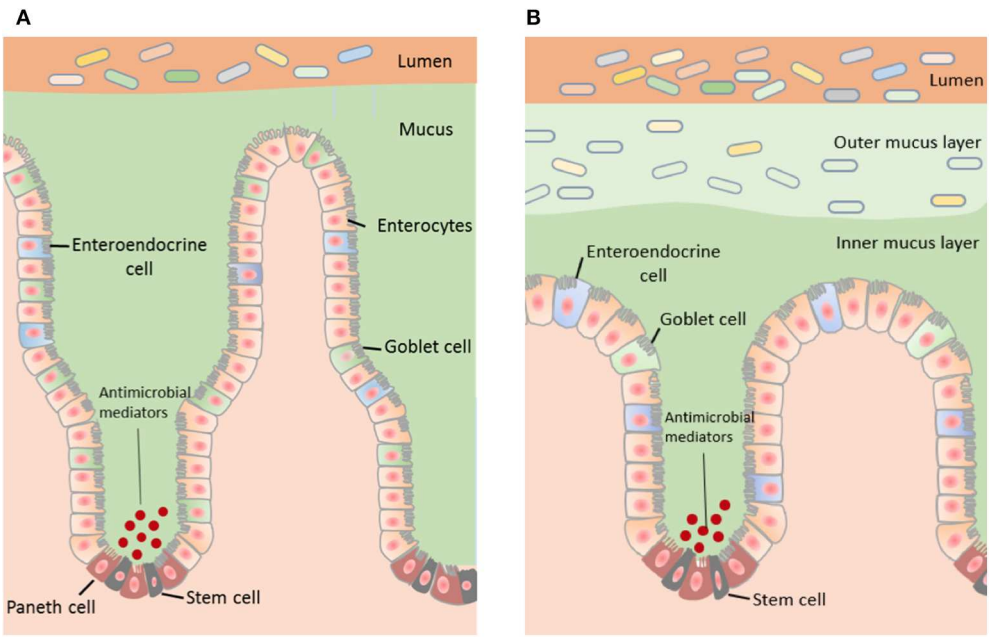

FIGURE 1 | The structure of the mucus layer varies with regional locations within the GI tract. **(A)** The small intestine contains a single layer of mucus, which is loosely attached to the epithelium and easily penetrable. Bacteria within the small intestine are primarily repelled from the epithelium by antibacterial modulators. **(B)** The distal colon contains two mucus layers; a stratified adherent inner mucus layer and loosely adhesive outer mucus layer. The inner mucus layer of the colon is essentially sterile and the outer mucus layer harbors the intestinal microbiota.

of the outer mucus layer is determined by the composition of the mucus-inhabiting bacteria. Interestingly, this group reported that the inner mucus layer of the proximal colon is also penetrable to bacteria (Ermund et al., 2013). In contrast, Kamphuis and colleagues reported that the two distal colonic mucus layers adhere to the fecal pellet rather than the intestinal epithelium in rodents and that the organization of the colonic mucus layers is dependent on the presence of fecal content (Kamphuis et al., 2017). Specifically, this study utilized fluorescence *in situ* hybridization and histological techniques in longitudinal sections to demonstrate that the fecal pellet is covered by a sterile mucus layer of variable thickness that is not attached to the epithelium. They also showed that within the proximal part of the proximal colon, which contains colon content prior to formation of a fecal pellet, the mucus layer is loosely organized and the bacteria in this region are in contact with the epithelial surface (Kamphuis et al., 2017).

The dissimilarities in the mucus layers of the colon reported may be due to methodological variations including the orientation of tissue sectioning and mucus staining techniques. Overall, multiple studies examining mucus properties carried out in both mice (Macfarlane et al., 2011; Motta et al., 2015; Welch et al., 2017) and humans (Swidsinski et al., 2007a) describe two mucus layers in the colon that include a firm mucus layer adjacent to the epithelium that is devoid of bacteria.

Commensal bacteria secrete mucinases and proteinases that continuously degrade the outer mucus layer contributing to its highly disorganized nature (Donaldson et al., 2016). Similarly, a role for bacteria in mucus thickness has been demonstrated in germ free mice which have a thinner inner colonic mucus layer. Simply adding components of the bacterial cell wall (e.g., lipopolysaccharide; LPS) is sufficient to increase mucus thickness in this model, highlighting a role for bacteria in regulating the structure of the outer mucus layer (Petersson et al., 2011). The continual release of mucus contributes to a dynamic process whereby the inner mucus layer is gradually converted to the irregular and less adherent outer mucus layer. This process involves Meprin β, an endogenous protease which aids mucus detachment (Wichert et al., 2017) and also bacteria penetration by increasing pore size in the outer mucus layer (Schutte et al., 2014).

Intestinal Mucus Composition

Mucus is primarily composed of branched glycoproteins (including mucins) that interact with the external environment and via their hydrophilic nature, influence mucus viscosity (Bergstrom and Xia, 2013). There are more than 20 subtypes of mucin identified in humans and their distribution varies throughout the GI tract. For example, the salivary glands produce MUC5B and MUC7 to lubricate food (Bobek et al., 1993; Nielsen et al., 1996; Khan et al., 1998; Thornton et al., 1999) and the mucus layer in the stomach contains MUC5AC (Ho et al., 1995; Atuma et al., 2001; Nordman et al., 2002). Although MUC5AC is not typically expressed in the large intestine, it has been detected in the distal colon along with MUC-2 during inflammation associated with ulcerative colitis and adenocarcinoma in patients (Forgue-Lafitte et al., 2007). It is well-established that the major glycoprotein within the intestinal mucus layer is mucin-2 (MUC-2 protein).

There are three major structural domains within the MUC2 protein; the N-terminal domain, a central large PTS (proline, threonine, and serine) domain and the C-terminal domain. Following translation, full-length MUC2 protein cores form dimers via disulfide bridges near their C-terminus within the endoplasmic reticulum (ER) of goblet cells. Within the Golgi apparatus, MUC2 proteins undergo O-linked glycosylation. In this process glycans such as xylose, mannose, N-acetylglucosamine, and N-acetylgalactosamine (O-GalNAc) are covalently attached to the hydroxyl group (-OH) of threonine and serine residues of the PTS domain (Godl et al., 2002). Glycans account for 80% of the total mass of the MUC2 protein and extend perpendicularly from the protein core giving the molecule a "bottle brush-like" appearance (**Figure 2**). O-Glycans can be modified via formation of linkages with sulfate, sialic acid, and fucose. These modifications play an important role in influencing interactions between the host microbial populations with mucus (Arike and Hansson, 2016).

A complex polymerization process occurs within the trans-Golgi network by which MUC2 protein dimers interact firstly as trimers and then are tightly bundled into MUC2 secretory granules (Godl et al., 2002; Ambort et al., 2012). High Ca^{2+} ion concentration alongside low pH enables mucus packing by masking negatively charged glycans on the MUC2 protein. During this process, concatenated ring structures are formed (Grubb and Gabriel, 1997; Choi et al., 2001; Ambort et al., 2012; Gustafsson et al., 2012b; Schutte et al., 2014).

Although the main component of mucus in the small intestine and the colon is mucin-2, a rich variety of other proteins largely originating from shredded epithelial cell debris that becomes trapped in the mucus are also present within the mucus biofilm, including IgG Fc-binding protein (FCGBP), Calcium activated chloride channel 1 (ClCA1), Zymogen granule membrane protein 16 (ZG16), Anterior gradient 2 (AGR2), and immunoglobulins (Johansson et al., 2008).

Mucus Expansion

After mucus secretion, the MUC2 protein complex expands dramatically to form a net-like structure (Ambort et al., 2012). Mucin expansion occurs due to increased pH and decreased Ca^{2+} levels driven by cystic fibrosis transmembrane regulator (CFTR) channels. CFTR-mediated secretion of $HCO3^{-}$ reduces Ca^{2+} levels which weakens the ring structure of the mucin complex and allows the densely packed MUC2 mucin to expand into large flat sheets (Ambort et al., 2012). The newly secreted mucus sheets are laid down on the epithelium by interacting with previously secreted mucus and subsequently attaching to the epithelium (Johansson and Hansson, 2016) (**Figure 2**). In the colon, expansion of the outer mucus layer is also triggered by bacteria that release glycosidases that sequentially cleave individual monosaccharides from mucin glycans (Johansson and Hansson, 2016) to further relax the tight-knit structure of mucin glycans (Johansson et al., 2008).

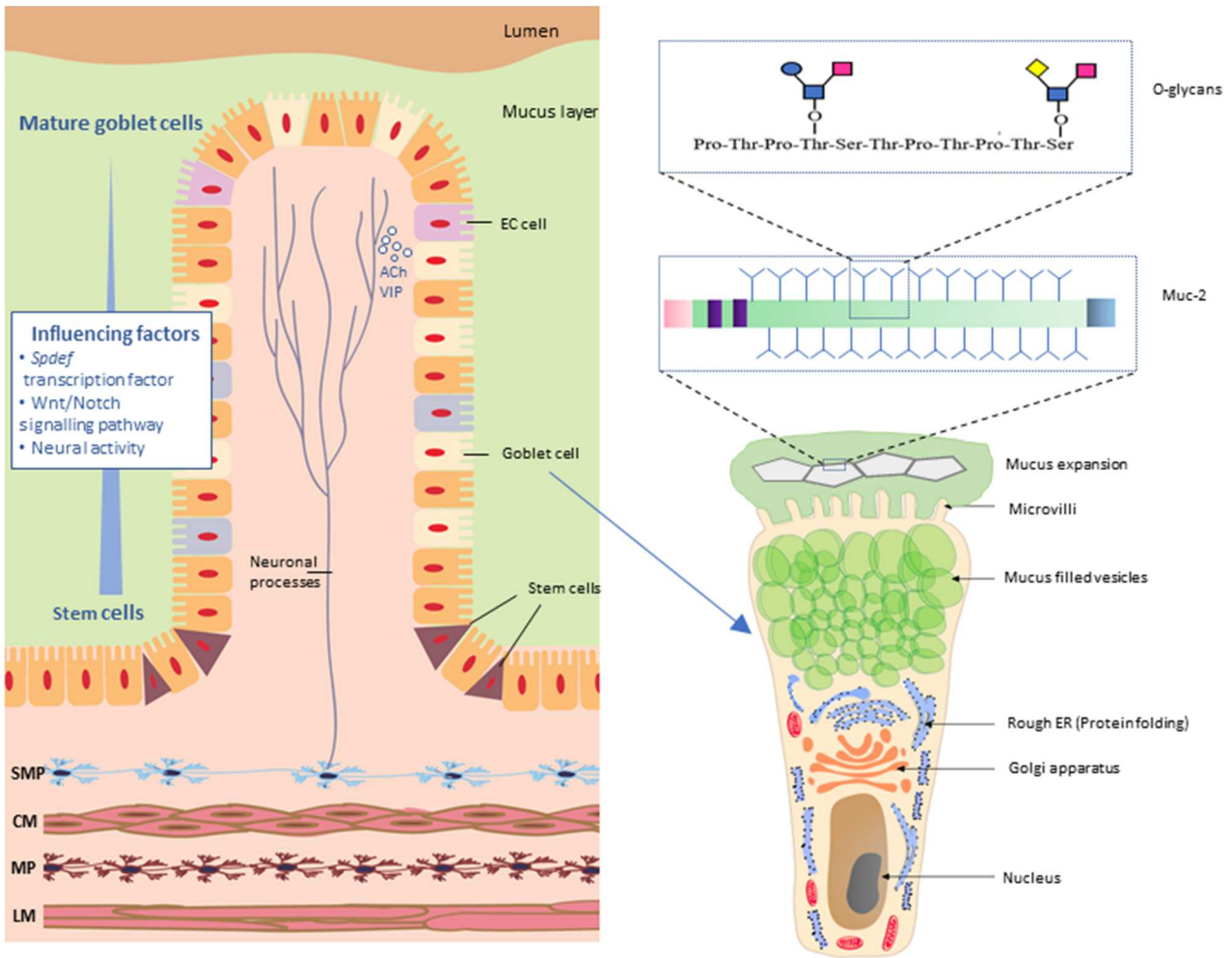

FIGURE 2 | Neuronal innervation of goblet cells in the intestinal mucosa. Neurons of the submucosal plexus (SMP) innervate goblet cells by release of neurotransmitters such as acetylcholine (ACh) and vasoactive internal peptide (VIP). Maturation of goblet cells is influenced by *SAM pointed domain-containing Ets transcription factor* (*Spdef*), Wnt/Notch signaling and neuronal activity. Mature goblet cells have a characteristic goblet shape. The apical region is distended by the presence of mucin granules, giving the cell the characteristic cup shape with other cellular organelles condensed in the basal "stem-like" region. Muc-2 protein comprises multiple O-glycans arranged in a "bottle brush" like formation. SMP, submucosal plexus; CM, circular muscle; MP, myenteric plexus; LM, longitudinal muscle; EC cell, enteroendocrine cells.

Mucus Secreting Goblet Cells

The intestinal epithelium consists of absorptive and secretory cell lineages including enterocytes, enteroendocrine cells (EECs), Paneth cells, and goblet cells. Goblet cells are specialized cells equipped with specific biological machinery for the secretion of mucus and are present throughout the entire length of the intestine (**Figure 2**). These cells, as their name suggests, are easily identifiable in histologically stained cross sections of the intestine due to their characteristic "goblet-like" shape. Intestinal epithelial cells, including goblet cells, arise from multipotential stem cells residing at the base of the intestinal crypts and subsequently migrate from the crypts to the top of the villus prior to eventually being shed into the lumen (Cheng and Leblond, 1974). In mice, this migratory process occurs over 2–3 days (Specian and Oliver, 1991). Differentiation of goblet cells is directly controlled by the transcription factor *SAM pointed domain-containing ETS transcription factor* (*Spdef*) (Noah et al., 2010) and also via a network of transcriptional factors regulated by the Notch and Wnt signaling pathways known to influence developmental and inflammation pathways (van Es et al., 2005; Clarke, 2006; Fre et al., 2009; Gersemann et al., 2009; Gregorieff et al., 2009; Kwon et al., 2011; Heuberger et al., 2014; Tian et al., 2015). Furthermore, enteric neural activity has been shown to influence the maturation and production of stem cells in the GI tract (Lundgren et al., 2011) which, in turn, suggests a role for the ENS in goblet cell proliferation and differentiation.

Goblet cell morphology changes dramatically during the cellular lifespan (Specian and Oliver, 1991). Immature goblet cells are larger and pyramidal in shape with cellular organelles dispersed throughout the cell and interspersed with mucus

granules in the apical cellular region. As these goblet cells migrate toward the colonic epithelial surface, they reduce in volume as a result of shedding cytoplasmic content and organelles. During this phase of volume reduction, goblet cells reduce contact with the basal laminar surface adjacent to the epithelium and simultaneously increase contact with the luminal surface of the GI tract. The goblet cells then rapidly produce and store mucus granules, resulting in the distention of the apical cellular region to produce the typical "cup" shape. The nucleus and other cellular organelles of the goblet cells are concentrated in narrowed stem-like subcellular regions located at the base of the cells (Specian and Oliver, 1991). These processes could be altered in neurological disorders. For example in Alzheimer's disease, the metalloprotease Meprin β, which cleaves amyloid precursor protein (Schönherr et al., 2016; Becker-Pauly and Pietrzik, 2017) also regulates mucus detachment from goblet cells in the small intestine (Wichert et al., 2017).

Mucus Interactions With Microbes

Microbial populations are spatially organized along the length of the intestine as well as from the luminal to mucosal axis (Palestrant et al., 2004). Mucus viscosity increases toward the distal region of the GI tract. This viscosity gradient along the length of the GI tract reportedly determines the spatial distribution of intestinal microbiota (Swidsinski et al., 2007b). The composition of bacteria adjacent to the mucosa is different to the bacterial populations that reside within the luminal content (Swidsinski et al., 2005). This mucosal to luminal bacterial distribution is likely driven by variations in oxygen levels and nutrient availability (Yasuda et al., 2015).

The mucus layer serves as a carbon and energy source, predominantly in the form of glycans, for mucus residing bacteria. As an adaptation to residing in a glycan-rich environment, these bacteria produce mucus-degrading enzymes such as glycosidase, sulphatase, and sialidases (**Table 1**) that cleave the mucus network to enhance the utilization of mucus as an energy source. A range of mucus-degrading bacteria present within the mucus, includes *Akkermansia muciniphila* (Derrien et al., 2004), *Bacteroides thetaiotaomicron* (Xu et al., 2003), *Bifidobacterium bifidium* (He et al., 2001), *Bacteroides fragilis* (Macfarlane and Gibson, 1991), and *Ruminoccous gnavus* (Png et al., 2010). These bacterial species cleave mucus O-glycans to produce monosaccharides (Berry et al., 2013) which can be further utilized by other mucus-residing bacteria including Lachnospiraceae (Nava et al., 2011), Clostridium cluster XIV (van den Abbeele et al., 2013), Enterobacteriaceae (Ashida et al., 2008), and *Clostridium difficile* (Ng et al., 2013). Further adaptation of bacteria has been identified in Lactobacillus (Etzold et al., 2014) and Bacteroides (Sicard et al., 2017) where the presence of multi-repeat cell-surface adhesins enable retention of the bacteria within the mucus layer. The syntrophic, symbiotic, and mutualistic interactions of the microbes in the mucus layer create the environment which drives microbial community selection and defines physical properties of the mucus layer.

Some mucus residing bacteria form mucosal biofilms, complex microbial communities embedded in a polymeric matrix. Techniques including fluorescent *in situ* hybridization and electron microscopic studies reported the presence of bacterial biofilms in the healthy colon of mice, humans and rats (Palestrant et al., 2004; Swidsinski et al., 2005; Bollinger et al., 2007; Macfarlane et al., 2011; Motta et al., 2015). Altered levels of biofilm associated bacteria such as *Bacteroides fragilis*, Enterobacteriaceae family were reported in Crohn's disease and inflammatory bowel disease (Masseret et al., 2001; Macfarlane and Dillon, 2007; DuPont and DuPont, 2011; Srivastava et al., 2017).

Therefore, the mucus associated bacterial biofilm also could play a role in these disorders. Alterations in these complex community structures could result in abnormal mucus invasion, epithelial adherence, and spatial distribution of bacterial species.

TABLE 1 | Predominant mucus-degrading bacteria and secreted digestive enzymes.

Bacteria	Mucus degrading enzyme	References
Akkermansia muciniphila	Glycosidase	Png et al., 2010; van Passel et al., 2011
Bacteroides thetaiotaomicron	Sulfatase, neuraminidase, α-fucosidase, β-galactosidase α- N-acetylgalactosaminidase β-N-acetylglucosaminidase	Xu et al., 2003
Rumminococcus gnavus	α-galactosidases	Png et al., 2010
Rumminococcus torques	α-N-acetylgalactosaminidase	Png et al., 2010
Bacteroides fragilis	Neuraminidase, sulfatase, protease, α-N-acetylgalactosaminidase, β-galactosidase, β -N-acetylglucosaminidase, α-fucosidases	Macfarlane and Gibson, 1991
Bacteroides vulgatus	Neuraminidase, α and β-galactosidases, α-fucosidase β-N-acetylglucosaminidase, α and β -N-acetylgalactosaminidase	Onderdonk et al., 1983; McCarthy et al., 1988
Adherent invasive *Escherichia coli*	Vat protease	Gibold et al., 2016
Giardia duodenalis	Cysteine protease	Amat et al., 2017
Entamoeba histolytica	Cysteine protease	Lidell et al., 2006

THE ENTERIC NERVOUS SYSTEM (ENS)

The digestive tract is innervated by the enteric nervous system (ENS), an intrinsic neuronal network that regulates GI functions (Furness et al., 2013) in addition to extrinsic innervation from the parasympathetic and sympathetic components of the autonomic nervous system (reviewed in Uesaka et al., 2016). Neuronal control of intestinal function is largely regulated by two ganglionated plexuses; the myenteric and submucosal plexus. The myenteric plexus predominantly regulates GI motility while the submucosal plexus regulates the secretion of water and electrolytes primarily via the neurotransmitters acetylcholine (ACh) and vasoactive intestinal peptide (VIP).

The ENS Influences Mucus Secretion

Mucus secretion is influenced by nervous system activity and occurs via two processes; (i) vesicle secretion and (ii) compound exocytosis. During vesicle secretion, mucus-secreting goblet cells release mucus content by fusion of the mucus granule membrane with the overlying plasma membrane (Lang et al., 2004). This process is regulated by vesicle exocytotic components like syntaxin, Munc 18, vesicle-associated membrane proteins (VAMP) and synaptosomal nerve-associated proteins (SNAP) proteins (Cosen-Binker et al., 2008). During compound exocytosis, all mucus granules are fused together and empty the mucus as a single unit. As yet, the molecular pathways regulating compound exocytosis have not been defined.

VIP and ACh are the two main secretagogues responsible for neurally-evoked mucosal secretion (Specian and Neutra, 1980; Neutra et al., 1984; Lelievre et al., 2007; Gustafsson et al., 2012a; Ermund et al., 2013). ACh induces mucus secretion by activating M3 muscarinic receptors located on goblet cells within the epithelium in both the small intestine and in the colon (Specian and Neutra, 1980; Neutra et al., 1984; Gustafsson et al., 2012b; Ermund et al., 2013). Exocytosis of mucus-containing granules is regulated by intracellular Ca^{2+} and Ca^{2+-}mobilizing agents (including acetylcholine; Birchenough et al., 2015). The activation of M3 muscarinic receptors mobilizes Ca^{2+} from intracellular stores to induce mucus secretion (Ambort et al., 2012).

Mucus release is differentially regulated in a region-specific manner in the GI tract. ACh specifically targets both crypt and villus-associated goblet cells in the small intestine (Birchenough et al., 2015). In contrast, in the colon, goblet cells located in crypts are responsive to ACh, but equivalent cells at the epithelial surface do not respond to ACh or the cholinergic agonist, carbachol (Gustafsson et al., 2012b). Release of the neuropeptide VIP enhances mucus secretion (Lelievre et al., 2007) via modulating CFTR-dependent secretions (Alcolado et al., 2014). Furthermore, VIP deficiency in mice results in reduced goblet cell number and reduced *muc-2* gene expression levels (Wu et al., 2015). A recent study displayed that mucosal VIP-containing neurons are in close proximity with ileal goblet cells and VPAC receptor antagonist alter the goblet cell numbers in the ileum (Schwerdtfeger and Tobet, 2020).

Gut Motility and Mucus Movement

In addition to its prominent action in regulating GI motility and peristalsis, the myenteric plexus plays a key role in mucus renewal. GI motility regulates mucus levels by propelling mucus to the distal GI tract. Myenteric neurons coordinate cyclic motility patterns known as migrating motor complexes (MMCs) that contribute to the "housekeeping" functions of the intestine by flushing undigested materials, mucus, and bacteria along the small intestine. Altered ENS regulation of motility can therefore also perturb mucus renewal. Interestingly, patients with irritable bowel syndrome (IBS) report lower MMC frequencies and show bacterial overgrowth in the small intestine (Pimentel et al., 2002) implicating alterations in the mucus environment.

ANIMAL MODELS OF MUCUS IMPAIRMENT

Preclinical models have demonstrated that abnormalities in GI structure and function are associated with altered mucus production. For example, colonic mucus layer thickness is decreased alongside progressive inflammation in a mouse model of colitis (Petersson et al., 2011). In the absence of an inner mucus layer, bacteria can penetrate deep into the epithelial crypts and interact with the colonic epithelium (Johansson et al., 2008) which can exacerbate disease. Furthermore, multiple studies report that alterations in mucus secretory processes result in an underdeveloped colonic inner mucus layer, often associated with sparsely filled goblet cells and an increased susceptibility to colitis (An et al., 2007; Park et al., 2009; Stone et al., 2009; Fu et al., 2011; Tsuru et al., 2013; Bergstrom et al., 2014).

Muc-2 Knockout Mice

Mice lacking the mucus protein MUC2 ($MUC2^{-/-}$ mice) lack an inner colonic mucus layer despite the presence of goblet cells and other mucus layer components. Interestingly, Rahman and colleagues showed changes in colonic innervation in mice expressing a point mutation in *Muc-2* (Rahman et al., 2015) highlighting interactions between mucus production and innervation of the GI tract. Knockout mice also exhibit altered intestinal cell maturation, migration, and abnormal intestinal crypt morphology (Velcich et al., 2002). These mice develop adenomas and rectal tumors as well as increased infiltration of neutrophils and lymphocytes, loose stools, diarrhea with blood, rectal prolapses, and fail to thrive (Velcich et al., 2002). In the longer term, these mice also show increased susceptibility to developing colon cancer (Velcich et al., 2002; van der Sluis et al., 2006).

Cystic Fibrosis

Patients with cystic fibrosis are commonly diagnosed with concomitant GI abnormalities including meconium ileus and distal intestinal obstruction syndrome (Colombo et al., 2011) due to an increase in secreted mucus volume, mucus dehydration, and increased viscosity that contributes to blockage of the small intestine. Both mucus buildup and reduced mucus movement occur in these patients due to dysregulated mucus secretion. Cystic fibrosis is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) channel important for mucus hydration. These mutations cause defective chloride ion transport out of epithelial cells and dehydration of mucus overlying the epithelium. In patients, mucus remains tightly attached to the small intestinal epithelium and peristaltic movements fail to propel the mucus forward within the GI tract. In keeping with these changes, an increased bacterial load has been observed in cystic fibrosis patients (O'Brien et al., 1993), likely due to the elevated volume and viscosity of mucus that provides an ideal environment for commensal microbes.

Mouse models expressing CFTR mutations also display severe intestinal dysfunction and a mucus layer that is firmly attached to the mucosal epithelium (Grubb and Gabriel, 1997; Seidler et al.,

2009; Frizzell and Hanrahan, 2012). Since a prominent role of mucus is to trap and transport bacteria to the distal regions of the gastrointestinal tract via peristalsis, animal models provide an excellent experimental tool to investigate the effects of mucus perturbation on microbial dysbiosis.

Hirschsprungs Disease

Extreme effects of neuronal loss on goblet cell function and on mucus layer properties have been observed in Hirschsprung disease, a life-threatening developmental disorder where the distal colon lacks enteric neurons due to the failure of neural crest cells to completely migrate during gastrointestinal development. Patients with Hirschsprung disease have a reduced mucin turnover rate, a decreased goblet cell population and reduced expression of *Spdef* and *Krueppel like factor 4* which drive goblet cell differentiation and maturation (Aslam et al., 1997a,b; Nakamura et al., 2018). These findings highlight the importance of the ENS in the development and function of mucus-producing goblet cells in the clinical setting.

Mouse models of Hirschprung Disease additionally provide evidence for neural-mucus interactions. For example, endothelin receptor B knockout mice (Ednrb$^{-/-}$ mice) along with mice expressing a mutation in the RET gene that encodes the receptor for the glial cell line-derived neurotrophic factor (GDNF) are well-characterized models which have been examined for alterations in mucus and goblet cell structure. Mice lacking endothelin receptor B, known for its role in angiogenesis and neurogenesis, show colonic aganglionosis resembling the clinical presentation. Ednrb$^{-/-}$ mice showed an increase in both goblet cell numbers and size as well as increased expression of *Spdef* and Math 1 transcription factors in the distal colon (Thiagarajah et al., 2014). In addition, the absence of *Ednrb* in mice alters mucus structure as evidenced by reduced permeability to 200 nm nanoparticles *in vitro* (Thiagarajah et al., 2014; Yildiz et al., 2015). Furthermore, significant differences in the commensal microbiome were also present in this model (Ward et al., 2012).

The absence of GDNF signaling in mice similarly results in a severely underdeveloped ENS. Furthermore, these mice have altered mucus composition and mucus retention (Porokuokka et al., 2019). Overall, these clinical and animal model data illustrate involvement of the nervous system in the regulation of goblet cell differentiation and maturation as well as influencing mucus properties.

NEUROLOGICAL DISORDERS AND MUCUS DYSFUNCTION

Patients with neurological disorders frequently present with coexistent bowel diseases but whether this is due to nervous system changes *per se* or additional downstream effects such as dysbiosis, immune dysregulation and/or altered mucus production is uncertain. Gut disorders are often associated with, and precede, the core diagnostic symptoms of autism, Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis (Pfeiffer, 2003; Buie et al., 2010; Preziosi et al., 2013; Coggrave et al., 2014). Severe gastrointestinal dysfunction can be debilitating, exacerbate core symptoms of neurological disease, and decrease quality of life. Thus, clarifying the role of the nervous system in mucus production and maintenance could improve understanding of the pathophysiology of neurological disease. Furthermore, modulating mucus properties to optimize probiotics and microbial engineering could provide additional "psychobiotic" therapeutic options for these disorders.

A major function of the intestinal mucus layer is to form a barrier between the intestinal epithelium and the luminal content to protect the intestine from pathogenic invasion. A number of biological pathways influence mucus production and volume: (i) stem cell proliferation and subsequent maturation of goblet cells is influenced by the SPDEF transcription factor and the Wnt/notch signaling pathways, as well as neural activity; (ii) multiple neurotransmission pathways directly activate mucus release from goblet cells, including via muscarinic receptors; (iii) motility driven by the enteric nervous system can also affect mucus renewal; (iv) vesicular signaling molecules govern mucus release; and (v) microbes are integral in maintaining mucus homeostasis (**Figure 3**).

Developmental Pathways

Key developmental pathways implicated in neurological disease are involved in goblet cell maturation, mucus production and release. For example, the Spdef and Wnt/Notch signaling pathways, known to be crucial for neuronal development in the brain, also influence stem cell maturation in the GI tract. As Spdef regulates the terminal differentiation of goblet cells and Paneth cells (Noah et al., 2010) alterations in these pathways would influence goblet cell turnover and numbers (Lo et al., 2017), therefore modulating mucus properties. The Wnt-beta catenin pathway is also associated with neurological disease (Sani et al., 2012; Zhang et al., 2012, 2014; Ferrari et al., 2014; Huang et al., 2015; Hoseth et al., 2018). This pathway stimulates the synaptic expression and localization of neuroligin-3, a synaptic adhesion protein associated with autism spectrum disorder (Medina et al., 2018). Wnt signaling pathways are also implicated in Parkinson's Disease via interactions with PARK genes (Berwick and Harvey, 2012). Although potential changes in goblet cell number and morphology or mucus properties have not been studied in animal models of autism or several other models of neurological disorders, we predict that Wnt-mediated pathways are altered in the gastrointestinal tract and affect mucus properties, thereby contributing to patient GI symptoms.

Protein Misfolding

Due to the high levels of protein produced, mucus production processes within goblet cells are susceptible to protein misfolding, retention in the endoplasmic reticulum (ER), and ER stress. Protein misfolding is known to trigger the unfolded protein response (UPR), which is associated with chronic inflammation and autoimmune changes in neurodegenerative diseases such as PD, Alzheimer's disease, and multiple sclerosis (Mhaille et al., 2008; Matus et al., 2011). Accordingly, protein misfolding could result in altered production and apoptosis of goblet cells, therefore affecting mucus properties.

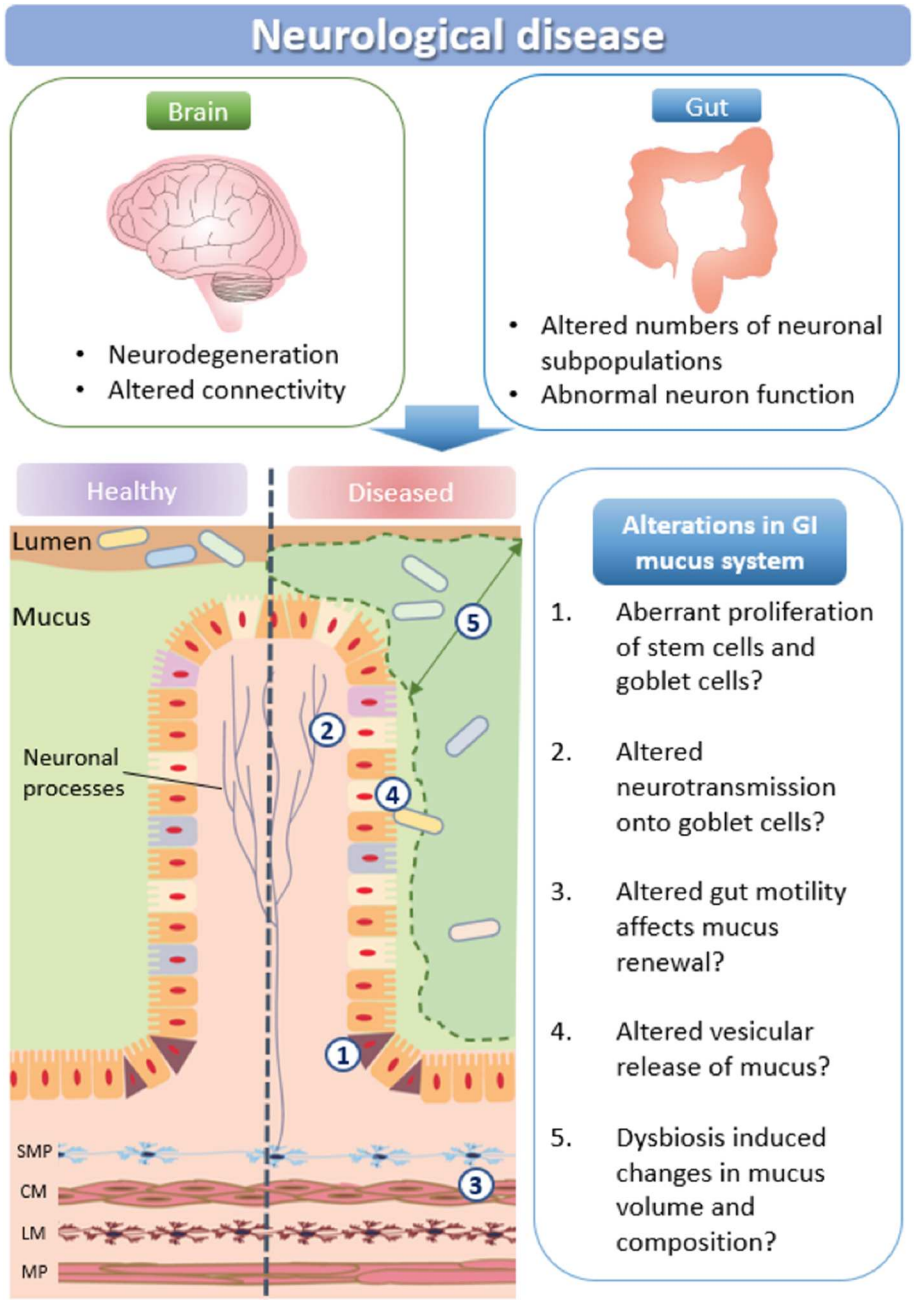

FIGURE 3 | How neurological disease may impact mucus production. Schematic representation of potential changes in mucus production and microbial communities in neurological disorders. SMP, submucosal plexus; CM, circular muscle; MP, myenteric plexus; LM, longitudinal muscle.

Vesicle-Associated Proteins

Biological pathways required for neurotransmission and mucus release share molecular components. Multiple neurological disorders are associated with gene mutations that impair neuronal communication via synapses, therefore mutations in the brain potentially affect mucus properties in the gastrointestinal tract. Examples of mucus release components that overlap with synaptic neurotransmitter systems include syntaxin, Munc 18, VAMP, and SNAP proteins. These vesicle-associated proteins are commonly expressed at neuronal synaptic membranes and have been identified as being mutated in neurological disorders (syntaxin; ASD, SNAP; ADHD, Munc 18; epilepsy/ASD (Guerini et al., 2011; Durdiaková et al., 2014; Hamada et al., 2017). Changes in the function of these proteins will not only contribute to brain disorders but may also disrupt vesicular secretion of mucus. Further investigation of mucus

TABLE 2 | Altered mucosal microbiome in patients with neurological disease.

Neurological disorder	Gastrointestinal dysfunction	Altered mucosal microbiome (↓ ↑ abundance)	References
Autism spectrum disorder	Constipation, diarrhea, functional abdominal pain, food allergies, bloating	↓ Akkermansia muciniphila ↓ Bifidobacteria species	Wang et al., 2011
		↑ Mucosa-associated Clostridiales (Lachnospiraceae and Ruminococcaceae) ↓ Dorea, Blautia, Sutterella	Luna et al., 2017
		↑ Burkholderia ↓ Neisseria	Kushak et al., 2017
		↑ Sutterella	Williams et al., 2012
		↓ Bacteroidetes ↑ Firmicutes (↑ *Ruminococcaceae* ↑ *Lachnospiraceae*)	Williams et al., 2011
Parkinson's disease	Constipation	↓ Faecalibacterium (*Blautia*, *Coprococcus*, and *Roseburia*) ↑ Pro-inflammatory Proteobacteria of the genus *Ralstonia*	Keshavarzian et al., 2015
		↓ *Dorea*, ↓ *Bacteroides*, ↓ *Prevotella*, ↓ *Faecalibacterium*, ↓ *Bacteroides massiliensis*, ↓ *Bacteroides coprocola*, ↓ *Stoquefichus* ↓ *Blautia glucerasea*, ↓ *Dorea longicatena*, ↓ *Bacteroides dorei*, ↓ *Bacteroides plebeus*, ↓ *Prevotella copri*, ↓ *Coprococcus eutactus*, ↓ *Ruminococcus callidus* ↑ *Christensenella,* ↑ *Catabacter,* ↑ *Lactobacillus,* ↑ *Oscillospira,* ↑ *Bifidobacterium,* ↑ *Christensenella minuta,* ↑ *Catabacter hongkongensis,* ↑ *Lactobacillus mucosae,* ↑ *Ruminococcus bromii,* ↑ *Papillibacter cinnamivorans*	Petrov et al., 2017
		↓ Prevotellaceae	Scheperjans et al., 2015
		↑ *Akkermansia muciniphila*	Heintz-Buschart et al., 2018
Alzheimer's disease	Constipation, incontinence	↑ *Akkermansia muciniphila* ↑ *Prevotella denticola*	Zhuang et al., 2018
		↓ Firmicutes and Bifidobacterium ↑ Bacteroidetes	Vogt et al., 2017
		↑ Escherichia/Shigella (pro-inflammatory) ↓ E. rectale (anti-inflammatory)	Cattaneo et al., 2017
Multiple sclerosis	Constipation, diarrhea	↑ *Methanobrevibacter* ↑ *Akkermansia muciniphila* ↓ *Butyricimonas*	Jangi et al., 2016
		↑ *Akkermansia muciniphila*,	Berer et al., 2017
		↓ Faecalibacterium	Cantarel et al., 2015
		↑ *Akkermansia muciniphila*, ↑ *Acinetobacter calcoaceticus* ↓ *Parabacteroides distasonis*	Cekanaviciute et al., 2017

Arrows indicate an increase or decrease in abundance of bacteria.

properties is therefore warranted in these models and in patients with neurological disorders that potentially express mutations in these and related synaptic genes.

Mucosa-Associated Microbial Dysbiosis

In neurological disease, changes in mucus properties could additionally alter commensal microbial populations. Dysbiosis has been reported for the mucus-residing microbiome in patients with various neurological disorders including autism, Parkinson's disease, Alzheimer's disease, and multiple sclerosis (**Table 2**). Because dysbiosis can alter gut barrier function (i.e., via altering mucus thickness), this could contribute to disease progression. Microbial populations influence mucus hydration by releasing enzymes that modify mucus structural networks. Microbes release enzymes that degrade mucus, and this enzymatic cleavage of mucin complexes expands and hydrates the mucus 3-dimensional structure. For example, increased release of mucin-degrading enzymes due to an overgrowth of mucus-residing

bacteria (such as *Akkermansia muciniphila*) increases mucus thickness and strengthens the protective mucosal barrier (Ottman et al., 2017). An additional effect of increasing mucus thickness may be reduced nutrient absorption. Such an increase could be beneficial (i.e., in the case of obesity) but detrimental in neurodegenerative diseases such as multiple sclerosis and Parkinson's Disease (Cani, 2018).

Autism

Autism spectrum disorder is a neurodevelopmental disorder characterized by impaired social interactions and restrictive and repetitive behavior. In 2018, 1 in 59 children are diagnosed with autism in the United Status. GI dysfunction is a major comorbidity for autism patients (Kohane et al., 2012; Chaidez et al., 2014; McElhanon et al., 2014) and includes symptoms such as abdominal pain, diarrhea, constipation, and bloating. Altered levels of mucosa-associated bacterial species are reported in autism patients with GI dysfunction with *Akkermansia muciniphila* Dorea, Blautia, *Sutterella Neisseria* having decreased abundance, while mucosa-associated Clostridiales (*Lachnospiraceae* and *Ruminococcaceae*), Burkholderia, *Ruminococcaceae, Lachnospiraceae,* and Sutterella have increased abundance (Wang et al., 2011; Williams et al., 2011, 2012; Kushak et al., 2017; Luna et al., 2017).

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease observed in people over 60 years of age (de Lau and Breteler, 2006). In addition, PD is increasingly correlated with GI disorders prior to the onset of characteristic motor symptoms such as tremor and coordination of complex movement. Although the pathophysiology of PD remains unclear, the accumulation of α-synuclein appears to cause neuronal death (Kirik et al., 2002; Braak et al., 2003). Parkinson's patients with colonic inflammation also showed α-synuclein deposits in their colon (Holmqvist et al., 2014). The mucosal biopsy samples of PD patients showed increased abundance of *Akkermansia muciniphila,* and Ralstonia, and a decrease in abundance of Faecalibacterium (Blautia, Coprococcus, Roseburia) and Prevotella (Keshavarzian et al., 2015; Scheperjans et al., 2015; Petrov et al., 2017; Heintz-Buschart et al., 2018).

Alzheimer's Disease

Alzheimer's disease is an increasingly prevalent neurodegenerative disease characterized by progressive cognitive decline and also reported to have comorbid GI dysfunction. Patients with Alzheimer's disease who also had symptoms indicative of IBS showed dysbiosis involving increased abundance of mucolytic bacteria including *Akkermansia muciniphila* and *Prevotella denticola* (Zhuang et al., 2018). Similarly stool samples of Alzheimer patients examined for targeted bacteria showed an increase in the abundance of *Escherichia/Shigella* (pro-inflammatory taxa) and a decrease in abundance of *E. rectale* (anti-inflammatory taxa) (Cattaneo et al., 2017). Microbial dysbiosis in Alzheimer's disease has been implicated in increasing gut permeability, which may influence systemic inflammation and impairment of the blood brain barrier (Vogt et al., 2017; Kowalski and Mulak, 2019).

Multiple Sclerosis

Multiple sclerosis involves an aberrant immune system that causes inflammation and results in demyelination in the central nervous system. Multiple studies in patients with multiple sclerosis have found increased abundance of mucosal bacteria including *Akkermansia muciniphila, Methanobrevibacter,* and *Acinetobacter calcoaceticus* and decreased abundance of *Butyricimonas,* Faecalibacterium, and *Parabacteroides distasonis* (Cantarel et al., 2015; Jangi et al., 2016; Berer et al., 2017; Cekanaviciute et al., 2017). Such alterations in the mucosal microbiome potentially favor the growth of pathogenic bacteria that alter the composition of the mucus layer and therefore may exacerbate core symptoms of these disorders (Camara-Lemarroy et al., 2018; Buscarinu et al., 2019)

CONCLUSION

In summary, multiple pathways relevant to mucus homeostasis may be impacted by nervous system impairments in neurological disease. Furthermore, altered mucus properties could contribute to the widespread observations of microbial dysbiosis in autism, Parkinson's Disease, Alzheimer's Disease, and multiple sclerosis, and potentially exacerbate core symptoms. Overall, this review highlights that mucus properties could be impaired in neurological disease and provides new avenues for clinically relevant research into GI dysfunction in these disorders.

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All authors contributed to the design and drafting of the final manuscript.

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Long-Term Exposure to Ceftriaxone Sodium Induces Alteration of Gut Microbiota Accompanied by Abnormal Behaviors in Mice

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Background: Growing evidence points out that a disturbance of gut microbiota may also disturb the gut–brain communication. However, it is not clear to what extent the alteration of microbiota composition can modulate brain function, affecting host behaviors. Here, we investigated the effects of gut microbiota depletion on emotional behaviors.

Methods: Mice in the experimental group were orally administered ceftriaxone sodium solution (250 mg/ml, 0.2 ml/d) for 11 weeks. The open-field test and tail-suspension test were employed for the neurobehavioral assessment of the mice. Fecal samples were collected for 16s rDNA sequencing. The serum levels of cytokines and corticosterone were quantified using enzyme-linked immunosorbent assays. The immunohistochemistry method was used for the detection of brain-derived neurotrophic factor (BDNF) and c-Fos protein.

Results: The gut microbiota for antibiotic-treated mice showed lower richness and diversity compared with normal controls. This effect was accompanied by increased anxiety-like, depression-like, and aggressive behaviors. We found these changes to be possibly associated with a dysregulation of the immune system, abnormal activity of the hypothalamic-pituitary-adrenal axis, and an alteration of neurochemistry.

Conclusions: The findings demonstrate the indispensable role of microbiota in the gut–brain communication and suggest that the absence of conventional gut microbiota could affect the nervous system, influencing brain function.

Keywords: gut microbiota, emotional behaviors, ceftriaxone sodium, anxiety, depression, aggressive behavior

INTRODUCTION

Gut microbiota, known as a reservoir of bacteria, not only plays an essential role in host digestion and energy metabolism but shapes host immunity (Aleshukina, 2012; Antonopoulos and Chang, 2016; Thursby and Juge, 2017). Recently, evidence of its influence extends well-beyond the gut, many studies have begun to report that the gut microbiota may be associated with the development

and progression of diseases affecting multiple organ systems such as liver, lung, and brain (Felix et al., 2018; Lee and Jayaraman, 2019; Yuan et al., 2019). Researchers believe that there is a potential connection between the gut and the central nervous system (CNS). Additional studies have defined this connection as a bi-directional communication covering multiple connections, such as immune response, the vagus nerve, and humoral components (Mayer et al., 2015). Recent evidence has unlocked a novel pivotal member, gut microbiota, which plays an important role in this communication. As a result, this concept, now known as the microbiota-gut-brain (MGB) axis, has been prompted and subsequently implicated in multiple disorders, such as digestive, neurological, and psychiatric diseases (Scriven et al., 2018; Iannone et al., 2019).

Antibiotics are one of the most commonly prescribed drugs worldwide. There has been an increasing concern that variations in the microbiota induced by antibiotics may have detrimental consequences for health (Kim et al., 2017). A growing body of evidence confirms the role of specific microbial compositions in the modulation of brain functions as well as host behaviors. To be specific, a complete absence of gut microbiota resulted in alteration of blood–brain barrier (BBB) permeability and brain neurochemistry with decreased social behaviors in mice (Braniste et al., 2014).

While the whole brain is vulnerable to external stimuli, two regions that influence stress responsivity and behavior have been considered as the most likely targets for gut microbiota (Luczynski et al., 2016). The first region is the amygdala, which seems to be involved in many forms of negative emotionality, including anxiety (Davis et al., 1994; Janak and Tye, 2015). After receiving input from disgust stimuli, the amygdala projects to the regions or sub-regions regulating anxious and defensive behaviors (Kovács, 2008). Usually, the activation of the amygdala is measured by c-Fos expression (Kovács, 2008). The second region, the hippocampus, is well-known as for emotion regulation. In this study, germ-free (GF) mice exhibited more anxiety-like behaviors, which were accompanied by higher brain-derived neurotrophic factor (BDNF) levels in the dentate region of the hippocampus (Sudo et al., 2004; Neufeld et al., 2011). Here, we explored the contribution of gut microbiota to the CNS via depleting bacteria with ceftriaxone sodium, a broad spectrum antibiotic.

MATERIALS AND METHODS

Study Design

Male BALB/c mice (6–8 weeks; Institute of Laboratory Animals of Sichuan Academy of Medical Sciences, Sichuan, China) were maintained (ten mice per cage) under a specific-pathogen-free (SPF) condition at 22–26°C, 40–60% humidity, and 12-h light-dark cycle. The mice were given 1 week to acclimate. All mice were fed with adequate food and clean water. At the end of adaptive phase, all mice (initial weight 23.55 ± 1.49 g) were randomly divided into two groups ($n = 20$ for each group) and given either sterile saline solution (the control group was defined as the CT group, 0.2 ml/d) or ceftriaxone sodium solution (Qilu Pharmaceutical, Shandong, China) (the antibiotic group was defined as the AB group, 250 mg/ml, 0.2 ml/d) intragastrically once a day for 11 consecutive weeks (details about drug dosages is included in **Supplementary Materials**). Mice were housed by group (10 mice per cage) from the first day of gavage to avoid interference between different groups. A battery of behavioral tests was administrated weekly, with 1 h of rest between each test.

Eleven weeks after ceftriaxone treatment, the mice in the AB group exhibited a remarkable difference in behavioral parameters. On the second day after the last behavioral experiment was performed, the mice were administered the final gavage exposure, and 1 h later, fresh blood and stool was sampled. The mice were inspected daily for changes in appearance and body weight. All experiments followed the guidelines of the Chinese Council on Animal Care and were approved by the Animal Care Advisory Committee of Sichuan University, Sichuan, China. The experimental design was shown in **Figure 1**.

Behavioral Tests

Two behavioral tests were carried out under following sequence (from 8 a.m. to 5 p.m.): open-field test (OFT) → tail-suspension test (TST). The OFT, which involves a low stress level, preceded the TST, which involves a high stress level (Di et al., 2017). Prior to each behavioral test, mice were habituated for at least 1 h to the testing room (Champagne-Jorgensen et al., 2020). The lighting condition was set at 15 lux for all behavioral tests (Dere et al., 2004).

The Open-Field Test

The equipment of the OFT was composed of a square arena 100 × 100 cm with 40 cm walls. The floor was subdivided into a center and periphery compartment with 25 squares. Mice were placed alternatively in the open field for at least 30 min and allowed to explore undisturbedly before the first test (Champagne-Jorgensen et al., 2020). In the formal test, mice were placed singly in the center of the open field and allowed to freely explore for 5 min. Relevant parameters (the total distance, the total time in the periphery, and center of the open field) were recorded by a video monitor. At the end of the test, mice were sent back to their home cages, and the test box was cleaned with 70% ethyl alcohol and air dried. The OFT has been proven to be efficient in detecting anxiety and selecting anxiolytic drugs (Kraeuter et al., 2019).

The Tail-Suspension Test

Mice were suspended in an upside-down position by the tail, so that they could not escape or touch nearby surfaces. The rationale for the test is that mice are under enormous stress, and, if they don't have the desire to live, they will develop a motionless posture quickly and maintain it for a longer period. The total duration of quiescence and activity during 5 min was scored, respectively (Młyniec and Nowak, 2012).

Abbreviations: MGB, microbiota-gut-brain; SPF, specific-pathogen-free; OFT, open-field test; TST, tail-suspension test; OTUs, operational taxonomic units; ELISA, enzyme-linked immunosorbent assay; BDNF, brain-derived neurotrophic factor; IL, interleukin; HPA, hypothalamic-pituitary-adrenal; CNS, central nervous system; BBB, blood–brain barrier; LPS, lipopolysaccharide; GF, germ-free.

Gut Microbiota Analysis

A TIANamp Bacteria DNA Kit (TIANGEN, China) was used to extract fecal DNA. Then, the extraction was eluted using elution buffer and stored at −80°C until PCR amplification detection by LC-Bio (Hangzhou, China). The V3-V4 region of the prokaryotic 16S rRNA gene was amplified with primers

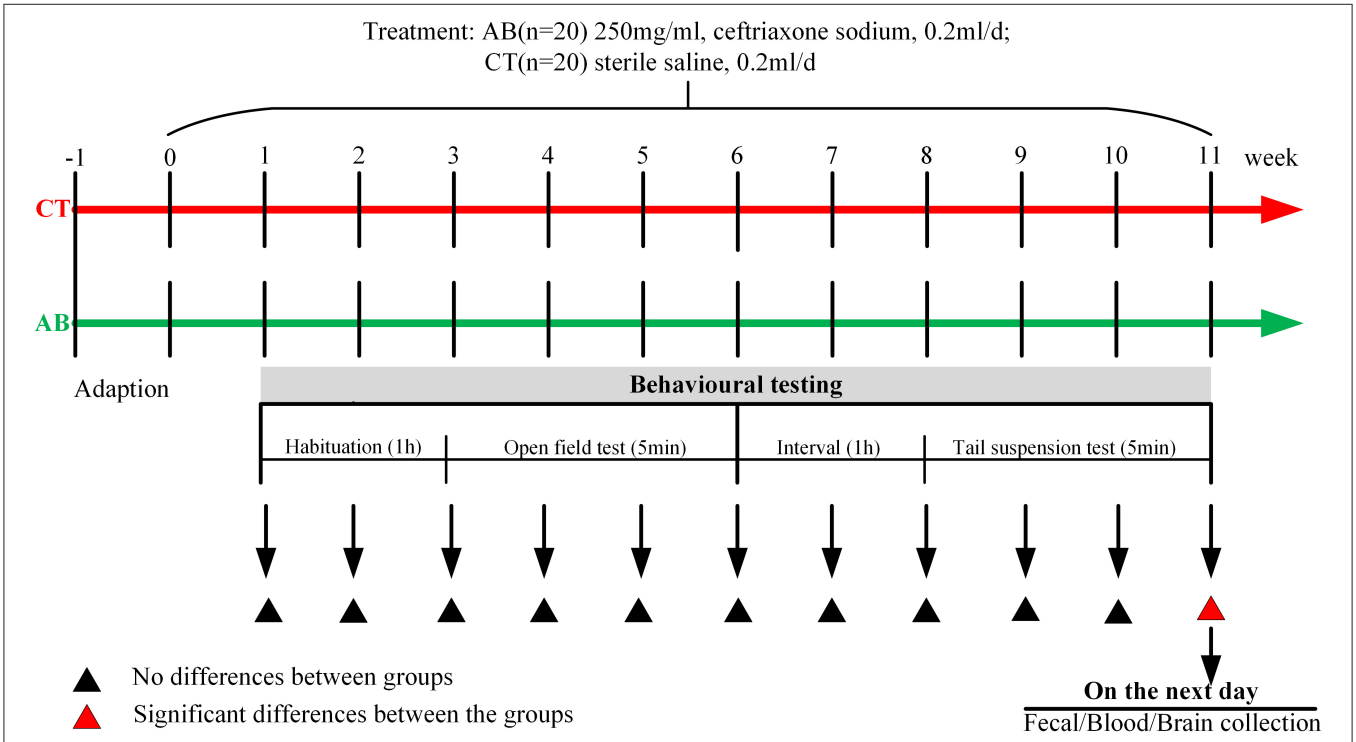

FIGURE 1 | Study design. After seven days of adaptation, the antibiotic (AB) group was given ceftriaxone and the control (CT) group was given saline by gavage once a day. The open-field test (OFT) and tail-suspension test (TST) were given to mice once a week. After 11 weeks of oral gavage, significant behavioral differences were noted between the groups. On the second day after the last behavioral experiment was performed, mice were administered the final gavage exposure, and 1 h later, fresh blood and stool was sampled.

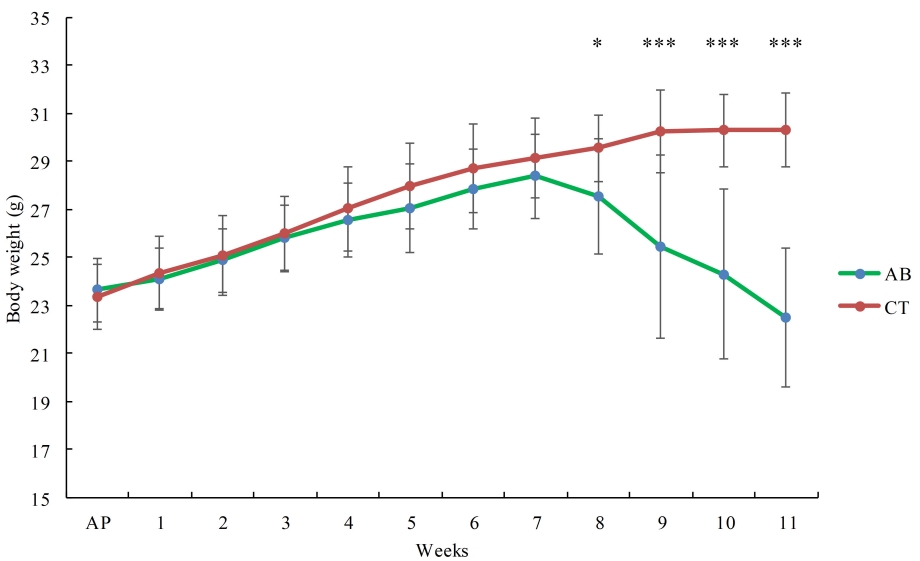

FIGURE 2 | Effect of ceftriaxone on body weight. AB, ceftriaxone administration for 11 weeks ($n = 14$); CT, saline treatment for 11 weeks ($n = 18$). AP: adaptive phase. *$P < 0.05$, ***$P < 0.001$.

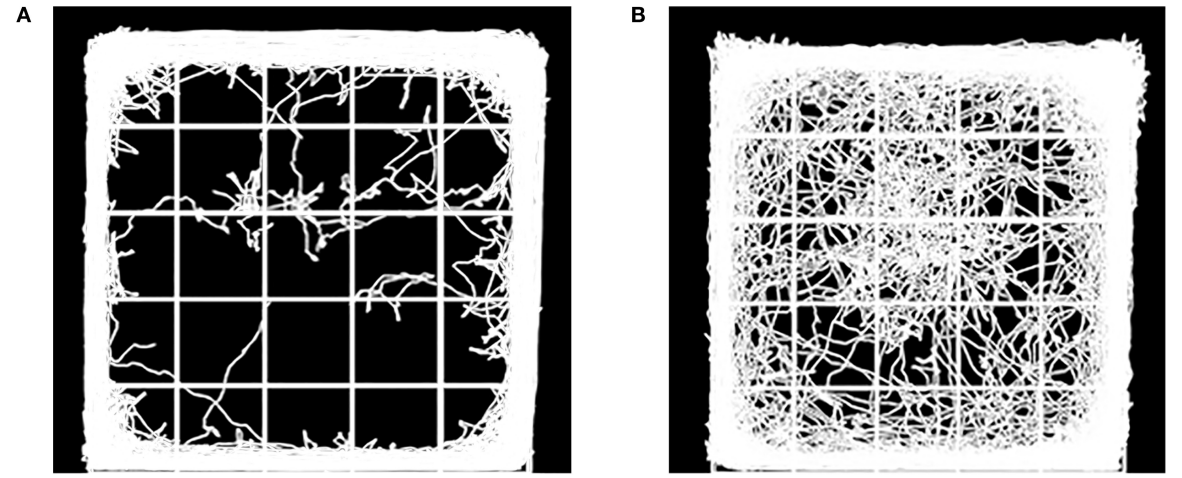

FIGURE 3 | Diagram showing movement of **(A)** AB group mice and **(B)** CT group mice in the open field. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$).

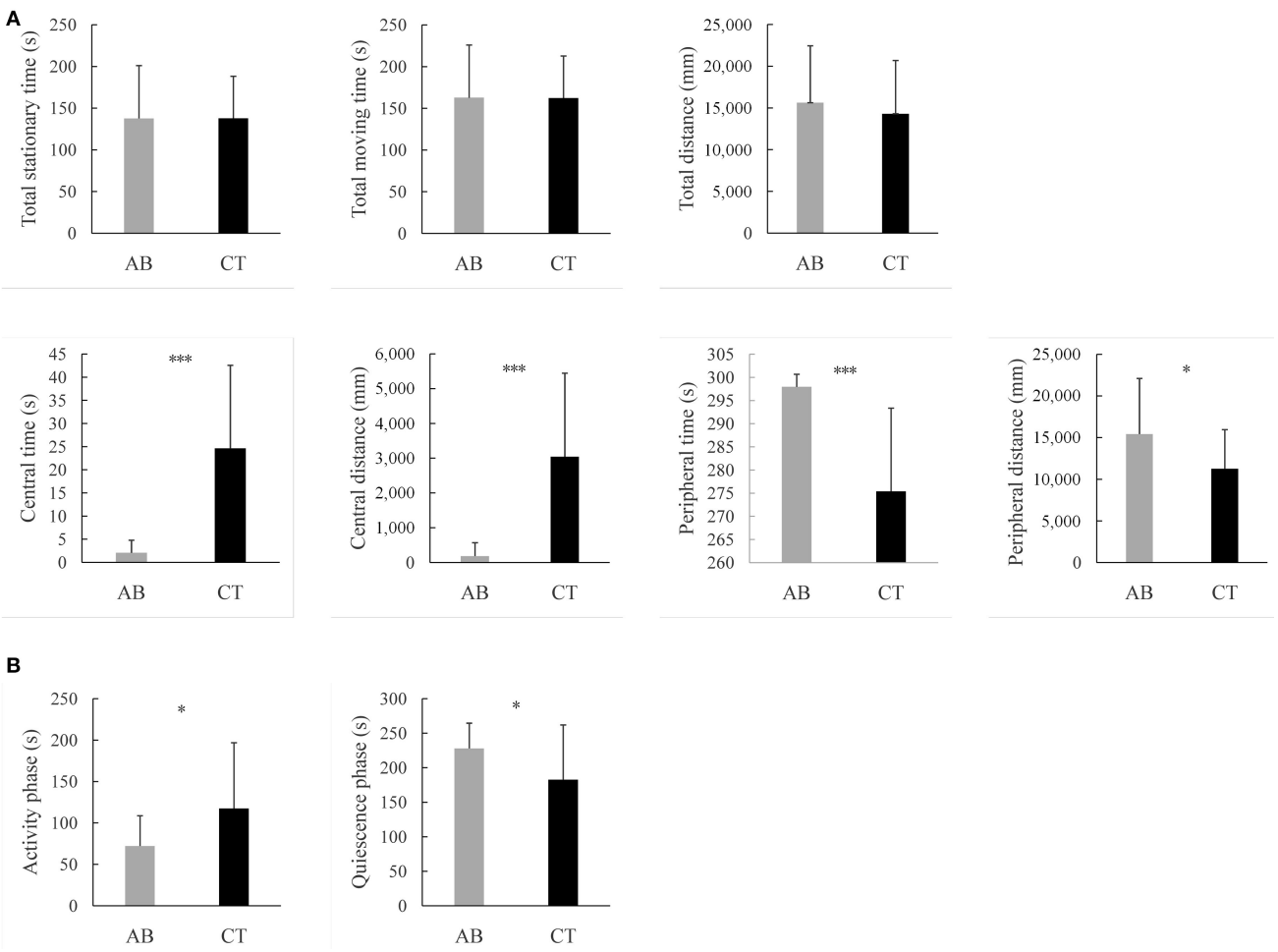

FIGURE 4 | Results of behavioral tests **(A)** in the open field test, **(B)** in the suspension tail test. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$). $^{*}P < 0.05$, $^{***}P < 0.001$.

338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Fadrosh et al., 2014). The detailed operation was performed as described previously (Li et al., 2019).

Serum Cytokine Assay

Cytokines secretion is usually induced in an inflammation or infection. Except for their effects on immunity, cytokines can also affect brain function and modulate host behaviors (Köhler et al., 2018). Research has suggested that serum IL-6 and IL-10 levels are putative biomarkers for several mood disorders (Wiener et al., 2019). Here, fresh blood was collected in sterile tubes, coagulated at room temperature, and centrifuged at 1000 × g for 10 min after the last ceftriaxone sodium treatment. The serum was stored at −70°C for later analysis. IL-6 and IL-10 were quantified by enzyme-linked immunosorbent assay (ELISA) (Neobioscience, Shenzhen Xinbosheng Biotechnology Co., Ltd, China). The detection limit of the assay was about 1 pg ml^{-1}. According to the manufacturer's protocol, the assay was performed in triplicate.

Serum Corticosterone Assay

Corticosterone is the end product of the hypothalamus-pituitary-adrenal (HPA) axis in rodents. Rising corticosterone levels suggest increases in HPA axis activity (Hiroshi et al., 2006). Serum corticosterone was measured by ELISA (Cusabio,Wuhan Huamei Biotechnology Co., Ltd, China). The detection limit of the assay was about 1 ng ml^{-1}. The assay was performed in triplicate according to the manufacturer's protocol.

Immunohistochemistry

The immunohistochemistry was used to assess expression of brain-derived neurotropic factor (BDNF) in the hippocampus and c-Fos in the amygdala. The whole process consisted of brain collection, sectioning, and immunolabeling. The details referred to previous literatures (Gareau et al., 2011).

Each maker was quantified by staining intensity and extent. We scored the staining intensity as follows: negative, weak, moderate, and strong (on a scale of zero to four). The staining extent was divided into five grades according to the percentage of positive cells in the region: negative, 0–25, 26–50, 51–75, and 76–100% (on a scale of zero to four) (Liu et al., 2011).

Semi-quantification of BDNF was calculated by multiplying the intensity score and fraction score in the CA1, CA3, and DG (dentate gyrus) regions of the hippocampus (Olympus, Tokyo, Japan, BX53). Similarly, semi-quantification of c-Fos was performed by calculating the intensity score and fraction score in the CeC, CeL, and CeM regions of the amygdala. The immunohistochemical analysis was performed blind.

Statistical Analysis

Data were expressed as the mean ± standard deviation or median (IQR) and analyzed by one-way ANOVA or Wilcoxon rank sum test in SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

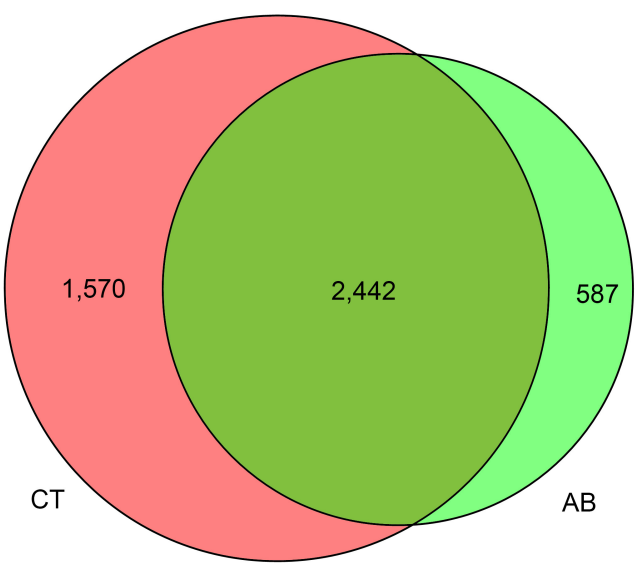

FIGURE 5 | Venn diagram of fecal bacteria. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$).

RESULTS

The Effect of Ceftriaxone Treatment on Body Weight

Mice in the AB group gained less weight than the CT group, with this difference increasing progressively over time. After gavage for 7 weeks, the weight of the AB group was significantly lower than that of the CT group ($p < 0.05$) (**Figure 2**).

The Effect of Ceftriaxone Treatment on Mice Behaviors

Mice Treated With Ceftriaxone Sodium Exhibited Anxiety-Like Behaviors

OFT is usually performed to assess locomotor activity and exploratory behavior (Kraeuter et al., 2019). The former was represented by the total distance traveled throughout the 5 min, and no differences were observed between the two groups. Previous studies suggest mice prefer staying close to the walls and travel more in the periphery field can be described as showing signs of anxiety (Crawley, 1985). Comparatively, mice with lower anxiety tend to spend more time in the central field. In this study, the AB group spent less time in the center as compared to the CT group ($p < 0.001$) after gavage for 11 weeks. Meanwhile, the AB group reduced movement in the center ($p < 0.001$) (**Figures 3**, **4A**). For details of the 11-week behavioral data analysis, see **Supplementary Figure 1**.

Mice Treated With Ceftriaxone Sodium Exhibited Depression-Like Behaviors

TST was often used for evaluating the ability to cope with a stressful situation (Kraeuter et al., 2019). Decreased duration of activity is considered a sign of depressive behavior (Castagne et al., 2011). In the test, the AB group showed decreased activity

during the 5 min and stopped escaping earlier than the CT group ($p < 0.05$) after gavage for 11 weeks (**Figure 4B**).

Mice Treated With Ceftriaxone Sodium Exhibited High Aggressive Behavior

Eight weeks after ceftriaxone administration, visible injuries were observed in the AB group, suggesting that aggressive behaviors had occurred. Four mice from the AB group were excluded from the experiment 10 weeks later due to serious injuries influencing mobility. In contrast, the CT group did not get injured throughout the experiment.

The Effect of Ceftriaxone Treatment on Gut Microbiota Composition

16S rDNA sequencing was used to identify alterations in gut microbiota after gavage for 11 weeks. Ceftriaxone administration induced a significant change in gut microbiota diversity. For the Venn diagram (**Figure 5**), the number of shared and unique OTUs indicate a similarity and difference of gut microbiota between groups, respectively (Ren et al., 2018). Based on this, there were 587 OTUs specific to the AB group and 1,570 specific to the CT group, accounting for 19.38 and 39.13% of the total OTU richness, respectively. All samples shared 2,442 OTUs at 97% similarity. The alpha diversity analysis revealed that the AB group had lower species diversity, richness, and evenness than that of the CT group by plotting Chao1, Shannon, Simpson, and Observed_species curves (**Figure 6**). A strong antibiotic effect was observed in beta diversity analysis. The PCA plot showed an appreciable separation between the two groups, indicating they had low similarity in gut microbiota composition. Likewise, the PCoA plot and MDS plot indicated the microbiota of AB group clustered separately from CT group (**Figure 7**).

According to abundance analysis, the dominant phyla in AB and CT groups were *Bacteroidetes* and *Firmicutes*, while the relative abundance of *Firmicutes* was lower in the AB group than that in CT group. Significant abundance differences were observed in the following phyla: *Proteobacteria* increased while five phyla decreased (*Firmicutes, Actinobacteria, Candidatus Saccharibacteria, Deferribacteres*, and *Candidatus_Melainabacteria*) in the AB group (**Figure 8A**, **Table 1**). At the genus level, ceftriaxone increased the

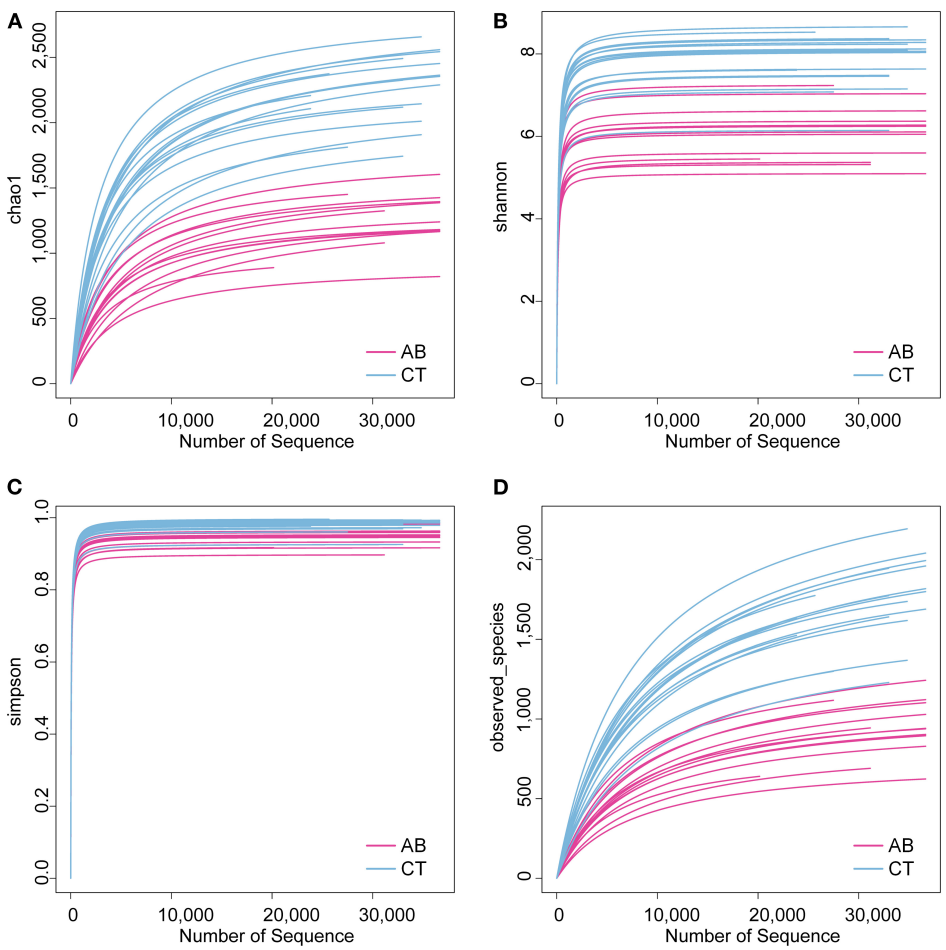

FIGURE 6 | Alpha diversity analysis for gut microbiota. **(A)** Chao1 curves for each group. **(B)** Shannon-Wiener curves for each group. **(C)** Simpson curves for each group. **(D)** Observed_species curves for each group. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$).

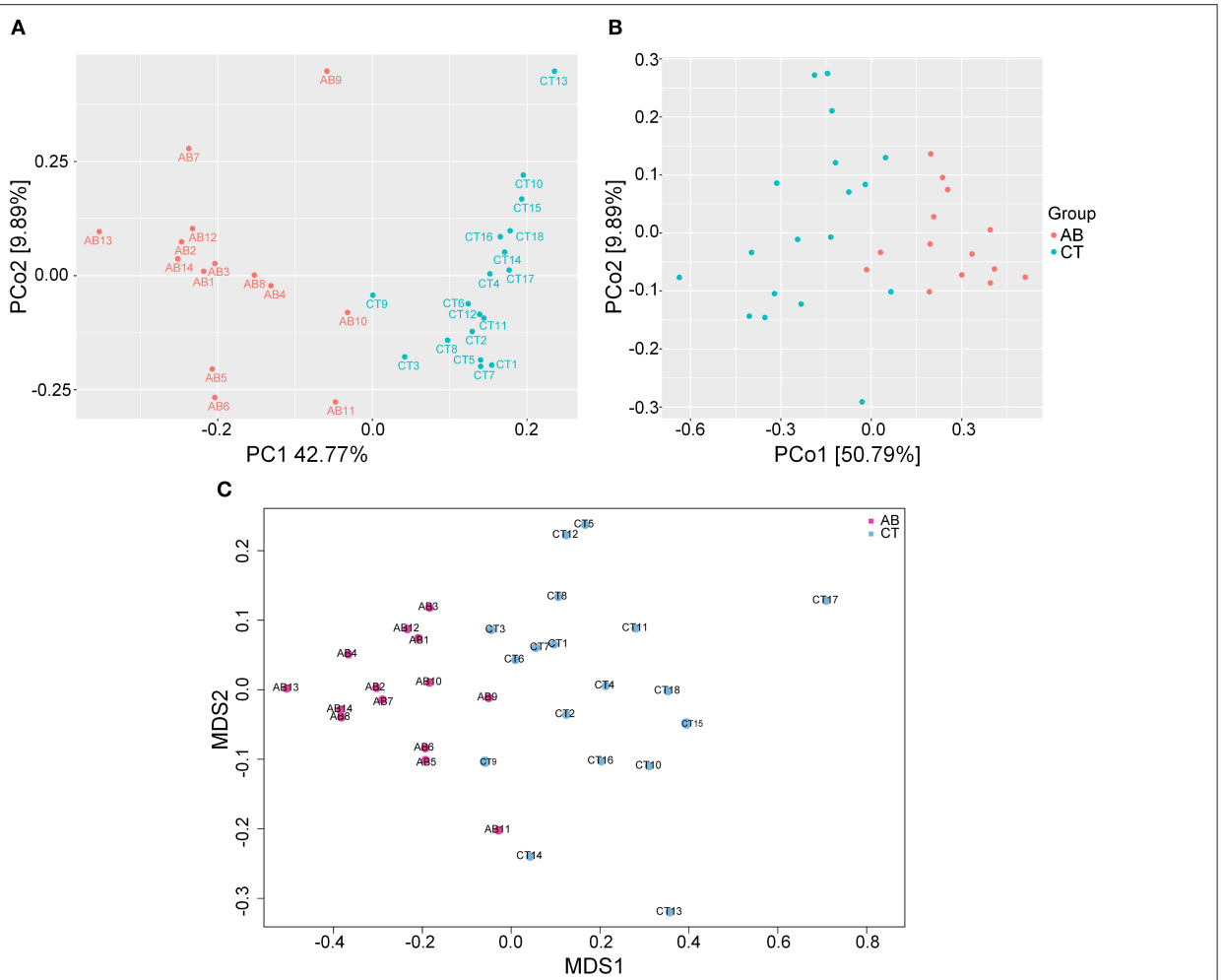

FIGURE 7 | Beta diversity analysis for gut microbiota. **(A)** PCA plot of weighted UniFrac distances between samples. **(B)** PCoA plot of weighted UniFrac distances between samples. **(C)** MDS plot of weighted UniFrac distances between samples. AB, antibiotic group (n = 14); CT, control group (n = 18).

proportion of *Proteobacteria, Porphyromonadaceae_unclassified, Escherichia*, and *Parabacteroides*, while *Lactobacillus, Acetatifactor, Bacteroidetes_unclassified, Barnesiella, Helicobacter, Prevotella, Alistipes,* and *Bacteroidales_unclassified* declined (**Figure 8B**, **Table 2**). Ten phyla and 21 genera were clustered by heatmaps, which demonstrated the relative abundance of species in different samples. In the CT group, the samples got closer to each other, indicating a higher similarity among them (**Figure 9**).

The Effect of Ceftriaxone Treatment on Serum Cytokines and Corticosterone

Ceftriaxone induced increased IL-6 and IL-10 in the AB group (IL-6: 51.82 ± 9.99 pg/ml and 43.21 ± 10.18 pg/ml for AB and CT, respectively) (IL-10: 274.81 ± 95.59 pg/ml and 173.12 ± 55.31 pg/ml for AB and CT, respectively) (**Figures 10A,B**). In addition, serum corticosterone was significantly higher in the AB group than in the CT group (10.16 ± 4.97 ng/ml and 5.39 ± 4.03 ng/ml for AB and CT, respectively) (**Figure 10C**).

The Effect of Ceftriaxone Treatment on Hippocampal Cell Proliferation and Neural Activity

A slight decrease of BDNF in the CA1, CA3, and DG regions of the hippocampus was observed in the AB group compared to the CT group (**Figure 11**, **Table 3**). Meanwhile, c-Fos expression increased in the amygdala of the AB group without a statistically significant difference (**Figure 12**, **Table 3**).

DISCUSSION

The gut, a vulnerable but vital organ, is affected by different factors easily. Antibiotics are one of the common causes leading to gut disturbance, especially given the broad spectrum of antibiotics. Consistently, little is known about adverse effects of these antibiotics on health except for drug resistance. But, recently, medications with antibiotic have been reported to enhance the risk of allergies, inflammatory bowel diseases, obesity, and even mental diseases (Harris and Baffy, 2017;

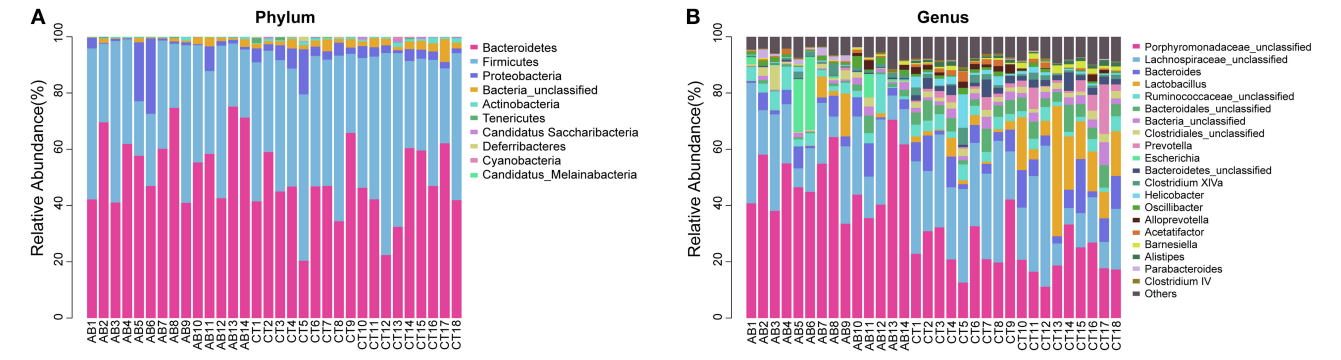

FIGURE 8 | Composition abundance of gut microbiota **(A)** at the phylum level and **(B)** the genus level. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$).

TABLE 1 | Relative abundance of gut microbiota at the phylum level.

Phylum	AB (%)	CT (%)	*P*-value
Candidatus_Saccharibacteria	0.00	0.27	0.006**
Actinobacteria	0.34	0.81	0.006**
Deferribacteres	0.00	0.15	0.008**
Bacteria_unclassified	1.17	2.62	0.005**
Candidatus_Melainabacteria	0.00	0.01	0.024*
Proteobacteria	4.89	4.13	0.034*
Firmicutes	36.51	46.12	0.047*
Bacteroidetes	56.97	45.58	0.064
Cyanobacteria	0.01	0.09	0.097
Tenericutes	0.11	0.23	0.379

*AB, antibiotic group (n = 14); CT, control group (n = 18). *P < 0.05, **P < 0.01.*

TABLE 2 | Relative abundance of gut microbiota at the genus level.

Genus	AB (%)	CT (%)	*P*-value
Porphyromonadaceae_unclassified	49.13	23.43	0.000***
Lachnospiraceae_unclassified	20.49	24.57	0.335
Bacteroides	4.32	7.07	0.082
Lactobacillus	1.86	9.23	0.028*
Ruminococcaceae_unclassified	4.21	3.45	0.408
Bacteroidales_unclassified	1.31	4.89	0.000***
Bacteria_unclassified	1.17	2.62	0.007**
Clostridiales_unclassified	1.80	2.03	0.673
Prevotella	0.10	3.14	0.010*
Escherichia	4.16	0.00	0.038*
Bacteroidetes_unclassified	0.32	2.65	0.000***
Clostridium XlVa	1.21	1.47	0.571
Helicobacter	0.24	1.93	0.029*
Oscillibacter	1.31	0.84	0.125
Alloprevotella	0.43	1.10	0.074
Acetatifactor	0.44	1.07	0.033*
Barnesiella	0.30	1.11	0.002**
Alistipes	0.00	1.00	0.000***
Parabacteroides	0.95	0.15	0.004**
Clostridium IV	0.40	0.40	0.953

*AB, antibiotic group (n = 14); CT, control group (n = 18). *P < 0.05, **P < 0.01, ***P < 0.001.*

Torres-Fuentes et al., 2017; Guo J. et al., 2019; Slykerman et al., 2019). Also, some investigators suggest that abnormal gut microbiota or some intestinal infections may be responsible for a series of metabolism or immunity-related diseases (Wang and Wang, 2016). The impact of intestinal dysbacteriosis induced by antibiotics on brain functions and behaviors piques interest and is yet to be elucidated. Therefore, this study was designed to determine whether long-term ceftriaxone exposure altered gut microbiota and thus affected host behaviors.

Ceftriaxone administration caused significant weight loss in the study. This is consistent with the previous finding that the weight gain of mice was delayed significantly following the ceftriaxone treatment (Miao et al., 2020). However, it was contradictory with previous findings that antibiotics result in weight gain in the animal production system (Angelakis, 2017). The disparate findings imply that the growth of animals may be impacted by the dosage, intervention time and, above all, types and properties of antibiotics.

Ceftriaxone could result in a significant gut microbiota dysbiosis by killing most of the normal flora and providing the living space for other potential pathogens (Cheng et al., 2019). The gut microbiota of mice was altered greatly in quantity and quality by the oral administration of ceftriaxone in this study. Similar to the result, other studies confirmed that oral ceftriaxone significantly decreased the quantity of fecal microbiota (Cheng et al., 2017, 2019; Guo et al., 2017; Miao et al., 2020). At the phylum level, the microbiota diversity of the AB group decreased, *Proteobacteria* became a dominant phylum, and the abundance of *Bacteroidetes, Firmicutes, Actinobacteria,* and *Deferribacteres* decreased. This result is supported by studies that ceftriaxone could characteristically decrease the alpha-diversity of the fecal microbiota accompanied with more *Proteobacteria* and less *Bacteroidetes* (Cheng et al., 2017, 2019; Miao et al., 2020). In some dysbiosis and related diseases, an increased *Proteobacteria* is perceived as a diagnostic characteristic since it is closely related to colon epithelial oxygenation as well as the disruption of the

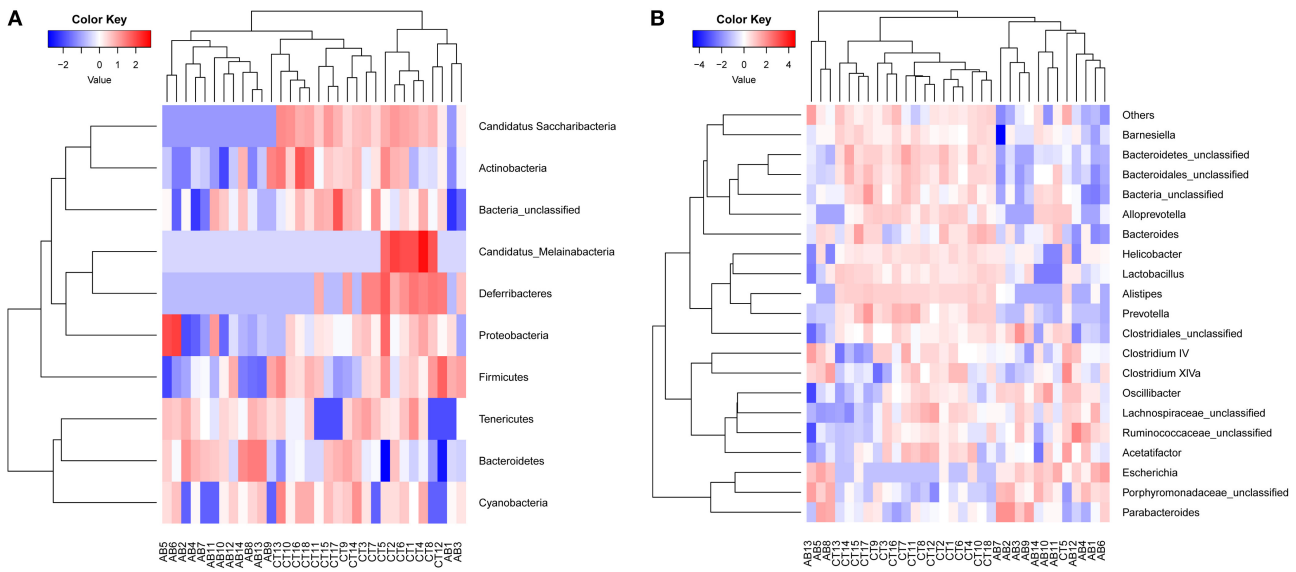

FIGURE 9 | Heat maps of gut microbiota **(A)** at the phylum level and **(B)** the genus level. Red and blue colors indicate high and low values of the percent of reads classified at that rank. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$).

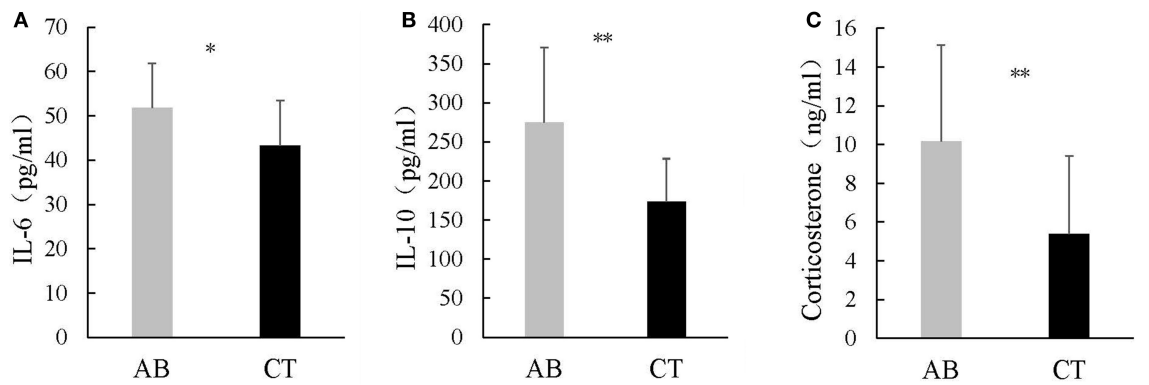

FIGURE 10 | Concentrations of **(A)** IL-6, **(B)** IL-10, and **(C)** corticosterone in serum. AB, antibiotic group ($n = 14$); CT, control group ($n = 18$). *$P < 0.05$, **$P < 0.01$.

gut anaerobic environment (Zhu et al., 2013; Shin et al., 2015; Miao et al., 2020). *Firmicutes* has become a controversial strain as some studies identified an increase in *Firmicutes* after ceftriaxone treatment, but others have demonstrated a declined *Firmicutes* in the ceftriaxone group (Cheng et al., 2017, 2019; Miao et al., 2020). Evidence from clinics has suggested that patients with depression often have decreased *Firmicutes* (Huang et al., 2018). Experimental findings further revealed that decreased *Firmicutes* led to a reduction in short-chain fatty acids, which are an important physiological basis for low-level inflammation during depression (Huang et al., 2018). *Bacteroidetes*, as an important microbe for short-chain fatty acids, almost disappeared from the feces of the mice during exposure to ceftriaxone (Miao et al., 2020). Significant alteration of fecal microbiota was also observed at the genus level: *Porphyromonadaceae, Escherichia,* and *Parabacteroides* dominated the gut microbiota of the AB group mice, while *Lactobacillus, Clostridiales, Acetatifactor, Bacteroidetes, Barnesiella, Helicobacter, Prevotella, Bacteroidales,* and *Alistipes* were lowered. In line with this, some researchers have proposed that decreased *Barnesiella* after ceftriaxone gavage is a common and sensitive gut microbiota of the BALB/c mice and can be used as an indicator for assessing the balance of the gut microbiota (Zhao et al., 2013). *Bacteroidetes* is closely associated with digestion and interacts with the host's immune system, affecting the growth of other bacteria (Karlsson et al., 2011). In addition, an increase in *Escherichia* prevalence after oral antibiotic treatment has been reported for vancomycin and imipenem (Stokes, 1949), amoxicillin, bismuth (Dawes and Foster, 1956), and metronidazole (Paege and Gibbs, 1961). It is difficult to discern whether an increase in *Escherichia* could be beneficial or harmful as *Escherichia* is both a commensal and pathogenic inhabitant of a host's gastrointestinal tract. But

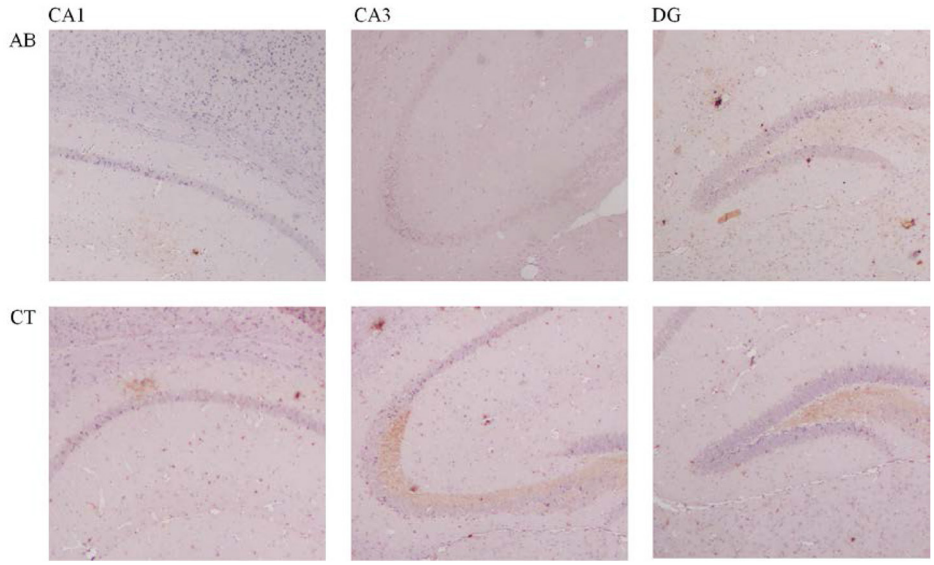

FIGURE 11 | Immunohistochemical results of BDNF in the hippocampus of mice (×400). AB, antibiotic group ($n = 14$); CT, control group ($n = 18$). CA1, field CA1 of hippocampus; CA3, field CA3 of hippocampus; DG, dentate gyrus.

TABLE 3 | Expression of BDNF in the hippocampus and expression of c-Fos in the amygdala.

		BDNF			c-Fos		
Group	***n***	**CA1**	**CA3**	**DG**	**CeM**	**CeL**	**CeC**
AB	14	1 (1.00–3.25)	3 (2.00–6.75)	3 (1.75–4.00)	6 (3.00–7.50)	6 (3.00–7.50)	2 (0.50–5.00)
CT	18	2 (1.00–4.00)	4 (2.25–8.25)	3 (2.00–4.00)	0 (0.00–4.50)	0 (0.00–4.50)	1 (0.00–9.00)
P-value		0.350	0.586	0.884	0.076	0.076	0.892

AB, antibiotic group; CT, control group; CA1, field CA1 of hippocampus; CA3, field CA3 of hippocampus; DG, dentate gyrus; CeM, central amygdaloid nucleus, medial division; CeL, central amygdaloid nucleus, lateral division; CeC, central amygdaloid nucleus, capsular part.

most of the time, *Escherichia* is considered a potential pro-inflammatory bacteria (Liu et al., 2019). Of particularly note, increased *Porphyromonadaceae* associates with mental deficits and cognitive disorders as well as anxiety-like behaviors in mice (Scott et al., 2017). *Lactobacillus* is known as a protective species against long-lasting metabolic disturbances and prevents gut dysbiosis, but was suppressed by ceftriaxone (Robles-Vera et al., 2018). Researchers also discovered that elevated *Parabacteroide* relates to the etiology of depression (Cheung et al., 2019). These results indicate, once again, that different bacteria may be involved in different functions or biological pathways.

Alterations in gut microbiota were accompanied by behavioral changes in the mice, including anxiety-like, depression-like, and aggressive behaviors. These behavioral changes cannot necessarily be a result of the direct toxic effect of ceftriaxone on the brain, since ceftriaxone is a non-absorbable antibiotic and usually given by injection. Previous studies have demonstrated the complex interaction between gut microbiota and the CNS; this is what is known as the MGB axis (Wang and Wang, 2016). Animal experiments support that absence or change in gut microbiota affects the HPA axis answering to stress, anxiety, and relevant behavior (Koopman and El Aidy, 2017; Lach et al., 2018; Chen et al., 2019). In addition, the rodents infected with intestinal pathogens showed anxiety-like behaviors, which can be partly explained by the activation of vagal afferents (Klarer et al., 2014). In one study of GF BALB/c with a high-anxiety level and NIH Swiss mice with a high exploratory ability, when the two groups exchanged each other's microbiota, the donor behavioral characteristics could be reproduced in recipients (Crumeyrolle-Arias et al., 2014). On the other hand, clinical trials suggest treatment with probiotics could control the stress response and improve anxiety symptoms by restoring the gut microbiota (Liu et al., 2015). Our further work will test whether probiotics could improve the abnormal behaviors. At present, two major types of probiotics are commonly used: *Bifidobacterium* and *Lactobacillus* (Logan and Katzman, 2005; Rao et al., 2009; Silk et al., 2009). According to Wang et al., *Lactobacillus fermentum* strain NS9 administration not only normalized the composition of gut microbiota but reduced the anxiety-like behavior induced by ampicillin (Wang et al., 2015). Furthermore, the antidepressant effect of *Bifidobacterium infantis* has also been identified in the rat separation model of depression (Desbonnet et al., 2010).

Immune dysregulation was demonstrated by high levels of serum cytokines. This is supported by the evidence that

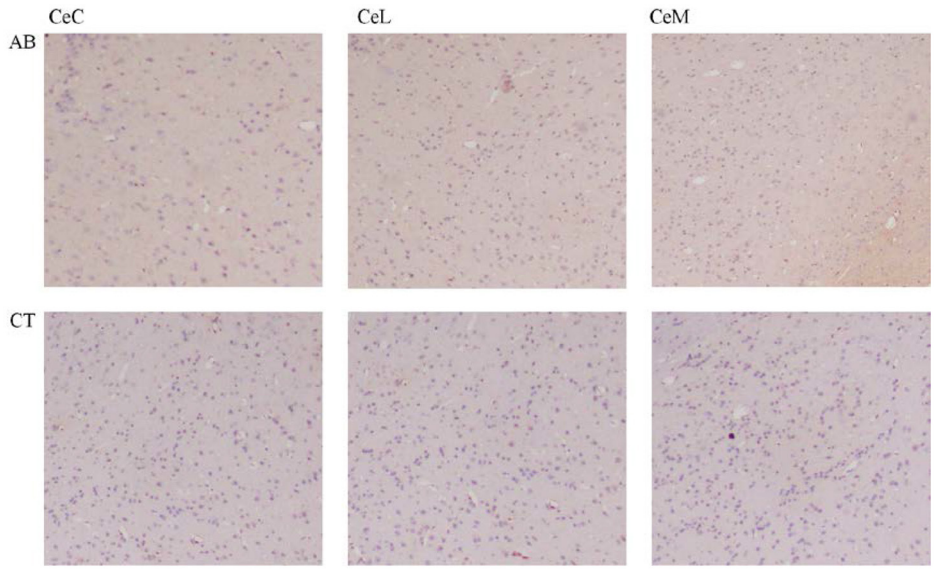

FIGURE 12 | Immunohistochemical results of c-Fos in the amygdala of mice (×400). AB, antibiotic group ($n = 14$); CT, control group ($n = 18$). CeC, central amygdaloid nucleus, capsular part; CeL, central amygdaloid nucleus, lateral division; CeM, central amygdaloid nucleus, medial division.

inflammatory factors associate with a profile of behavioral changes (Capuron and Miller, 2011; Salim et al., 2012; Felger and Lotrich, 2013). Vagal sensory neurons express receptors for cytokines, so the inflammatory factors could directly activate the vagal afferents (Reardon et al., 2018). One study proposes that anxiety is related to inflammation; for example, mice infected with *Schistosoma mansoni* showed a reduction in behaviors such as exploration and grooming (Sulaiman et al., 1989). In addition, abnormal emotions, such as anxiety and neophobia, could happen following bacterial infection or as a response to bacterial products (Capuron and Miller, 2011). Anxiety levels increased when humans were exposed to lipopolysaccharide (LPS) (Grigoleit et al., 2011). Of the numerous cytokines, IL-6 is perceived as an atypical proinflammatory cytokine, having been demonstrated to show elevated levels in depressed animals and patients (Jiang et al., 2020; Lamers et al., 2020). In a study, IL-6 knockout mice became resistant to the development of depression-like symptoms (Monje et al., 2011). The underlying mechanisms involve in two pathways, the HPA axis and neurotransmitter metabolism, both of which are affected by increased IL-6 in depression (Ting et al., 2020). Furthermore, anxious patients also had higher serum levels of IL-6 than common people (Tang et al., 2018; Zou et al., 2020). In addition to impact on HPA axis activity, IL-6 could cross the blood–brain barrier, as they affect the uptake and release of mood-relevant neurotransmitters, including dopamine, 5-HT, noradrenaline, and gamma-aminobutyric acid (Zalcman et al., 1994; Clement et al., 1997; Anisman et al., 2008; Miller, 2009). IL-10, a prototypical anti-inflammatory cytokine, was closely related to depression (Li et al., 2020). Lower IL-10 has been observed in depression, while IL-10 was elevated after antidepressant treatment (Dai et al., 2020; Lee et al., 2020). In contrast, studies have reported higher IL-10 in depressive patients and decreased IL-10 after treatment for depression (Köhler et al., 2018; Himmerich et al., 2019; Wang et al., 2019; Brunoni et al., 2020). One explanation for increased IL-10 is that it is an anti-inflammatory response to correct an inflammatory activation caused by higher levels of proinflammatory cytokines (Bhattacharya and Drevets, 2017). In this way, higher IL-10 levels, as observed in our study, may be associated with the development of abnormal patterns. On the other hand, previous studies found a high dose of IL-10 may induce anxiety in the OFT (Harvey et al., 2006), two other behavioral tests for anxiety detection (Munshi et al., 2019). Taken together with these experiment evidences, the abnormal pattern of mice may be a direct result of increased inflammatory mediators.

Elevated corticosterone, one marker of HPA axis activation, was observed in the mice of the AB group (Borrow et al., 2019). Several studies indicate that the disturbance of gut bacteria affects the HPA axis. Specifically, adrenocorticotrophin and corticosterone levels for GF mice were higher than of mice bearing conventional microbiota (Crumeyrolle-Arias et al., 2014). Besides, the hyperactivity of the HPA response in GF mice could be partially reversed by gut microbiota transplantation (Huo et al., 2017). Probiotics, such as *Bifidobacterium* species, have been demonstrated to be efficient in restoring HPA axis function (Moya-Pérez et al., 2017).

The BDNF level showed a decreasing trend in the hippocampus of the AB group. According to the previous study, BDNF can maintain and promote development, differentiation and regeneration of neurons as well as affect learning and memory (Bercik et al., 2011). The hippocampus provides the brain with a spatiotemporal framework within which various sensory, emotional, and cognitive components are integrated (Yang and Wang, 2017). Literature has reported that hippocampus degeneration with diminished BDNF leads

to a decline in cognition (Deltheil et al., 2008). Recently, gut microbiota is thought to directly affect BDNF expression. The GF mice showed a decreased BDNF in the cortex and hippocampus (Bercik et al., 2011). This coincides with the thesis that reduction of BDNF after gut dysbiosis possibly leads to impairment of cognitive function (Frohlich et al., 2016). Contrary to the findings, some studies observed an increased BDNF in the amygdala and hippocampus when gut microbiota imbalance induced a decline in spatial memory (Desbonnet et al., 2015). In addition, *Bifidobaterium adolescentis* shows a promising anxiolytic and antidepressant property as it up-regulated BDNF expression by restoring the balance of gut microbiota (Guo Y. et al., 2019). A slight increase of c-Fos was observed in the amygdala of the AB group. C-Fos serves as a component of transcription factor AP-1 and biomarker of neuronal activation, playing a major role in processing emotion and motivation (Baulmann et al., 2000; Roberts et al., 2019). Abnormal activation of c-Fos in the brain may be related to gut disorders; for example, as compared to uninfected mice, a significantly increased c-Fos was observed in mice infected with *Campylobacter jejuni* (Goehler et al., 2008). Meanwhile, a study indicated c-Fos activation following immune activation; this finding was in accord with our findings that cytokines increased with increasing c-Fos (Lyte et al., 2006).

CONCLUSION

In general, we found that mice exposed to 11 weeks of ceftriaxone sodium treatment had a lower diversity and abundance of gut microbiota and showed more behavioral changes as compared to mice that were given normal saline. Dysregulation of the nerve-endocrine-immunological network may be a potential mechanism underlying abnormal behaviors induced by impaired gut microbiota. The study revealed the unknown side effects of antibiotics to a certain extent. Follow-up studies rebalancing the gut dysbacteriosis are required to further confirm the relationship between gut microbiota and brain function.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI, SRA accession: PRJNA592623.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Advisory Committee of Sichuan University.

AUTHOR CONTRIBUTIONS

ZZ and HY put forward the hypothesis, and BW, CT, LZ, and ML guided and supervised the experimental investigation and practices. LM, HW, and JL were responsible for data collection and analysis. YY provided pathologic diagnosis. ZZ was the main executer and involved in the entire research process, from proposal, planning and execution to implementation and composition. 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/fcimb.2020.00258/full#supplementary-material

Supplementary Figure 1 | Behavioral data analysis for eleven weeks. **(A)** Data from the open field test and **(B)** tail suspension test. AB: antibiotic group ($n = 20$), CT: control group ($n = 20$). Four mice of AB group were kicked out at the tenth week of gavage due to serious injuries influencing mobility.

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