GUT MICROBIOTA IN THE OCCURRENCE, DEVELOPMENT AND TREATMENT OF GUT-BRAIN DISORDERS

EDITED BY: Zongxin Ling, Qixiao Zhai, Liwei Xie and Tingtao Chen
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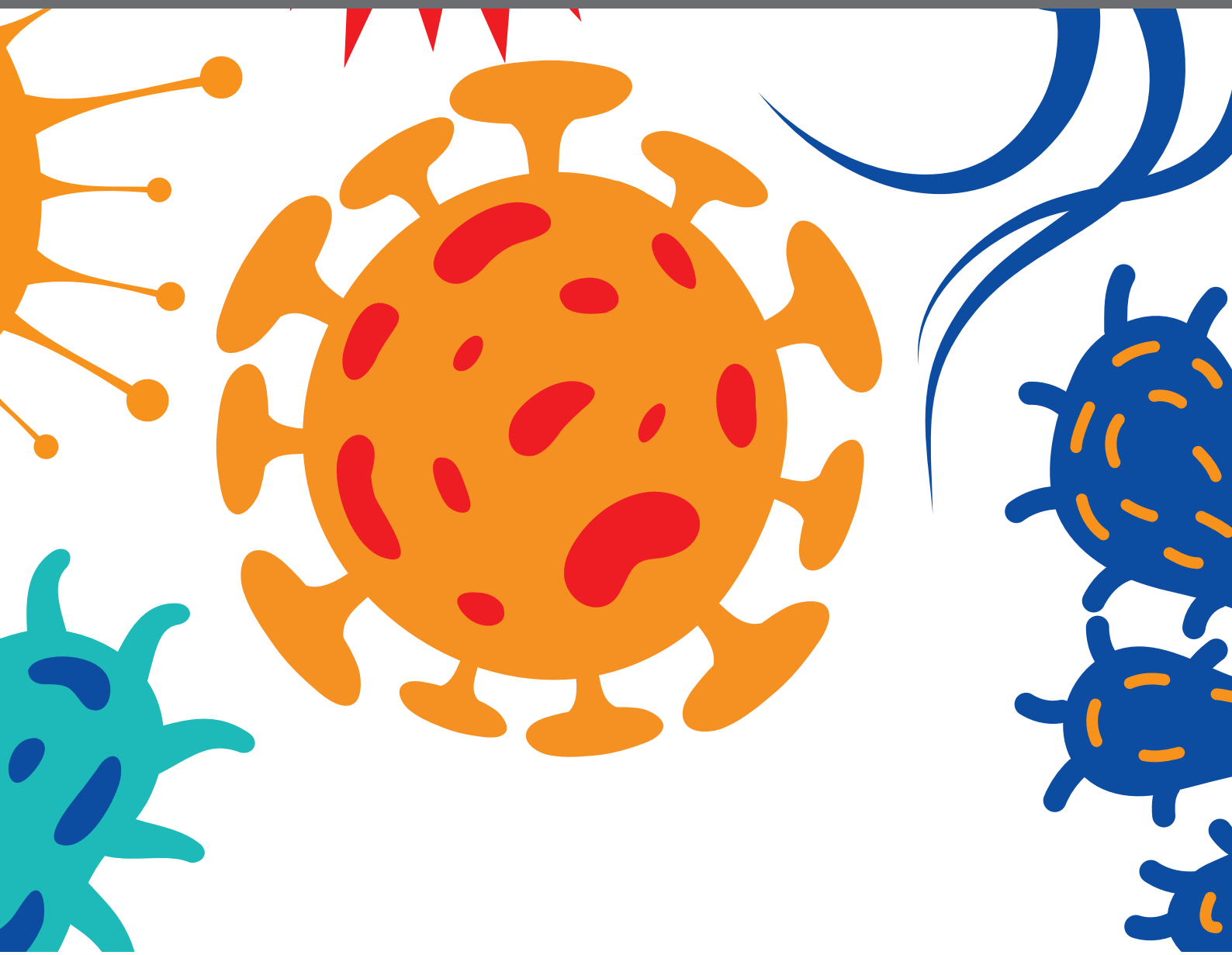

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GUT MICROBIOTA IN THE OCCURRENCE, DEVELOPMENT AND TREATMENT OF GUT-BRAIN DISORDERS

Topic Editors:
Zongxin Ling, Zhejiang University, China
Qixiao Zhai, Jiangnan University, China
Liwei Xie, Guangdong Academy of Science, China
Tingtao Chen, Nanchang University, China

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EDITORIAL
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Editorial: Gut Microbiota in the Occurrence, Development and Treatment of Gut-Brain Disorders

Shengjie Li, Jing Wei and Tingtao Chen**

The Institute of Translational Medicine, Nanchang University, Nanchang, China

Keywords: gut microbiota, psychiatric disorders, gut-brain axis, stroke, fecal microbiota transplantation, psychobiotics, prebiotics

Editorial on the Research Topic

Gut Microbiota in the Occurrence, Development and Treatment of Gut-Brain Disorders

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Andrew T. Gewirtz, Georgia State University, United States

****Correspondence:***
Tingtao Chen
chentingtao1984@163.com
Jing Wei
315794984@qq.com

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Increasingly, it is recognized that symbiotic microorganisms, specifically the microbiota resident in the gut, may influence neurodevelopment and programming of social behaviors among animal species and humanity (Sherwin et al., 2019). Studies have shown that the gut microbiota-derived signals can transfer to the brain and may alter brain function *via* the microbiota-gut-brain axis, a bidirectional communication pathway between the gut and brain (Dinan and Cryan, 2017). Various neurodevelopmental diseases, including autism, depression, anxiety, and Alzheimer's disease (AD), are correlated with gut microbiota dysbiosis and altered metabolic activities. Observations across preclinical and clinical data elucidate that targeting gut microbiota through dietary or live bacteria interventions can promise beneficial therapeutic effects on the associated behavioral symptoms in psychiatric disorders (Dinan and Cryan, 2017). Collectively, these results suggest that the connection between gut microbiota and the brain plays a vital role in the occurrence, development, and treatment of gut-brain disorders.

Although several communication pathways contribute to the gut microbiota on brain physiology and behavior, the deep mechanisms by which enteric bacteria communicate with the brain and how the gut microbiota influences the brain causally are still needed to be illustrated. Moreover, further understanding of the roles and mechanisms of psychobiotics in treating various gut-brain disorders may help generate new therapeutic strategies for neural disorders in humans.

This Research Topic brings nine articles summarizing the regulation of intestinal microbes to gut-brain disorders in mammals, the potential mechanisms of targeting microbiota in the treatment of neurological disorders *via* the gut-brain axis, and the beneficial effects of psychobiotics on some neurodegeneration diseases.

In their review article, Chen et al. provide an overview on the current knowledge related to the potential microbial mechanisms and metabolic effects in the progression of Hepatic Encephalopathy (a neurological disorder that occurs in individuals with chronic liver diseases) *via* the perspective of gut-brain axis. They also discuss novel therapeutic strategies (e.g., fecal microbiota transplantation (FMT) and providing specific probiotics) that maintain intestinal homeostasis is vital for treating liver disease-related neurological disorders. Tang et al. extend these concepts to Hippocampus-dependent neurodegenerative

diseases. Their review outlines the recent findings on the relationship between intestinal microbes and the hippocampus's plasticity, neurochemicals, and function. They also highlight the advances in modulating hippocampal structure and behavior using probiotics, prebiotics, and diet through the gut microbiota-hippocampus axis. Cognitive impairment, characterized by conditions where a huge range of mental deficits are expressed, is reported to be an early symptom of the preclinical AD spectrum (Xu et al., 2021). *Porphyromonas gingivalis*, the key periodontal pathogen that could be detected in the brain of AD patients, can induce cognitive impairment and dysbiosis of the gut and then contribute to the occurrence and development of AD (Diaz-Zuniga et al., 2020). As described by Chi et al., *P. gingivalis* infection of oral origin causes the gut microbiota dysbiosis, neuroinflammation, and glymphatic system impairment in mice, resulting in cognitive decline through disturbing the microbiota-gut-brain axis. Moreover, they discuss the potential molecular basis of *P. gingivalis* infection-induced neurological diseases, including how it affects brain functions and disturbs the gut barrier.

Depression following stroke, also known as post-stroke depression (PSD), is a common mood disorder with one symptom of the altered gut microbiome and its metabolites, indicating that gut microbiome may participate in the development of PSD. A study presented in the issue comparing the profile of gut microbiome and fecal metabolomics in rats suffering PSD found that the microbial and metabolic phenotypes were changed significantly in PSD rats (Jiang et al.). Furthermore, the changed gut microbes were highly consistent with their behavioral performance and correlated with the disturbances of fecal metabolomics that mainly assigned to lipid metabolism in PSD rats. Although studies focused on the neurological aspects of stroke that were accumulating in the last decades, researchers should explore deep insights into the relationship between gut microbiota and stroke. In their article, Zhao et al. summarize recent progress in the interactions between gut microbiota and ischemic stroke (the most common stroke events caused by the blockage of blood flow), including how stroke affect gut microbiota composition and how these changes reversely influence stroke outcome and prognosis. Moreover, they discuss that modulating gut microbiota by psychobiotics or prebiotics are helpful in the post-stroke therapy, such as specific functional bacterial species (e.g., *Bifidobacterium longum*), natural products (e.g., *Panax Notoginsenoside* extract) and metabolic compounds (e.g., lactulose). They also suggest that concerning gut microbiota will provide novel avenues to treat post-stroke disorders and remind clinicians for exceptional care in post-stroke management.

Microbiota-based therapeutic strategies have demonstrated the potential to alleviate symptoms of neurological disorders in various preclinical models. As described by Yuan et al., lactulose supplementation has been used to treat neurological outcomes due to its ability to improve neurological function, suppress inflammation in the brain and gut, regulate microbiota and associated metabolic disturbance, and inhibit harmful bacteria *in vivo*. Another study performed by Zhang et al. suggests that *Sophora alopecuroides* L.-derived alkaloids can improve depression-like behaviors and depression-related indicators through modulating gut microbiota, which reveals the mechanism of action of alkaloids in the treatment of brain disorders.

Manipulation of the gut microbiota by FMT is an emerging therapeutic strategy that has been shown to improve cognitive function and brain development disorders (Vendrik et al., 2020). A clinical trial performed by Li et al. suggests that FMT can relieve gastrointestinal and autism symptoms without inducing any severe complications by improving the gut microbiota in children with autism spectrum disorders. Moreover, this study highlights a specific microbiota intervention that targets *Eubacterium coprostanoligenes* to enhance the FMT response. Washed microbiota transplantation (WMT), another version of modified FMT, is demonstrated to be safer, more precise and more quality-controllable than FMT (Zhang et al., 2020). In this issue, Wang et al. report that WMT partially rescues the alterations of behaviors, microbiota composition, and brain structures by light-induced stress.

In conclusion, this Research Topic provides readers with an overview of the potential role of gut microbiota and its metabolic profiles in the occurrence, development, and treatment of associated neurological disorders. However, the precision medicine of gut-brain conditions *via* the microbiota-gut-brain axis remains in the distant future until the physiological and molecular mechanisms underlying these connections could be deeply elucidated. And more clinical researches should be carried out to support the new therapeutic strategies targeting gut microbiota for neural disorders in humans.

AUTHOR CONTRIBUTIONS

SL, JW, and TC wrote and revised this article. All authors made a substantial, direct and intellectual contribution to this work, and approved it for publication.

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REVIEW
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The Role of Intestinal Bacteria and Gut–Brain Axis in Hepatic Encephalopathy

*Zefeng Chen, Jingsheng Ruan, Dinghua Li, Min Wang, Zhiwei Han, Wenxia Qiu and Guobin Wu**

Guangxi Medical University Cancer Hospital, Nanning, China

Hepatic encephalopathy (HE) is a neurological disorder that occurs in patients with liver insufficiency. However, its pathogenesis has not been fully elucidated. Pharmacotherapy is the main therapeutic option for HE. It targets the pathogenesis of HE by reducing ammonia levels, improving neurotransmitter signal transduction, and modulating intestinal microbiota. Compared to healthy individuals, the intestinal microbiota of patients with liver disease is significantly different and is associated with the occurrence of HE. Moreover, intestinal microbiota is closely associated with multiple links in the pathogenesis of HE, including the theory of ammonia intoxication, bile acid circulation, GABA-ergic tone hypothesis, and neuroinflammation, which contribute to cognitive and motor disorders in patients. Restoring the homeostasis of intestinal bacteria or providing specific probiotics has significant effects on neurological disorders in HE. Therefore, this review aims at elucidating the potential microbial mechanisms and metabolic effects in the progression of HE through the gut–brain axis and its potential role as a therapeutic target in HE.

Keywords: bile acid, ammonia, neurotransmitter, blood–brain barrier, neuroinflammation, gut microbiota, hepatic encephalopathy

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Edited by:
Tingtao Chen,
Nanchang University, China

Reviewed by:
Matthew McMillin,
University of Texas at Austin, United States
Tiziano Balzano,
Centro Integral en Neurociencias A.C. HM CINAC, Spain
Xin Fang,
First Affiliated Hospital of Nanchang University, China
Gerard M. Moloney,
University College Cork, Ireland

****Correspondence:***
Guobin Wu
wuguobin@gxmu.edu.cn

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INTRODUCTION

Hepatic encephalopathy (HE), typically divided into three types [type A resulting from acute hepatic failure (ALF), type B resulting from portosystemic bypass or shunting, and type C resulting from cirrhosis], is a neurological complication that occurs in individuals with chronic liver diseases (Montagnese et al., 2018). In this condition, the body's metabolic processes are interrupted by hepatic dysfunction, ammonia, bile acids, and other substances that cross the blood–brain barrier (BBB) with increased permeability, accumulate in the brain, eventually causing neurological disorders. The impaired lymphatic system cannot, however, eliminate harmful substances, which may eventually aid the entire process (Ochoa-Sanchez and Rose, 2018; Hadjihambi et al., 2019). Mild HE (MHE) is a subclinical HE (SHE) that lacks the clinical manifestations associated with HE. Routine mental and neurological tests are normal in SHE. The diagnosis of MHE depends on psychometric and neurophysiological tests. It is very challenging to give accurate clinical diagnosis before overt symptoms set in, thus, leading to a decrease in the quality of life and survival time (Bajaj, 2008; Wijdicks, 2016). Learning ability of HE is not completely reversible and new cognitive decline occurs after treatment in some patients (Riggio et al., 2011; Hopp et al., 2019).

Intestinal microbiota are associated with human digestion and exhibit direct or indirect links to human health (Premkumar and Dhiman, 2018). New treatment modalities aim at regulating the balance of gut microbiota for relieving or curing related diseases. Studies have documented that intestinal bacteria are closely associated with emotional and cognitive–behavioral functions. The gut–liver–brain axis comprehensive treatment concept can be used to manage cognitive–behavioral disorders in HE (Oliphant and Allen-Vercoe, 2019). Intestinal microbiota significantly contribute to the pathogenesis of autism, Alzheimer's diseases, Parkinson's disease and other central nervous system (CNS) diseases (Zhu et al., 2020). The gut–brain axis is also involved in the progression of nervous system dysfunction. This review elucidates on the various mechanisms involved, and how intestinal microbiota and its metabolites facilitate the progression of liver diseases to HE through the gut–brain axis, and the potential therapeutic options for HE by regulating intestinal microbial community composition.

LIVER DISEASE IMPACTS INTESTINAL HOMEOSTASIS THROUGH THE GUT–LIVER AXIS

Maintenance of intestinal homeostasis is dependent on an intact intestinal mucosal barrier, a healthy intestinal microenvironment, and a delicate balance between nutrition and metabolites. Intestinal dysfunction in patients with HE occurs as liver function deteriorates. Small intestinal bacterial overgrowth and bacterial translocation are the essential features for intestinal homeostatic imbalance in patients with severe liver disease. Gut–liver axis is a pathway for bi-directional communication between the intestine and the liver. Regular operation of the gut–liver axis requires an intestinal mucosa barrier and a healthy liver function. The intestinal barrier is the first barrier against bacteria and their metabolites entering the blood. In some patients with liver disease, the intestinal barrier is destroyed, depending on disease severity (Simbrunner et al., 2019; Albillos et al., 2020; Gerova et al., 2020).

The liver is the body's largest immune organ. It eliminates toxic substances and bacterial metabolites from the intestines. Enterohepatic circulation of bile acids and urea plays a vital role in the gut–liver axis. The liver and gallbladder secrete primary bile acids into the gut. Various microbial species, including *Lactobacillus, Bifidobacterium* and *Enterococcus*, secrete bile salt hydrolase (BSH) and bile acid dehydratase enzymes that catalyze primary bile acids into secondary bile acids. Most circulating bile acids (about 95%) are taken up by the enterocytes and are recycled into the liver through the portal vein. Bile acids are then discharged into the intestines through the biliary tract after liver metabolism (Long et al., 2017; Mertens et al., 2017).

Elevated blood ammonia (hyperammonemia) levels cause mitochondrial dysfunction, oxidative or nitrative stress and cause apparent damage to the nervous systems such as brain permeability disorders, nerve conduction abnormalities, and the alteration of glucose metabolism in the human brain (Fan et al., 1990; Jayakumar and Norenberg, 2018). The intestinal tract is the primary source of ammonia. Intestinal bacteria decompose protein into ammonia by producing urease. Intestinal ammonia can be absorbed into the bloodstream. After ammonia is transported into the portal vein, it enters the liver and is re-synthesized to urea. This process is called enterohepatic circulation of urea. Urea enterohepatic circulation maintains a low concentration of ammonia in human blood (Wright et al., 2011). When enterohepatic circulation is cut off, the levels of ammonia and bile acids in the blood increase.

If intestinal metabolites in the blood are difficult to be broken down in the liver, or circulate directly through the collateral systems, hence, bypassing the liver, it leads to an increase in the concentration of toxic substances or neurotransmitters in the CNS. Gut bacteria release their components, including lipopolysaccharides (LPSs), peptidoglycan (PGN), bacterial lipoproteins (BLPs), mannans, and bacterial DNA into the blood. Lipopolysaccharide is the main component of gram-negative bacteria that triggers systemic inflammation. Moreover, high amounts of LPS increase BBB permeability and neuroinflammation, causing a large number of bacterial metabolites to get into the brain quickly, thus promoting the occurrence of HE (Hemmi and Akira, 2002; de Jong et al., 2016).

INTESTINAL MICROBIOTA COMMUNICATE WITH CENTRAL NERVOUS SYSTEM THROUGH GUT–BRAIN AXIS

Intestinal bacteria start to colonize the human body after birth. Maternal bacteria colonize the fetus's intestinal tract during delivery. As the infants mature, their gut microbiota composition improves and resembles that of healthy adults (Perez-Mu Oz et al., 2017). However, some studies have documented that the fetus obtains its gut microbiome or is exposed to microbial products and metabolites from maternal microbiota, which plays a vital role in the fetus's immune system or metabolism. (Gomez et al., 2016; Younge et al., 2019). Therefore, to maintain a healthy brain function, it is important to understand the relationships between CNS and intestinal microbiome. Germ-free (GF) mice are standard animal models used to study how intestinal microbiota affect the nervous system. Apparent differences in neurological development and neurotransmitter concentration exist between mice with typical microbiota and GF mice, which, however, show that commensal bacteria regulate and control the cognitive and motor functions of the nervous system (Mitsuharu et al., 2013; Principi and Esposito, 2016).

The enteric nervous system (ENS) connects intestinal microbiota with the CNS and is an essential communication pathway for the gut–brain axis. Intestinal microbiota also regulate the development and function of the ENS. Colonizing the intestinal microbiota of conventionally raised mice in GF mice can change the anatomical structure of the ENS and

improve the intestinal transport function, which is associated with the intestinal microbial metabolite 5-HT and microorganism activation 5-HT4 receptors in the ENS. Therefore, gut microbiota affects the CNS through the ENS (De Vadder et al., 2018).

Commensal gut microbiota and their metabolic products communicate with the CNS by mediating the activity of the vagus nerve and by regulating endocrine and immune pathways, which in turn exert an impact on cognitive, motor, and nervous system development. Intestinal bacteria affect the structure and function of the CNS. The affected structure and functions involve neurogenesis, myelination, glial cell function, synaptic pruning and BBB permeability of the CNS (Mika and Fleshner, 2015; Bonaz et al., 2018; Heiss and Olofsson, 2019; Nabhani and Eberl, 2020). Beneficial bacteria have been developed as intestinal microecological agents for clinical treatment because they play a significant role in CNS function.

CHANGES AND INFLUENCE OF GUT MICROBIOTA IN HEPATIC ENCEPHALOPATHY

Intestinal microbiota disorder is characterized by low intestinal microbiota diversity, overgrowth of harmful microbiota, and disruption of beneficial microbiota in HE. Compared to healthy controls, intestinal microbiota of cirrhosis patients has an abundance of 75,245 genes according to quantitative metagenomics (Qin et al., 2014). *The genus Bacteroidetes decreases with a decrease in liver function* (Haraguchi et al., 2019). Some intestinal microbiota have been correlated with the pathological mechanisms, processes and outcomes of HE (Bajaj, 2014; Iebba et al., 2018; Sung et al., 2019). For instance, the translocation of *Stenotrophomonas pavanii* and *Methylobacterium extorquens* into the peripheral blood system enhances the risk of HE (Iebba et al., 2018).

Cognitive and motor disorders originate from different structures of an impaired CNS. Psychometric HE score and diffusion kurtosis imaging (DKI) have been used to evaluate cognition and brain microstructure changes of patients with liver cirrhosis, respectively. Compared to healthy individuals, DKI parameters of gray matter and white matter have been found to be significantly decreased in cirrhosis. Psychometric HE score was found to be low and positively correlated with DKI parameters in cirrhosis, indicating that decreased brain microstructural complexity and cognitive impairment in patients with liver cirrhosis may have a potential correlation. (Chen et al., 2017). Thus, the link between microbiota changes and structural brain lesions enhances the understanding of HE. Ahluwalia et al. used magnetic resonance spectroscopy (MRS) and diffusion tensor imaging (DTI) to determine the association between the changes seen in the CNS and microbiota in HE. *Enterobacteriaceae* and *Autochthonoustaxa* were found to be positively and negatively correlated with astrocyte swelling, respectively. Based on the analysis of DTI images, *Porphyromonadaceae* is associated with neuronal damage (Ahluwalia et al., 2016). Moreover, *stool Alcaligenaceae* has been correlated with poor cognition in OHE (Bajaj, 2014). Identifying specific gut microbiota provides new strategies for clinical diagnosis, treatment, and eventually weighing the prognosis of HE. A summary of the above studies is presented in **Table 1**. The table shows the progression, outcomes, and specific microbiome in HE. And microbiota-associated mech-anisms involved in the pathogenesis of HE are showed in **Figure 1**.

BLOOD–BRAIN BARRIER PERMEABILITY

Brain edema is a common characteristic in HE that promotes neurological deterioration (Cudalbu and Taylor-Robinson, 2019).

TABLE 1 | The connection between gut microbiome and HE (positive relation ↑ and negative relation ↓).

Author	HE	Specimen	Method	Bacterial species
(Iebba et al., 2018)	Risk	Fecal	16S sequencing and NMR metabolism	*Bacteroides coprocola*↑ *Bifdobacterium longum*↑ *Bacteroides faecis* ↓ *Bacteroides coprophilus* ↓
		Blood		*Stenotrophomonas pavanii*↑ *Methylobacterium extorquens*↑ *Clostridium indolis*↓
(Sung et al., 2019)	Mortality	Fecal	16S sequencing	*Lactobacillus*↑ *Bacteroides* ↓ *Clostridium incertae sedis*↓ *Clostridium XI* ↓
	Recurrence	Fecal	16S sequencing	*Veilonella*↑ *Phascolarctobacterium* ↓ *Fusobacterium* ↓
(Zhang et al., 2013) (Ahluwalia et al., 2016)	Astrocyte swelling	Fecal	16s sequencing, MTPS	*Autochthonous taxa*↓ *Enterobacteriaceaeand*↑ *S. salivarius*↑
(Ahluwalia et al., 2016)	Neuronal damage	Fecal	MTPS	*Prevotellaceae*↑ *Veillonellaceae*↑ *Porphyromonadaceae*↑/↓

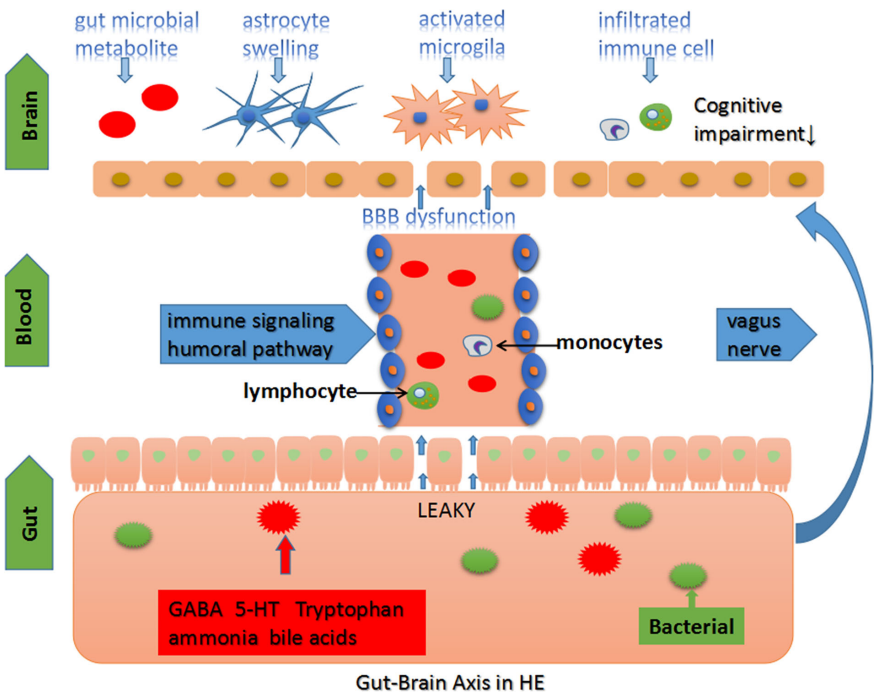

FIGURE 1 | The gut-brain axis in HE. The homeostasis of intestinal microbiota is affected in severe hepatic disease and portal shunt disease. Gut-origin substances are delivered to the brain through the immune, humoral and vagus nerve pathway (D'Mello et al., 2009; Cawthon and de La Serre, 2018). Chronic intestinal inflammation and "leaky gut" promote gut microbiota metabolite and bacterial translocation into the circulatory system leading to systemic inflammation and body metabolic disorders (Seo and Shah, 2012). The brain microenvironment loses stability, followed by BBB dysfunction. Moreover, multiple factors disturb the CNS function, including changes in brain structure, neurotransmitters, and other substance concentrations, leading to cognitive impairments in HE (Jones, 2003; Banks, 2006; Dhanda and Sandhir, 2015; Jayakumar and Norenberg, 2018; DeMorrow, 2019).

Permeability of the BBB increases in patients and animal models of HE (Dhanda and Sandhir, 2018; Weiss et al., 2019). The BBB, which is a crucial regulatory interface in the gut–brain axis, modulates the transportation of immune cells, inflammatory molecules, and intestinal bacterial metabolites, thereby, stabilizing the CNS microenvironment (Banks, 2006). Occludin and claudin-5 are key tight junction proteins that play an important role in regulating BBB permeability. Compared to mice with healthy gut microbiota, BBB permeability was found to be increased in GF mice, which relates to the expression of occludin, and claudin-5. Transplantation of healthy gut microbiota from pathogen-free mice was shown to ameliorate the changes in GF mice (Braniste et al., 2014).

BBB damage in HE patients is associated with the swelling of astrocytes, endothelial cell damage, and the opening of tight junctions. Ammonia and inflammation are responsible for BBB dysfunction in HE (Erickson et al., 2012; Marta and Jan, 2012). Hyperammonemia triggers brain edema by disrupting the glutamate or glutamine cycle in astrocyte (Norenberg and Martinez-Hernandez, 1979). Key connexins form a gap junction between astrocytes. In rats with bile duct ligation (BDL) or hyperammonemia, elevated blood ammonia levels are associated with gap junction dysfunction, which is significantly improved after ammonia-lowering treatment. However, the treatment effect is not mediated by increasing the expression of key connexins (Hadjihambi et al., 2017). Moreover, ammonium chloride was shown to down-regulate claudin-12 gene expression in a brain capillary endothelial cell culture model (Bélanger et al., 2007). Specific membrane transporters form the structural basis for BBB functions and the transportation of specific substances in and out of the brain. P-glycoprotein and Mrp2 are the ATP-binding cassette (ABC) transporters expressed in the brain endothelial cells (ECs) (Ebinger and Uhr, 2006). Expression and function of the ABC transporter affect drug distribution in the brain and prevents the accumulation of endotoxins in the nervous system. Moreover, hyperammonemia increases the expression of P-glycoprotein and Mrp2 by activating the NF-kB pathway in the BBB (Zhang et al., 2014). In ALF, the expression and function of ABC transporters in the BBB are also altered (Fan and Liu, 2018).

A high concentration of LPS results in robust inflammatory responses. Lipopolysaccharides bind brain endothelial cell membrane receptors, including TLR-2, TLR-4, and CD14, causing the release of cytokines and inflammatory mediators. However, a lower concentration of LPS enhances innate immune functions (Singh and Jiang, 2004; Dauphinee and Karsan, 2006). After injection of LPS, ALF mice were found to further aggravate hepatic injury and develop symptoms of liver coma. Moreover,

BBB is permeable to immunoglobulin G (IgG), which may be modulated by the up-regulation of MMP9 (Chastre et al., 2014). MMPs are proteases that degrade extracellular matrix (ECM), which can easily lead to an increase in vascular permeability. In BDL rats, MMP9 levels were found to be enhanced in the cortex, hippocampus, and striatum (Dhanda and Sandhir, 2018). Moreover, in LPS-induced systemic inflammatory responses, MMP9 activity was shown to be regulated by Cyclooxygenases-1 and -2 (COX1/COX2), which are critical regulators of innate immune responses (Aid et al., 2010). Banks and colleagues, using *in vitro* BBB models and animal inflammatory models, postulated that LPS-induced disruption of the BBB may be dependent on COX (Banks et al., 2015).

Gut microbial metabolites affect the physiological state of the BBB by producing SCFAs. GF mice exhibited decreased BBB permeability after Clostridium tyrobutyricum transplantation that mainly produces butyrate and after oral gavage of sodium butyrate (Braniste et al., 2014). In addition, propionate can protect the BBB by binding the receptor FFAR3 expressed in the human brain endothelium against oxidative stress (Hoyles et al., 2018).

NEUROINFLAMMATION AND IMMUNE REGULATION

Neuroinflammation regulates mood and behavior in patients by regulating the basal ganglia, cortical reward, and motor circuits. Anxiety-related areas of the brain are also affected (Felger, 2018; Cabrera-Pastor et al., 2019a). Neuroinflammation of the hippocampus and cerebellum is a key pathological feature of HE that leads to cognitive impairment. Hippocampal volume is decreased in cirrhosis patients. Neuroinflammation affects the expression of hippocampal glutamate receptors and GABAergic tone in the cerebellum, inducing spatial memory or movement disorder (Hassan et al., 2019; Lin et al., 2019).

Peripheral inflammation and chronic hyperammonemia collectively promote neuroinflammation in liver disease. Liver and intestinal function disorders promote the release of peripheral inflammatory factors, which can pass the BBB and directly affect brain functions (Banks, 2005; Rodrigo et al., 2010; Luo et al., 2015). Microglia are the primary immune cells in CNS, and excessive activation of microglia is the primary source of inflammatory factors that cause neuronal damages (Jaeger et al., 2019). The mechanisms of microglial activation include: i. brain infiltration of peripheral immune cells (D'Mello et al., 2009); and ii. activation of blood cytokine receptors in endothelial cells (Dantzer et al., 2008). Chronic hyperammonemia can also cause neuroinflammatory reactions (Rodrigo et al., 2010; Hernández-Rabaza et al., 2016; Balzano et al., 2020) Injecting extracellular vesicles of hyperammonemic rats into the control group causes neuroinflammatory reactions and dyskinesia (Izquierdo-Altarejos et al., 2020) The use of anti-TNFa therapy, which does not pass the BBB, prevents the neuroinflammatory responses induced by hyperammonemia (Balzano et al., 2020). Therefore, the pro-inflammatory effect of hyperammonemia may be mediated by peripheral inflammation.

Intestinal microorganisms are essential factors that cause systemic inflammation and immune activation of liver diseases. Apart from activating microglia in the brain, intestinal bacteria regulate microglia maturation and homeostasis, which corresponds to microglial defects in mice lacking short-chain fatty acids (SCFAs) receptor FFAR2 (Erny et al., 2015). Lipopolysaccharide is a commonly used *in vitro* inflammatory model of glial cells and animal neuroinflammatory modeling agent. Lipopolysaccharides administration transiently elevates blood levels of interleukin6 (IL6) and tumor necrosis factor-alpha (TNFa) (Labrenz et al., 2019). Pro-inflammatory factors combine with the receptors expressed in Cerebral Endothelial Cells (CECs) to produce a secondary messenger, which induces oxidative stress and neuroinflammation (Azhari and Swain, 2018). Compared to mice with healthy microbiota, there is a significant expression of inflammatory factors in the cortex of GF mice. Moreover, GF mice showed robust neuroinflammation and glial cell activation after receiving intestinal microbiota from cirrhotic mice when compared to mice receiving healthy microbiota. These changes were, however, not caused by liver diseases. These experimental results suggest that an imbalance of intestinal bacterial microbiota drives the development of neuroinflammation in cirrhosis mice and may contribute to the occurrence of HE (Liu et al., 2020).

Microbiota stimulates the vagus nerve to affect brain function in a situation where the intestinal barrier is injured by inflammation. Vagal afferent terminals that are located below the intestinal barrier directly receive the signal produced by microbiota to influence host behavior (Cawthon and de La Serre, 2018). Beneficial microorganisms and probiotic species produce bioactive compounds that regulate host mucosal immune or inflammatory responses. The process is, however, advantageous in improving the inflammation signals received from the peripheral system to the CNS. *Lactobacillus* inhibits TNF production by converting the L-histidine in food into histamine, which improves anti-inflammatory or immunoregulatory functions through the H2 receptor (Hemarajata et al., 2013). Furthermore, inhibition of TNFa formation may also protect against acute ammonia intoxication (Pozdeev et al., 2017).

Gut microbes are involved in immune regulation in HE patients (Martín et al., 2014). Probiotic supplementation plays a beneficial role in the immune function of HE individuals by increasing serum neopterin levels and producing reactive oxygen species (Horvath et al., 2016). Single microbial strains play specific modulatory roles in the body's immune system (Surana and Kasper, 2014; Geva-Zatorsky et al., 2017). The BBB prevents immune cells from freely entering the nervous system, where immune cells are more likely to enter the nervous system, as seen in the brain of dead cirrhosis patients after autopsy. In an animal model of liver inflammation, microglia activated by TNFa signals were shown to produce MCP1 and CCL2, and recruited monocytes expressing CCR2 into the brain, resulting in a significant infiltration of activated monocytes into the brain (D'Mello et al., 2009). However, there are specific immune system changes observed with MHE, such as increased activation of B lymphocytes and all subtypes of

CD4+ T lymphocytes (Mangas-Losada et al., 2017). These changes contribute to neuroinflammation and nervous system disorders.

Suppression/regulation of neuroinflammation is crucial for restoring memory and motor ability in patients with liver cirrhosis or HE. Patients with chronic liver disease, such as steatohepatitis, may have neuropsychological symptoms and cognitive impairment before reaching liver cirrhosis (Felipo et al., 2012; Grover et al., 2012; Mondal et al., 2020). Thus, neuroinflammation in patients with chronic liver disease may have occurred in the early stages of the disease. Balzano et al. analyzed brain tissue samples from patients with different degrees of steatohepatitis and cirrhosis. As disease severity progressed, microglia and astrocytes in the brain were gradually activated and mild steatohepatitis was found to be a pathological feature of neuroinflammation (Balzano et al., 2018). Prompt detection of symptoms and timely treatment may reduce HE cases as well as hospitalization rates.

INTESTINAL BACTERIA METABOLITES IN THE GUT–BRAIN AXIS

Ammonia

Hyperammonemia patients with or without cirrhosis have a motor and cognitive dysfunction, suggesting that ammonia affects the brain function through underlying mechanisms (Balzano et al., 2020). Ammonia-induced central nervous system toxicity is the main mechanism of HE. Excessive production of ammonia by gut bacteria such as *S. salivarius* contributes to increased ammonia levels in the blood and astrocyte edema (Zhang et al., 2013).

The primary therapeutic approaches of hyperammonemia include reducing ammonia production and promoting ammonia metabolism (Rose, 2012). Some studies have reported that hyperammonemia can be reduced by modifying intestinal microbiota. *Bacillus Lactis* consumes intestinal ammonia and increases overall survival in chronic and ALF mice (Nicaise et al., 2008). Fecal microbiota transplantation (FMT) was shown to attenuate hyperammonemia in HE animal models, which is an accessible and useful treatment option for patients (Kang et al., 2015). Shen et al. modified intestinal microbes to reduce urease activity, and transplanted them into the intestines of mice with liver injury. There was a significant reduction in mice morbidity and mortality (Shen et al., 2015). Moreover, Kurtz et al. modified the oral probiotic *Escherichia coli nissle 1917* in order to create a strain (*SYNB1020*) that produces l-arginine and consumes NH3 in the *vitro* system. *SYNB1020* was shown to decrease systemic hyperammonemia in a mouse model of thioacetamide (TAA)-induced liver injury. Phase I clinical trial showed a significant clinical effect, indicating the further clinical application of *SYNB1020* for hyperammonemia-related diseases (Kurtz et al., 2019).

Ammonia induces peripheral inflammation leading to cognitive disorders. Decreasing blood ammonia levels is beneficial for recovering cognitive impairment (Balzano et al., 2020). Karababa et al. reported that ammonia attenuates inflammatory responses in an astrocyte-dependent manner in co-cultured astrocytes and microglia treated with LPS. Neurosteroids secreted from astrocytes may contribute to the anti-inflammatory effects of ammonia, which may be one of the potential mechanisms for the absence of microglia reactivity in cerebral cortex of patients with liver cirrhosis and HE (Karababa et al., 2017).

Bile Acids

Bile acids promote lipid digestion as well as absorption and modulate cellular metabolic activities by binding nuclear receptor, including Farnesoid X Receptor (FXR), Pregnane X Receptor (PXR), Vitamin D Receptor (VDR), and the Glucocorticoid Recptor (GR) (Vítek and Haluzík, 2016). Serum bile acids are elevated during cirrhosis. In an HE animal model, activated apical sodium-dependent BA transporter (ASBT) was shown to promote intestinal bile acid reabsorption, which contributed to increased serum bile acid levels (Xie et al., 2018). However, the homeostasis of the bile acid pool has an intricate connection with intestinal bacteria. Fecal bile acid profile is modulated by gut microbiota in cirrhosis. Chenodeoxycholic (CDCA) and *Enterobacteriaceae* show a strong positive correlation. Meanwhile, *Ruminococcaceae* and Deoxycholic acid (DCA) had a positive correlation. After treatment with rifaximin, *Veillonellaceae*, the ratio of primary and secondary BA levels decreased in six early cirrhotics (Kakiyama et al., 2013).

Bile acids directly or indirectly affect BBB permeability. In BDL rat or rat brain microvessel endothelial cell treated with bile acids, the BBB tight junction was damaged by the activation of Rac1 and the downstream phosphorylation of the tight junction protein occludin (Quinn et al., 2014). Sphingosine-1-Phosphate Receptor 2 Signaling regulated by brain bile acids promotes neuroinflammatory responses in HE, leading to microglial activation and elevated CCL2 expression, thus indirectly and ultimately affecting BBB permeability (McMillin et al., 2017). As BBB permeability increases, unconjugated bile acids may passively diffuse into the brain. Serum bile acid levels have no apparent distinction in cirrhosis with or without HE. However, bile acid levels were found to be increased in the cerebrospinal fluid, while toxic bile acids accumulated in the brains of the BDL mouse models. Therefore, nervous system disorders are associated with the toxic effects of bile acids in HE (Weiss et al., 2016; DeMorrow, 2019).

FXR, activated by bile acids and mainly expressed in the neurons, causes HE-related CNS disturbances. Regulation of bile acids may be a potential strategy for the treatment of HE. FXR knockout mice had a high level of hepatitis development, causing a lower concentration of butyrate in the colon. Butyrate supplements can reverse dysfunctional bile acid synthesis and hepatitis (McMillin et al., 2016; Xie et al., 2018). Bile acids act on the nervous system through nuclear receptors, and can also activate the TGR5 membrane receptor to alleviate

neuroinflammation in AOM-induced type A HE mice. The TGR5 receptor is generally expressed in human and rodent tissues and is also up-regulated in multiple animal models with liver injury. TGR5 receptors play an active role in regulating liver inflammation, cholestasis, and fibrosis. The TGR5 receptor was found to be up-regulated in the cortex, thus, improving neurological decline in HE mice after activation by TGR5 (Duboc et al., 2014; Keitel et al., 2019). These findings imply the dual role bile acids play in the progression of HE.

Short-Chain Fatty Acids

Short chain fatty acids produced by intestinal microorganisms, including butyrate, propionate and acetate, protect the integrity of the intestines and reduce intestinal inflammation. Butyrate, as the main component of SCFAs modulates protein tight junctions to enhance gut barrier function. Its abnormal levels are associated with liver disease severity (Brahe et al., 2013; Stilling et al., 2016; Jin et al., 2019).

SCFAs can cross the BBB; therefore, they play a regulatory role in the gut–brain axis (Joseph et al., 2017). In healthy individuals, *Ruminococcaceae* and *Faecalicatena fissicatena* are positively correlated with SCFAs, which are both, however, decreased in cirrhosis patients. SCFAs provide energy for colonic epithelial cell metabolism. However, the ability to convert carbohydrates into SCFAs is diminished in cirrhosis patients (Brahe et al., 2013; Jin et al., 2019). There is a further reduction of SCFAs in cirrhosis with HE. Butyrate has a negative correlation with inflammatory markers and serum endotoxin (Juanola et al., 2019). SCFAs bind the G-protein-coupled receptor 43 (GPR43) to promote the regression of inflammation (Maslowski et al., 2009). Furthermore, SCFAs downregulate system inflammation and regulate neutrophils, macrophages, and other immune cells (Millard et al., 2002; Vinolo et al., 2011). They also have a strong anti-inflammatory effect on microglial and astrocyte models *in vitro;* therefore, SCFAs may have some potential for regulating neuroinflammatory processes (Huuskonen et al., 2004; Stilling et al., 2016).

Neurotransmitter

Gamma-Aminobutyric Acid

GABA is an important bioactive compound and a crucial inhibitory neurotransmitter in the nervous system. It is mainly produced in the gut by *Bifidobacterium* and *Lactobacillus*, although the GABAergic neurons also produce a small amount of GABA (Yunes et al., 2016; Strandwitz et al., 2019). *Lactobacillus* can regulate GABA concentrations and the expression of GABA receptors in the CNS through the gut–brain axis (Barros-Santos et al., 2020; Chen et al., 2020). In feces, the increased abundance of *Bifidobacterium longum* enhances the risk of HE (Iebba et al., 2018). Elevated GABA levels are associated with physiological and psychological processes in HE (Jones, 2003). When liver failure occurs, serum and brain GABA levels are elevated. GABA can exert pre- and post-synaptic inhibition, leading to motor and consciousness disorders (Kaupmann et al., 1997; Kullmann et al., 2005). Antagonists of the GABA receptor complex can improve the clinical symptoms of HE animals and electroencephalographic abnormalities (Bosman et al., 1991).

Gut ammonia is considered as an essential factor in elevated GABAergic tone. A study by Cauli et al. found out that hyperammonemia selectively increased the GABAergic tone of the cerebellum, ventral thalamus, and the ventromedial thalamus in hyperammonemic rats (Cauli et al., 2009a). The underlying mechanism by which ammonia increases GABA concentration is associated with GABA transaminase activity or neuronal tricarboxylic acid cycle (Palomero-Gallagher and Zilles, 2013). Moreover, Fried et al. reported that ammonia enhances the release of GABA from enteric glia, subsequently altering intestinal neurotransmission, resulting in intestinal motility disorders and an increase in gut ammonia levels (Fried et al., 2017). Studies have also established that changes in GABAA receptor density are up-regulated in hyperammonemia models. In hyperammonemia, elevated GABA concentration and GABAA receptor density correlate to promote CNS disorders, although the expression of the GABAA receptor subunit is not consistent. For instance, GABAA receptor subunit a1 was found to be increased while the alpha-5 subunit was reduced in the hyperammonemia rat model (Palomero-Gallagher and Zilles, 2013; Hernández-Rabaza et al., 2016).

The benzodiazepine receptor (BZR) is part of the GABAA receptor complex; hence exogenous and endogenous benzodiazepine substances bind to GABAA receptor, causing an allosteric regulation of the receptor, thus increasing GABA transport. Ammonia has also been shown to associate with BZR ligands, causing CNS function disorders. Therefore, decreasing ammonia concentration can improve enhanced GABA-ergic (Helewski et al., 2003; Jones, 2003). Clinically, the benzodiazepine receptor is used as the target for improving GABA-ergic. Flumazenil is a benzodiazepine receptor blocker for the treatment of HE. Increased benzodiazepine receptor ligand significantly enhances GABA inhibition in HE brain (Ahboucha and Butterworth, 2005). In HE rat models, BZR levels were not altered in normal rat plasma upon antibiotic intervention. It, however, increased BZR precursors, which may either arise from gut bacteria, increased BZR synthesis in the brain, or enhanced GABA-ergic neurotransmission to promote HE (Yurdaydin et al., 1995).

Glutamate

Glutamate is an excitatory neurotransmitter that regulates nervous system development through NMDA and AMPA receptors (Martinez-Lozada and Ortega, 2015). When ammonia levels increase in the brain, glutamate binds ammonia, forming glutamine under the catalytic activity of glutamine synthetase. Accumulation of glutamine and ammonia is associated with brain edema. Studies have found that the extracellular concentration of glutamate increases due to abnormal uptake, transport and release of glutamate. Learning and memory impairment is associated with the abnormal glutamate-NO-cGMP metabolic pathway in the brain (Dabrowska et al., 2018; Cabrera-Pastor et al., 2019b). Even though different concentrations and duration of ammonia have

different effects on the expression of glutamate receptors, acute hyperammonemia associated mortalities are mediated by activated NMDAR (Monfort et al., 2002; Kosenkov et al., 2018). NMDAR antagonists were shown to effectively reduce hyperammonemia or ALF induced mortalities in rats (Cauli et al., 2009b; Cauli et al., 2014). Glutamate produced by diet or bacteria cannot be used by the CNS because of BBB. Studies have shown that glutamate and glutamate receptors affect the gut-brain axis in other diseases, such as inflammatory bowel disease (IBD) (Baj et al., 2019). In addition, probiotics and prebiotics can adjust the NMDA/AMPA ratio to affect cognitive functions in middle-aged rats (Romo-Araiza et al., 2018). However, it has not been established whether specific intestinal bacteria alterations affect glutamatergic transmission in HE patients.

5-Hydroxytryptamine

Gut tract is the leading site for 5-HT synthesis. High colonic and blood 5-HT levels are associated with specific gut microbiota metabolites (Roshchina, 2010), although the mechanism of 5-HT synthesis that is regulated by microbiota has not been established. Indigenous spore-forming bacteria (Sp) from mouse and human microbiota act on colonic enterochromaffin cells (ECs) to produce 5-HT (Yano et al., 2015). Moreover, probiotics can stimulate the gut–brain axis and increase 5-HT and serotonin transporter (5-HTT) expression, which may promote brain development and function (Ranuh et al., 2019).

Dysfunction of 5-HT receptor and excess serotonergic brain activity is involved in HE development (Apelqvist et al., 1998; Dhanda and Sandhir, 2015; Khiat et al., 2019). In hyperammonemia mice, the 5-HT2B receptor was found to be up-regulated in the brain and had no response to 5-HT. Moreover, the dysfunction of the 5-HT2B receptor was also observed in ammonia treated astrocytes *in vitro* (Yue et al., 2019). 5-HT(1A) is also involved in cognitive–behavioral disorders in HE, while its activation can, reverse nervous system dysfunctions (Magen et al., 2010). However, there was no difference in 5-HT of GF mice before and after FMT (Maslowski et al., 2009). Decreasing peripheral 5-HT absorption can help improve CNS disease. Oral Selective serotonin reuptake inhibitors (SSRIs) improve depression and decreases mortality rates in patients with chronic liver disease. This therapeutic effect is achieved by activating the vagus nerve dependent gut-brain signaling (Mullish et al., 2014; Neufeld et al., 2019).

5-HT concentration depends on the level of tryptophan in the brain (Maslowski et al., 2009). Free tryptophan (TRP), which is the precursor of the neurotransmitter 5-HT, increases only in HE, with no changes in hepatitis and cirrhosis. However, tryptophan is an essential amino acid that competes with other amino acids to cross the BBB. Thus, elevated serum free tryptophan levels invariably increases its availability to the brain and to the activity of serotonergic neurons (Herneth et al., 1998; Lozeva-Thomas, 2004; Saleem et al., 2008). Dietary tryptophan restriction improves neuroinflammation by impairing encephalitogenic T cell responses (Sonner et al., 2019). Probiotic treatment of hyperammonemia rats was shown to significantly decrease 5-HT metabolism (Luo et al., 2014).

EFFECTS OF CLINICAL TREATMENT ON THE INTESTINAL METABOLOME IN HEPATIC ENCEPHALOPATHY

Treatments for HE target disease causing agents, control infections, reduce absorption of intestinal ammonia, and correct the metabolic dysfunction caused by liver diseases. Several drugs, including antibiotics and laxatives, are used to treat HE. Probiotics and other drugs are also used in clinical practice. Clinical therapeutic drugs may or may not alter the intestinal metabolome to achieve therapeutic effect. We discuss the effects of several commonly used drugs on the intestinal microbiota of HE patients.

Antibiotics

Rifaximin is a common antibiotic used to treat patients with HE. It improves hyperammonemia, endotoxemia, and cognitive dysfunction (Kaji et al., 2017). Other antibiotics such as neomycin are not recommended because of their side effects (Patidar and Bajaj, 2013). Although rifaximin has bactericidal and bacteriostatic effects, it does not change the abundance of dominant intestinal bacteria in HE patients. In addition, it does not control the abundance of Gram-negative bacteria. It decreases blood endotoxin levels through unknown mechanisms. It is postulated that it can regulate the metabolism of intestinal bacteria or stabilize intestinal barrier functions (Kaji et al., 2017; Montagnese et al., 2018).Other studies have shown that it has immunomodulatory effects as it reduces inflammation by regulating bacteria. Rifaxmin was shown to improve the immune system in 59% of MHE patients (Mangas-Losada et al., 2019).

Lactulose

Various drugs are used to reduce blood ammonia levels. Lactulose is the most commonly used ammonia-reducing drug. It is an unabsorbable disaccharide that is used as a laxative because it triggers the production of large amounts of ammonia in stool (Montagnese et al., 2018). Lactulose alone or in combination with rifaximin is widely used in the treatment of HE. Clinical studies have shown that lactulose improves patients' cognitive functions and quality of life (Wang et al., 2019). In rats with CCL4-induced ALF, lactulose improved the plasticity of the nervous system (Yang et al., 2015). It also inhibits intestinal bacterial overgrowth, translocation and intestinal resistance. This decreases systemic inflammatory responses and hyperammonemia in HE rats. In patients with liver cirrhosis, clinical doses of lactulose promote the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* (Montagnese et al., 2018).

Probiotic

Probiotic treatment is a new adjuvant therapy for HE. Clinical studies have shown that probiotics can prevent the occurrence and recurrence of HE in patients with cirrhosis (Lunia et al., 2014; Venigalla et al., 2015). Probiotics comprise various bacteria which directly improve the composition of intestinal microbiota, thereby, conferring therapeutic effects. Probiotics reduce bacterial ammonia production and the absorption of intestinal ammonia and other toxins (Solga, 2003). In patients with

compensatory cirrhosis taking multiple probiotic strains for 6 months, their stool was found to be rich in probiotic strains, including *Lactobacillus brevis*, *Lactobacillus salivarius* and *Lactococcus lactis*. In addition, probiotics may boost the production of short-chain acids by increasing the abundance of multiple bacteria, including *Calibacterium prausnitzii*, *Syntrophococcus sucromutans* and *Alistipes shahii* (Horvath et al., 2020).

Fecal Microbiota Transplant

FMT is an emerging treatment approach that is aimed at rebuilding intestinal microbiota to treat diseases, and is gradually being generalized for the treatment of various intestinal dysfunction diseases, such as inflammatory bowel disease (IBD) (Monfort et al., 2002; Bibbo et al., 2017). A few animal experiments have shown that FMT has obvious protective effects on CCL4-induced ALF rats (Wang et al., 2017). This beneficial effect is not only observed in the improvement of cognitive function, but can also improve the markers of disease activity associated with the gut-liver-brain axis disorder. FMT was shown to significantly reduce neuroinflammatory responses in CCL4-induced cirrhotic mice. It also provided effective protection in HE by restoring normal intestinal permeability and improving liver damage indicators. TOLL-like receptors are important mediators of inflammatory responses. Hepatic TLRs and serum ammonia levels were found to be significantly down-regulated in cirrhosis rats after FMT (Wang et al., 2017; Liu et al., 2020). Although clinical trials of FMT are ongoing, we discussed its effectiveness and safety in clinical treatment based on the published results.

Recurrent HE leads to hospitalization. In an open and randomized clinical trial, it was determined whether the therapeutic effect of FMT enema in cirrhosis patients with recurrent HE after pretreatment with antibiotics is better than standard of care (SOC). Compared to SOC, a reasonable choice of donor FMT can significantly improve cognitive functions in patients and reduce incidences of serious adverse events. In a randomized, single-blind, placebo-controlled phase 1 clinical trial, compared to placebo, oral FMT capsules showed significant safety in cirrhosis patients with recurrent HE (Bajaj et al., 2017; Fuchs and Puri, 2020). Fecal transplantation improves liver functions in a number of liver diseases (Lechner et al., 2020). Restoring liver functions reduces the impact of various factors on the nervous system, prevents or defers neurological disorders in patients with liver diseases, and improves the quality of life for patients. Although clinical trials involving different liver diseases have just begun, FMT is an effective treatment method for liver diseases and their complications.

SUMMARY

Intestinal microbes have been implicated in shaping the nerves and immune systems or other fundamental process during growth. The occurrence of numerous diseases is accompanied by significant changes in microbial communities. As cirrhosis progresses, the composition of intestinal microbiome is altered. Harmful intestinal bacteria promote the occurrence of complications related to liver cirrhosis, including endotoxemia, infection, organ failure, and death. Gut bacteria regulate numerous metabolic processes and physiological functions by secreting different metabolites. Many intestinal metabolites (such as bile acids) are necessary for the human body and undergo enterohepatic recycling, while intestinal metabolic wastes (such as ammonia) are excreted from the body after hepatic metabolism. When these substances exceed physiological concentrations, they produce clinical manifestations of toxicity. They pass the BBB with increased permeability, destroy the nervous system microenvironment, nerve conduction, and even directly lead to coma and death. Intestinal intervention may be a treatment option for all stages of liver disease as it reduces the exposure of the liver and nervous system to intestinal toxins.

Intestinal microbiota is closely associated with CNS function, including brain structure, gene expression, and substance metabolism. Understanding the function of intestinal microbiota in host behavior will promote the management of mental and psychological diseases. Therapies that balance intestinal microbiota are critical for correcting central nervous activity and function in patients with CNS dysfunction due to abnormal intestinal microbiota composition. Such therapies can be designed to target species associated with disease progression. Probiotics or fecal transplantation can be used to manipulate the intestinal microbiome to improve hyperammonemia and endotoxemia. Proper selection of donor FMT reduces hospitalization rates, improves cognition and malnutrition in patients with cirrhosis. It also improves the prognosis of HE patients. Consequently, the underlying mechanisms through which microbes modulate CNS *via* the gut–brain axis should be studied. Liver function alterations in patients with cirrhosis are difficult to reverse. Maintaining intestinal homeostasis to treat liver disease-related nervous system damage is a new potential treatment method. Reasonable intestinal intervention combined with drug treatment may achieve mutually beneficial effects.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Roles of Gut Microbiota in the Regulation of Hippocampal Plasticity, Inflammation, and Hippocampus-Dependent Behaviors

Wen Tang[1†], Zhaoyou Meng[2†], Ning Li[1], Yiyan Liu[3], Li Li[4], Dongfeng Chen[1] and Yang Yang[1*]*

[1] Department of Gastroenterology, Daping Hospital, Army Medical University, Chongqing, China, [2] Department of Neurology, Xinqiao Hospital, Army Medical University, Chongqing, China, [3] College of Basic Medicine, Army Medical University, Chongqing, China, [4] Department of Gastroenterology, The First People's Hospital in Chongqing Liangjiang New Area, Chongqing, China

The study of the gut microbiota-brain axis has become an intriguing field, attracting attention from both gastroenterologists and neurobiologists. The hippocampus is the center of learning and memory, and plays a pivotal role in neurodegenerative diseases, such as Alzheimer's disease (AD). Previous studies using diet administration, antibiotics, probiotics, prebiotics, germ-free mice, and fecal analysis of normal and specific pathogen-free animals have shown that the structure and function of the hippocampus are affected by the gut microbiota. Furthermore, hippocampal pathologies in AD are positively correlated with changes in specific microbiota. Genomic and neurochemical analyses revealed significant alterations in genes and amino acids in the hippocampus of AD subjects following a remarkable shift in the gut microbiota. In a recent study, when young animals were transplanted with fecal microbiota derived from AD patients, the recipients showed significant impairment of cognitive behaviors, AD pathologies, and changes in neuronal plasticity and cytokines. Other studies have demonstrated the side effects of antibiotic administration along with the beneficial effects of probiotics, prebiotics, and specific diets on the composition of the gut microbiota and hippocampal functions, but these have been mostly preliminary with unclear mechanisms. Since some specific gut bacteria are positively or negatively correlated to the structure and function of the hippocampus, it is expected that specific gut bacteria administration and other microbiota-based interventions could be potentially applied to prevent or treat hippocampus-based memory impairment and neuropsychiatric disorders such as AD.

Keywords: gut microbiota, hippocampus, learning and memory, senile plaque, inflammation, Alzheimer's disease

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Zongxin Ling,
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Andrey Santos,
Campinas State University, Brazil
Esther Nistal,
Universidad de León, Spain

****Correspondence:***
Yang Yang
yyang_tmmu@163.com
Dongfeng Chen
chendf1981@126.com

[†]These authors have contributed equally to this work

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INTRODUCTION

The human microbiome is established early in life, and consists of approximately 3.8×10^{13} symbiotic microorganisms (Lukiw, 2016; O'Hagan et al., 2017). In the gastrointestinal tract, the colonized gut microbiota is a complex and dynamic community of microorganisms that can communicate with the host to influence the brain and behavior (Liang et al., 2015; Hu et al., 2020). Under normal conditions, aging is associated with changes in higher brain functions such as learning and memory, as well as dysbiosis in the gut microbiome (Daulatzai, 2014; Distrutti et al., 2014). One hundred years ago, the Nobel Prize winner Elie Metchnikoff proposed that cognitive decline and senility might be delayed by manipulating the intestinal microbiome with host-friendly bacteria (Scott et al., 2017). However, no significant progress showing that the bacterial constituents of the gut microbiota can influence brain function has been made over the past decade (O'Hagan et al., 2017). The term gut-microbiota-brain axis or gut-brain-axis is used to describe the relationship between the gut and the brain (Bienenstock and Collins, 2010).

The hippocampus, consisting of the cornu ammonis (CA) 1, CA2, CA3, dentate gyrus (DG), and subiculum, is the center of learning and memory (Lisman et al., 2017; Hainmueller and Bartos, 2018). Interestingly, although engrams (memory traces) in CA1 and CA2 do not stabilize over time, reactivation of engrams in the DG can induce recall of artificial memories even after weeks (Hainmueller and Bartos, 2018). Moreover, the hippocampus has also been implicated in depression and anxiety, and hippocampal neurogenesis has been implicated in cognitive processes (Toda et al., 2018). Since the gut microbiota has been shown to play a role in the pathology of Alzheimer's disease (AD) and other memory disorders, we reviewed the current progress on the gut microbiota's influence on the structure and function of the hippocampus and hippocampus-based learning and memory.

IMBALANCED GUT MICROBIOTA IN ALZHEIMER'S DISEASE SUBJECTS AND MODEL ANIMALS

AD is the most common neurodegenerative disorder, ultimately resulting in dementia, and the hippocampus is one of the affected brain regions (Moodley and Chan, 2014). Several clues from human fecal studies have shown that gut microbiota composition is different between AD patients and healthy controls (HCs). For example, AD patients showed lower abundance of *Eubacterium* but higher abundance of *Escherichia/Shigella* (Cattaneo et al., 2017), along with obvious changes in *Bacteroides*, *Actinobacteria*, *Ruminococcus*, *Lachnospiraceae*, and *Selenomonadales* (Zhuang et al., 2018). Other studies showed that among AD patients, patients with amnestic mild cognitive impairment, and HCs, the fecal microbial diversity was changed, showing a reduced proportion of phylum *Firmicutes* but enriched *Proteobacteria*. These results indicated that distinct microbial communities, especially enriched *Enterobacteriaceae*, were associated with AD (Zhuang et al., 2018; Liu et al., 2019). Furthermore, gene-targeted analysis of human gut microbiota in AD fecal samples found some unique gut bacterial sequences that were rarely seen in controls, highlighting the significant difference in the gut microbial genotypes between the AD patients and healthy human populations (Paley et al., 2018).

AD model rodents have been frequently used to explore alterations in the gut microbiota in AD. In the feces of AD mice, the microbiota composition and diversity were changed, with short-chain fatty acid composition (Zhang et al., 2017) and the amount of trypsin reduced when compared to wild type (WT) mice (Brandscheid et al., 2017). Additionally, the composition and diversity of the gut microbiota changed greatly with aging and AD pathology. Impaired spatial memory appeared in 6-month-old APP/PS1 AD model mice and was further aggravated in the 8-month-old mice. This was consistent with the accumulation in amyloid plaque and the remarkable shift in gut microbiota compared to WT mice. The abundance of *Helicobacteraceae*, *Desulfovibrionaceae*, *Odoribacter*, and *Helicobacter* increased significantly, while that of *Prevotella* decreased significantly (Shen et al., 2017). At 3 months of age, the fecal bacterial profiles did not show significant differences between the AD mice and control mice; however, at 6 months, the abundance of *Turicibacteriaceae* and *Rikenellaceae* increased in both groups, and an increase in *Proteobacteria* abundance was seen in AD mice after 6 months, particularly that of the genus *Sutterella* (*Betaproteobacteria*); the inflammation-related family *Erysipelotrichaceae* was more abundant in 24-month-old AD mice than in WT mice (Bauerl et al., 2018). These results indicated that AD pathology shifted gut microbiota composition towards an inflammation-related bacterial profile during aging, and suggested that these changes could contribute to disease progression and severity (Bauerl et al., 2018). Importantly, recent studies showed that when the gut microbiota from AD patients was transplanted into AD mice, the recipient mice showed more severe cognitive impairment and activated microglia in the hippocampus, and these effects could be effectively inhibited by transplantation of healthy human gut microbiota (Shen et al., 2020).

Thus, in both AD patients and AD model animals, significant changes in the gut microbiota have been reported, some of which increased while others decreased (**Figure 1** and **Table 1**), indicating that manipulation of the gut microbiota may be a promising intervention for the prevention or treatment of AD.

Abbreviations: AD, Alzheimer's disease; AGEs, advanced glycation end products; Aβ, β-amyloid; *B. breve* A1, *Bifidobacterium breve strain* A1; BDNF, brain-derived neurotrophic factor; *C. butyricum*, *Clostridium butyricum*; CA, cornu ammonis (hippocampus); Caf, cafeteria diet; CREB, cAMP-response element-binding protein; DG, dentate gyrus; DW2009, *Lactobacillus plantarum C29*-fermented soybean supplement; FMT, fecal microbiota transplantation; FOS, fructooligosaccharides; GF, germ free; GSPE, grape seed polyphenol extract; HC, healthy control; IL-6, interleukin-6; LJ, *Lactobacillus johnsonii* CJLJ103; lncRNAs, long non-coding RNAs; LPS, lipopolysaccharide; LW-AFC, an herbal medicine prepared from traditional Chinese medicine LiuweiDihuang decoction; MHE, minimal hepatic encephalopathy; OTUs, operational taxonomic units; PC, microbiota principal component; pCREB, phosphorylation CREB; p-Tau, phospho-tau protein; SPF, specific pathogen free; TLR, Toll-like receptor; TTK, Tetragonia tetragonioides Kuntze; VSL#3, a probiotic mixture comprising 8 Gram-positive bacterial strains; WT, wild type.

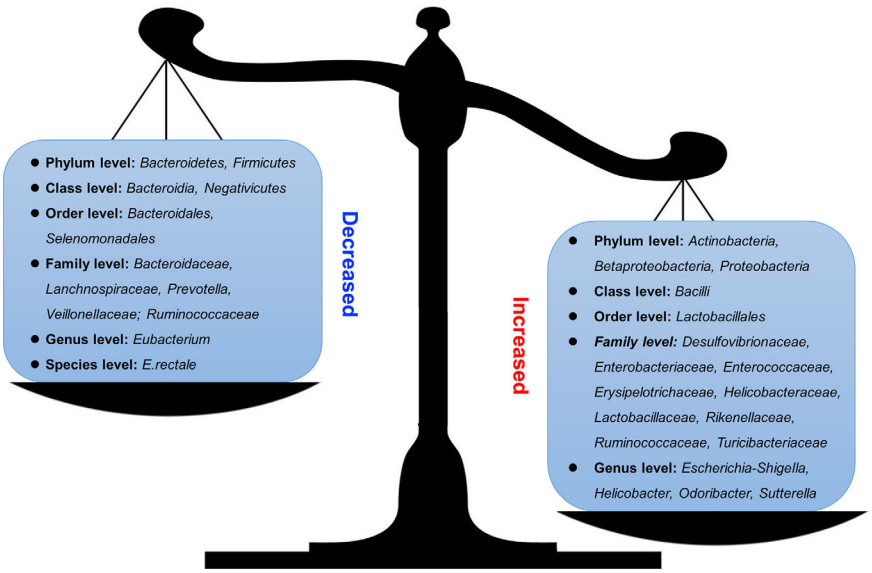

FIGURE 1 | Altered gut microbiota in AD patients and model mice. Some differences have been noticed regarding the changes of gut microbiota in AD patients or mouse models. For example, *Bacteroidetes* and *Firmicutes* decreased (left) at Phylum level while *Actinobacteria*, *Betaproteobacteria*, and *Proteobacteria* increased (right).

TABLE 1 | Altered gut microbiota in Alzheimer's disease (AD) patients and mice.

Object	Increased/enriched	Decreased	References
AD patients	*Escherichia-Shigella*	*Eubacterium, E. rectale*	(Cattaneo et al., 2017)
AD patients	*Proteobacteria, Actinobacteria, Enterobacteriaceae*	*Firmicutes*	(Liu et al., 2019)
AD patients	*Actinobacteria, Bacilli, Lactobacillales, Ruminococcaceae, Enterococcaceae, Lactobacillaceae*	*Bacteroidetes, Negativicutes, Bacteroidia, Bacteroidales, Selenomonadales, Lanchnospiraceae, Bacteroidaceae, Veillonellaceae*	(Zhuang et al., 2018)
AD mice	*Helicobacteraceae, Desulfovibrionaceae, Odoribacter, Helicobacter*	*Prevotella*	(Shen et al., 2017)
AD mice	*Turicibacteriaceae, Rikenellaceae, Proteobacteria, Sutterella, Betaproteobacteria, Erysipelotrichaceae*	*Ruminococcaceae, Lachnospiraceae. Bacteroidaceae*	(Bauerl et al., 2018)

HIPPOCAMPAL NEUROCHEMICALS AND NEUROPLASTICITY ARE REGULATED BY THE GUT MICROBIOTA

Changes in neurochemicals form the basis of structural and functional plasticity of the hippocampus. An early analysis of the cerebral metabolome revealed that the concentrations of 38 metabolites differed significantly between germ-free (GF) mice and WT mice, indicating that intestinal microbiota is closely related to brain health and disease and its functions, such as development, learning, memory, and behavior (Matsumoto et al., 2013). Kawase et al. reported that compared to specific pathogen-free (SPF) mice, hippocampal amino acids and neurochemicals in GF mice at postnatal week 7 were significantly changed, showing lower concentrations of L-Ala, L-Arg, L-Gln, L-Ile, L-Leu, L-Phe, L-Val, and GABA, but higher concentrations of Ser (Kawase et al., 2017). Another study showed that GF mice showed higher hippocampal levels of creatine, N-acetyl-aspartate, lactate, and taurine but lower levels of succinate than SPF mice (Swann et al., 2017). Furthermore, the hippocampus of GF mice showed an increase in synapse-promoting genes and markers of reactive microglia and synaptic density, all of which could be reversed by colonization with human *Bifidobacterium* species or conventional murine microbiota, indicating that *Bifidobacteria* are involved in the establishment of functional neural circuits in the hippocampus (Luck et al., 2020). Interestingly, one hippocampal microRNA (miRNA) study using GF, conventional, and GF colonized mice showed an increase in miR-294-5p expression in GF animals but normalized expression following colonization, indicating that the gut microbiota plays an important role in modulating small RNAs that influence hippocampal gene expression (Moloney et al., 2017). Similarly, one study showed that in the hippocampus of GF mice, 1355 lncRNAs were upregulated and 875 lncRNAs were downregulated. Further analysis revealed that most of their target genes were highly associated with cardiac hypertrophy, nuclear factors of activated T cells, gonadotropin-releasing hormone,

calcium, and cAMP-response element-binding protein (CREB) signaling pathways (Zhou et al., 2020).

The brain-derived neurotrophic factor (BDNF) regulates activity-dependent synaptic plasticity and psychiatric disorders (Bjorkholm and Monteggia, 2016; Leal et al., 2017), while CREB regulates genes related to neuronal differentiation, synaptic plasticity, learning, and memory (Sharma et al., 2019). Studies have shown that both hippocampal BDNF and CREB are regulated by the gut microbiota. The anticancer flavonoid quercetin, a secondary plant metabolite, has been shown to increase gut microbial diversity and relative abundance of *Glutamicibacter*, *Facklamia*, and *Aerocorrus*; increase hippocampal BDNF; and improve learning and memory (Lv et al., 2018). Zeng et al. used microarray analysis and revealed that the absence of the gut microbiota from birth was associated with decreased hippocampal CREB but an increase in phosphorylated CREB (pCREB), which could be restored by microbiota colonization in adolescence; hippocampal pCREB expression could be reduced by removal of the gut microbiota from SPF mice using antibiotics (Zeng et al., 2016). Additionally, oral administration of *Lactobacillus johnsonii CJLJ103*, a member of the human gut microbiota, may alleviate cholinergic memory impairment by increasing BDNF expression and pCREB in the hippocampi (Lee et al., 2018). Interestingly, gut microbiota-induced hippocampal BDNF expression might be mediated by the vagus nerve, since it could be regulated by subdiaphragmatic vagotomy (O'Leary et al., 2018). A recent study showed that when fecal microbiota transplantation (FMT) was conducted on aged and young rats, the young rats showed impairment in cognitive behavior, a decrease in dendritic spines and expression of BDNF, N-methyl-D-aspartate receptor NR1 subunit, and synaptophysin, but an increase in the expression of advanced glycation end products (AGEs) and receptors for AGEs. At the phylum level, FMT decreased the relative abundance of *Bacteroidetes*, while increasing the relative abundance of *Actinobacteria.* At the genus level, FMT rats showed lower levels of *Prevotella*, *Bacteroides*, *Parabacteroides*, and higher levels of *Sutterella* (Li et al., 2020).

Furthermore, studies have shown that the morphology and neurogenesis of the hippocampus are regulated by the gut microbiota. Convincing evidence comes from studies of GF animals. Luczynski et al. reported that compared to the control mice, GF mice showed significant hippocampal expansion with shorter pyramidal neurons, and less-branched, stubby mushroom- spines and granule cells (Luczynski et al., 2016). Indirectly, Val-Laillet et al. found that a Western diet (fat 33%, refined carbohydrate 49%) induced a decrease in microbiota activity and hippocampal neurogenesis but increased cell proliferation, higher working memory and reference memory scores, accompanied by a smaller hippocampal granular cell layer volume (Val-Laillet et al., 2017) Similarly, Möhle et al. found that antibiotics, which could severely deplete the intestinal microbiota, significantly decreased hippocampal neurogenesis (Mohle et al., 2016).

Probiotics, diets, and obesity also play roles in the regulation of the hippocampus, which might be mediated by the gut microbiota. Distrutti et al. reported that treatment of aged rats with VSL#3, a probiotic mixture comprising eight gram-positive bacterial strains, increased the abundance of *Actinobacteria* and *Bacteroidetes* and modulated the expression of CD11b (a marker for microglia), BDNF, syntaxin, and drebrin in the hippocampus (Distrutti et al., 2014). VSL#3 has also been shown to prevent diet-induced microbiota deficits by increasing the abundance of some taxa such as *Streptococcus*, *Lactobacillus*, and *Butyrivibrio*, which were decreased by the cafeteria (Caf) diet. Meanwhile, hippocampal-dependent place tasks were also regulated by these treatments (Beilharz et al., 2018). However, in the hippocampus, the Caf diet increased the expression of many neuroplastic genes and serotonin receptor 5-HT1A, which are the best predictors of place memory, and are related to the microbiota principal component (PC) 1 (Beilharz et al., 2018). For obese humans, hierarchical clustering with magnetic resonance imaging analysis revealed a specific gut microbiota-brain map profile, and the Shannon index was linked to R2* and fractional anisotropy of the hippocampus (Fernandez-Real et al., 2015). Moreover, changes in waist circumference in obese humans are associated with iron deposition in the hippocampus, and these changes are linked to shifts in the gut microbiome (Blasco et al., 2017).

Taken together, the current findings suggest that the gut microbiota can be regulated by antibiotics, probiotics, diets, and obesity. They further affect hippocampus-dependent behaviors by acting on neurochemicals, neurotrophic factors, transcriptional factors, neurogenesis, and plasticity of pyramidal and granular cells. These findings are summarized in **Figure 2** and **Table 2**.

ALTERATIONS IN THE GUT MICROBIOTA AFFECT HIPPOCAMPUS-DEPENDENT LEARNING AND MEMORY

Numerous studies have revealed that the gut microbiota may affect hippocampus-dependent learning, memory, and behavior. Probiotics regulate learning and memory through action on the gut microbiota. When old (15–17 months) mice were treated with a multi-species live *Lactobacillus* and *Bifidobacteria* mixture (*Lactobacillus acidophilus CUL60*, *L. acidophilus CUL21*, *Bifidobacterium bifidum CUL20*, and *B. lactis CUL34*), the spatial navigation, as shown by the results of a water maze, was moderately improved and the long-term object recognition memory was dramatically improved (O'Hagan et al., 2017). These results indicate that chronic dietary supplements with multi-species live microorganisms have beneficial effects on memory. Kobayashi et al. showed that oral administration of *Bifidobacterium breve strain A1 (B. breve A1)* to AD mice reversed the impaired behavior in a Y-maze test and the reduced latency in a passive avoidance test. Further gene profiling analysis revealed that *B. breve A1* administration suppressed the expression of hippocampal inflammation and immune-reactive genes that were induced by amyloid beta (Aβ) (Kobayashi et al., 2017). Additionally, in a mouse model of

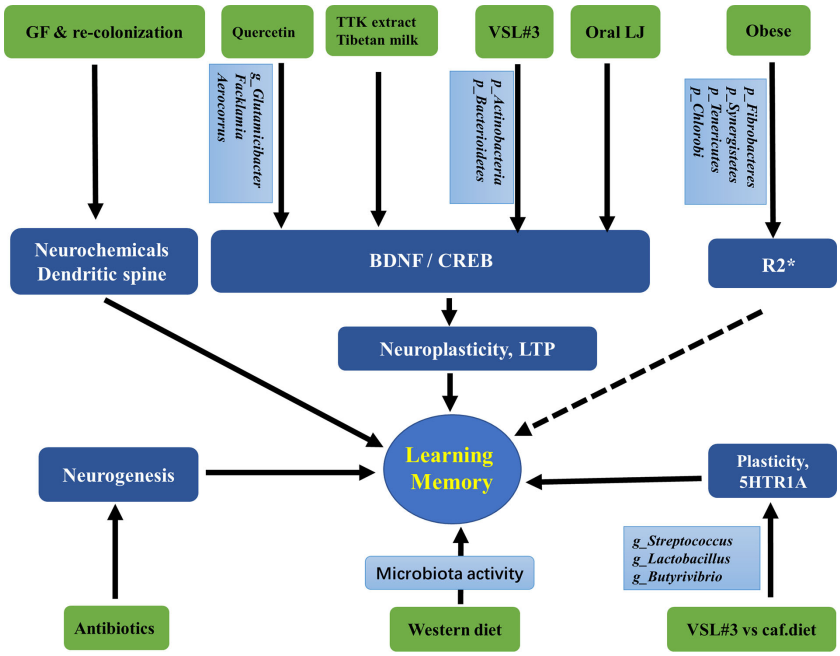

FIGURE 2 | Associations between gut microbiota and the hippocampus-dependent plasticity and behaviors. GF, Germ free; LJ, *Lactobacillus johnsonii CJLJ103*; VSL3#, a probiotic mixture comprising 8 Gram-positive bacterial strains; caf, cafeteria; LTP, long-term potentiation, the cellular mechanism of synaptic plasticity; TTK, Tetragonia tetragonioides Kuntze extract; R2*, a validated magnetic resonance imaging (MRI) marker of brain iron content which can be rapidly measured under clinical conditions. The taxonomic group of bacteria: Phylum, Class, Order, Family, Genus, Species, were marked with p, c, o, f, g, s.

TABLE 2 | Associations between gut microbiota and the hippocampal-dependent behaviors.

Treatment	Related microbiota	Hippocampal target	Behavior	References
quercetin	*Glutamicibacter*, *Facklamia*, *Aerocorrus*	BDNF	learning and memory	(Lv et al., 2018)
GF mice and colonization		CREB, pCREB	anxiety-related and passive behaviors	(Zeng et al., 2016)
oral administration	*Lactobacillus johnsonii CJLJ103* (LJ)	BDNF, pCREB	cholinergic memory	(Lee et al., 2018)
FMT	*Prevotella, Bacteroide, Parabacteroides, Sutterella*	dendritic spines, BDNF, NMDA receptor, synaptophysin, AGEs and receptor	cognitive behavior	(Li et al., 2020)
germ free		hippocampal expansion, neurons, dendritic spine		(Luczynski et al., 2016)
Western diet	microbiota activity	decreased neurogenesis, increased proliferation	working and reference memory	(Val-Laillet et al., 2017)
antibiotics		neurogenesis		(Mohle et al., 2016)
probiotic mixture VSL#3	*Actinobacteria* and *Bacterioidetes*	BDNF, neuronal plasticity, LTP, inflammation		(Distrutti et al., 2014)
probiotic mixture VSL#3 vs cafeteria diet	*Streptococcus*, *Lactobacillus*, *Butyrivibrio*	neuroplasticity, serotonin receptor (5HT) 1A	place memory	(Beilharz et al., 2018)
obese	*Fibrobacteres*, *Synergistetes, Tenericutes RA#, Chlorobi RA*	right hippocampus R2*		(Blasco et al., 2017)
obese	diversity	lowest R2*		(Fernandez-Real et al., 2015)

GF, germ free; FMT, fecal microbiota transplantation; LTP, Long-term potentiation; VSL#3, a probiotic mixture comprising 8 Gram-positive bacterial strains (Streptococcus thermophilus DSM24731, Bifidobacterium breve DSM24732, Bifidobacterium longum DSM24736, Bifidobacterium infantis DSM24737, Lactobacillus acidophilus DSM24735, Lactobacillus plantarum DSM24730, Lactobacillus paracasei DSM24733, Lactobacillus delbrueckii subspecies Bulgaricus DSM24734).

vascular dementia, *Clostridium butyricum* treatment was shown to increase the diversity of intestinal bacteria, improve spatial learning and memory dysfunction, and morphological changes in hippocampal granule cells. It also activated the BDNF-PI3K/Akt pathway in the hippocampus (Liu et al., 2015).

Plant extracts may affect learning and memory through action on the gut microbiota. In a d-galactose-induced aging mouse model, tuna oil administration restored the diversity of the gut microbiota, showing significant changes in 27 key operational taxonomic units; it also alleviated aging and memory deterioration

and changed the expression of proteins related to synaptic repair and signal transduction (Zhang et al., 2018). Additionally, treatment of LW-AFC, an herbal medicine prepared from the traditional Chinese medicine LiuweiDihuang decoction, was given to senescence-accelerated mouse prone 8 (SAMP8) mice, which resulted in improvement of cognitive impairments including spatial learning and memory, active avoidance response, and object recognition memory capability. This was accompanied by significant changes in operational taxonomic units (OTUs; eight increased and 12 decreased) in the gut microbiota. Further examinations showed that there were seven OTUs significantly correlated with all three types of cognitive abilities (three negative and four positive correlations) at the order level, including *Bacteroidales*, *Clostridiales*, *Desulfovibrionales*, and *CW040* (Wang et al., 2016). Tetragonia tetragonioides Kuntze (TTK) extract was also shown to protect against short-term and special memory loss, which might involve the upregulation of the hippocampal pCREB/pAk/pGSK-3β pathway, expression of BDNF and CNTF, and cytokines such as TNF-α and IL-1β. These changes were accompanied by a decrease in *Clostridiales*, *Erysipelotrichales*, and *Desulfovibrionales* but an increase in *Lactobacillales* and *Bacteroidales* (Kim et al., 2020). Such cognition-improving effects were seen in Tibetan fermented milk-treated APP/PS1 AD mice, which showed an increase in intestinal microbial diversity and increased abundance of *Bacteroides, Faecalibacterium* spp. Mucispirillum, and *Ruminiclostridium;* cognitive function was negatively correlated with *Mucispirillum* abundance and positively correlated with *Muribaculum* and *Erysipelatoclostridium* abundance (Liu et al., 2020). These results are summarized in **Table 3**.

GUT MICROBIOTA AND HIPPOCAMPAL INFLAMMATION

Inflammation in the hippocampus is key to the vulnerability and recovery from psychiatric disorders. Several studies have reported that the gut microbiota may change the hippocampal inflammatory response and the related behaviors. For example, in obese mice, alterations in the gut microbiota could be ameliorated by *B. pseudocatenulatum CECT 7765* accompanied by reduced Toll-like receptor 2 (TLR2) protein or gene expression in the hippocampus (Agusti et al., 2018). An early study showed that exposure to magnesium deficient diet induced changes in gut microbiota composition that was positively correlated to the levels of hippocampal interleukin-6 (IL-6) (Winther et al., 2015). Beilharz et al. found that a diet with saturated fatty acid and sugar but lacking polyunsaturated fatty acid significantly impaired hippocampal-dependent place recognition memory accompanied by altered composition of gut microbes. Further analysis revealed that the strongest relationship was detected between hippocampal IL-1b, TLR4, PPARGC1A, PLA24GA, PTGES2, and microbiota PC2 or PC3 (Beilharz et al., 2016), indicating the existence of a gut-microbiota-hippocampal inflammation-behavior axis. Teasaponin, the major active component of tea, has been shown to attenuate gut microbiota alterations induced by a high-fat diet, prevent recognition memory impairment, and improve neuroinflammation deficits (indicated by levels of TLR4, MyD88, p-JNK, NF-κB, IL-1β, IL-6, and TNF-α) in the hippocampus (Wang et al., 2017). Furthermore, treatment of aged rats with VSL#3 induced a robust change in the composition of intestinal microbiota, with an increase in the abundance of *Actinobacteria* and *Bacteroidetes*; modulated expression of inflammatory genes, such as CD68 mRNA and CD11b mRNA in hippocampal slices; and decreased expression of markers of microglial activation (Distrutti et al., 2014).

The Gram-negative facultative anaerobe *B. fragilis*, which constitutes an appreciable proportion of the human gastrointestinal gut microbiome that secretes an unusually complex mixture of neurotoxins, including extremely proinflammatory lipopolysaccharides (LPS) (Zhao and Lukiw, 2018). Unexpectedly, Zhang et al. reported abundant LPS immunoreactivity in the AD-affected hippocampus, indicating that a major source of proinflammatory signals in the AD brain may originate from the gut microbiome due to intestinal mucosa barrier and blood-brain barrier dysfunction (Zhang et al., 2017). It has been shown that LPS-induced changes in *Firmicutes* commensals and depletion *Proteobacteria* opportunistic organisms were reversed to control levels by FMT in male rats, and LPS mice treated with FMT showed better spatial memory in

TABLE 3 | Gut microbiota and hippocampus-dependent memory.

Treatment	Gut microbiota	Behavior	References
Lactobacillus and *Bifidobacteria* mixture	*Lactobacillus acidophilus CUL60*, *L. acidophilus CUL21*, *Bifidobacterium bifidum CUL20* and *B. lactis CUL34*	spatial navigation, long-term object recognition memory	(O'Hagan et al., 2017)
oral administration	*Bifidobacterium breve strain A1*	Y maze, passive avoidance	(Kobayashi et al., 2017)
	Clostridium butyricum	spatial learning and memory	(Liu et al., 2015)
tuna oil	microbiota diversity	memory	(Zhang et al., 2018)
LW-AFC	operational taxonomic units (*Bacteroidales*, *Clostridiales*, *Desulfovibrionales* and *CW040*)	spatial learning and memory, active avoidance, object recognition memory	(Wang et al., 2016)
Tetragonia tetragonioides Kuntze extract	decrease in *Clostridiales*, *Erysipelotrichales*, and *Desulfovibrionales* but increase in *Lactobacilales* and *Bacteroidales*	short-term and special memory	(Kim et al., 2020)
Tibetan fermented milk	*Bacteroides*, *Faecalibacterium* spp. Mucispirillum, *Ruminiclostridium;* *Muribaculum*, *Erysipelatoclostridium*	cognitive function	(Liu et al., 2020)

LW-AFC: an herbal medicine prepared from traditional Chinese medicine from LiuweiDihuang decoction.

behavioral tests (Li et al., 2018). A recent study by Mohammadi et al. showed that a probiotic formulation (*Lactobacillus helveticus R0052* and *Bifidobacterium longum R0175*) reversed LPS-induced elevation of both the circulating and hippocampal levels of proinflammatory cytokines, and attenuate the effect of LPS on memory (Mohammadi et al., 2019). Furthermore, LPS were shown to drive an NF-kB-miRNA-mediated deficiency in gene expression that contributes to alterations in synaptic architecture, synaptic deficits, amyloidogenesis, innate immune defects, and progressive inflammatory signaling, all of which are characteristics of AD-type neurodegeneration (Zhao and Lukiw, 2018).

Many factors are involved in the pathogenic gut microbiota-related systemic inflammation, due to increased LPS and proinflammatory cytokines, barrier dysfunction, and dysfunctional vago-vagal gut-brain axis (Daulatzai, 2014). The colitis mice showed impaired memory, increased fecal and blood levels of LPS, an increase in *Enterobacteriaceae*, but a decrease in *Lactobacillus johnsonii*. These changes in behaviors and LPS production could be induced by treatment with *E. coli* isolated from the feces of colitis mice accompanied with NF-κB activation and TNF-α expression as well as suppressed BDNF expression in the hippocampus of mice. However, all these changes could be reversed by treatment with *Lactobacillus johnsonii* (Jang et al., 2018). This was further demonstrated by oral administration of *Lactobacillus brevis OW38* to aged mice showing reduced LPS levels in colon fluid and blood and reduced ratio of *Firmicutes* to *Bacteroidetes* or *Proteobacteria* to *Bacteroidetes*, which was significantly higher in aged mice than in young mice. Treatment with OW38 in aged mice inhibited the expression of inflammatory markers (such as TNF and IL-1β) and NF-κB activation, and suppressed the expression of senescence markers (p16, p53, and SAMHD1) in the hippocampus of aged mice (Jeong et al., 2016). These results strongly demonstrated that gut microbiota disturbance could induce hippocampal inflammation and memory impairment. Moreover, it has been reported that when FMT is conducted, young recipient rats show impairment in cognitive behavior but an increase in expression of proinflammatory AGEs and their receptor, accompanied by changes in gut microbiota composition (Li et al., 2020). Specifically, *Lactobacillus plantarum* decreased the expression of hippocampal TLR4 (Mohammed et al., 2020).

Taken together, the alterations in the gut microbiota may change the inflammatory status in the hippocampus and hippocampus-dependent behaviors, which could be improved by probiotics, microbiota transplantation, or diet management. These results are summarized in **Figure 3** and **Table 4**.

GUT MICROBIOTA AND HIPPOCAMPAL ALZHEIMER'S DISEASE PATHOLOGIES

Human microbiota may strongly influence the pathology of AD, the deposition of Aβ, and formation of neurofibrillary tangles in the hippocampus (Kohler et al., 2016). The effects of aging and the risk of neurodegenerative diseases can be reduced by probiotics, or by combining probiotics and prebiotics known as synbiotics, which can significantly modify the composition of the gut microbiome (Lye et al., 2018). Long-term (6 months) antibiotic treatment of 2-week-old AD mice induced shifts in gut microbial composition and diversity, a decrease in Aβ plaque deposition, but an increase in soluble Aβ in the brain of AD mice, suggesting that gut microbiota diversity could regulate host innate immunity mechanisms that are related to Aβ amyloidosis (Minter et al., 2016). Moreover, early postnatal (days 14–21) antibiotic treatment resulted in long-term alterations in gut microbial genera (predominantly *Lachnospiraceae* and *S24-7*) and reduced brain Aβ deposition in aged AD mice, accompanied by reduced plaque-localized microglia and astrocytes (Minter et al., 2017). A recent study showed that when 3xTg-AD mice in the early stage of AD were treated with the SLAB51 probiotic formulation, the gut microbiota and their metabolites changed significantly, and the impaired neuronal proteolytic pathways (the ubiquitin proteasome system and autophagy) were partially recovered. Cognitive function improved and the accumulation of Aβ aggregates was reduced (Bonfili et al., 2017).

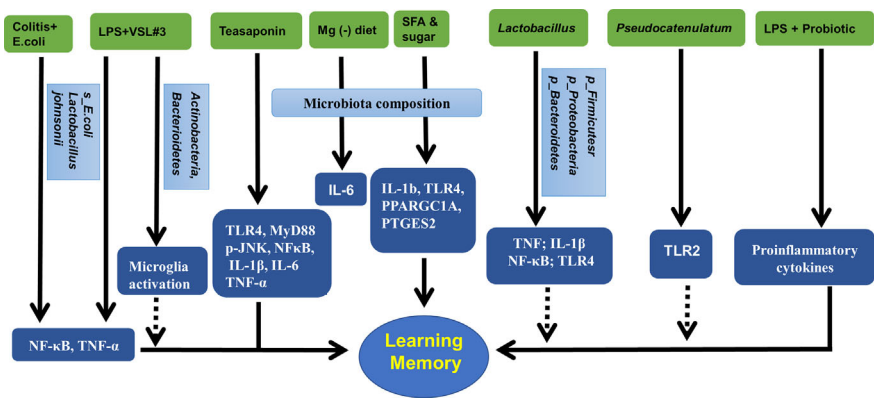

FIGURE 3 | Gut microbiota, hippocampal inflammatory targets, and memory. Mg (-), magnesium deficient diet; SFA, saturated fatty acid; VSL3#, a probiotic mixture comprising 8 Gram-positive bacterial strains; LPS, lipopolysaccharide.

TABLE 4 | Gut microbiota and hippocampal inflammatory target.

Treatment	Gut microbiota	Hippocampal inflammatory target	Behavior	References
Pseudocatenulatum		TLR2		(Agusti et al., 2018)
magnesium deficient diet	microbiota composition	IL-6		(Winther et al., 2015)
saturated fatty acid and sugar	microbiota PC2 and PC3	IL-1b, TLR4, PPARGC1A, PTGES2	place memory	(Beilharz et al., 2016)
teasaponin	gut microbiota	TLR4, MyD88, p-JNK, NFκB, IL-1β, IL-6, TNF-α	recognition memory	(Wang et al., 2017)
VSL#3	microbiota composition (increase in *Actinobacteria* and *Bacteroidetes*)	CD68, CD11b, microglia activation		(Distrutti et al., 2014)
LPS and FMT	*Firmicutes phylum, Proteobacteria phylum*		spatial memory	(Li et al., 2018)
LPS and probiotic formulation	*Lactobacillus helveticus R0052*, *Bifidobacterium longum R0175*)	proinflammatory cytokines	memory	(Mohammadi et al., 2019)
LPS		NF-κB, microRNA		(Zhao and Lukiw, 2018)
Colitis, *E.coli* and *Lactobacillus johnsonii*	*Enterobacteriaceae, Lactobacillus johnsonii*	NF-κB, TNF-α, LPS	memory behavior	(Jang et al., 2018)
Lactobacillus brevis OW38	*Firmicutes* or *Proteobacteria* to *Bacteroidetes* ratio	TNF, IL-1β, NF-κB; LPS		(Jeong et al., 2016)
FMT	microbiota composition	pro-inflammatory AGEs and their receptor	cognitive behavior	(Li et al., 2020)
Lactobacillus Plantarum		TLR4		(Mohammed et al., 2020)

PC, microbiota principal component; FMT, fecal microbiota transplantation; LPS, lipopolysaccharide; VSL#3, a probiotic mixture comprising 8 Gram-positive bacterial strains.

In APP/PS1 mice, quercetin treatment increased gut microbial diversity and relative abundance of *Glutamicibacter*, *Facklamia*, and *Aerocorrus*; it also improved learning and memory in the Morris water maze test. Hippocampal BDNF levels were increased but Aβ plaques and p-Tau decreased; further analysis revealed that hippocampal p-Tau at ser396 was negatively correlated with *Aerococcus*, but p-Tau at ser404 was negatively correlated with *Facklamia* (Lv et al., 2018). Curcumin has also been shown to improve spatial learning and memory abilities and reduce Aβ plaque in the hippocampus of APP/PS1 mice. These changes may be related to the altered abundance of *Bacteroidaceae*, *Prevotellaceae*, *Lactobacillaceae*, and *Rikenellaceae* at the family level, and *Prevotella*, *Bacteroides*, and *Parabacteroides* at the genus level (Sun et al., 2020). Additionally, as mentioned above, the administration of TTK extract and Tibetan fermented milk also improved memory loss and reduced the deposition of hippocampal Aβ that involved changes in gut *Clostridiales*, *Erysipelotrichales*, *Desulfovibrionales*, *Lactobacillales*, *Bacteroides*, *Faecalibacterium* spp. Mucispirillum, and *Ruminiclostridium* (Kim et al., 2020; Liu et al., 2020). Additionally, mice treated with a ketogenic diet for 16 weeks showed significantly increased abundance of putatively beneficial gut microbiota (*Akkermansia muciniphila* and *Lactobacillus*), and reduced putatively proinflammatory taxa (*Desulfovibrio* and *Turicibacter*). These changes facilitated the clearance of Aβ, and reduced the risk of AD (Ma et al., 2018). Moreover, oral administration of grape seed polyphenol extract (GSPE) resulted in an increase in two phenolic acids, 3-hydroxybenzoic acid and 3-(3-hydroxyphenyl) propionic acid in rats. This treatment also interfered with the assembly of Aβ peptides into senile plaques, suggesting an important contribution of the intestinal microbiota to the protective activities of GSPE in AD (Wang et al., 2015). In a population-based cross-sectional cohort study, a very intriguing discovery was that the Mediterranean diet, which contains an unusually large quantity of *Lactobacilli*, seemed very effective in preventing AD (Jin et al., 2018). Furthermore, it has been reported that in APP/PS1mice, prebiotic fructooligosaccharide (FOS) treatment altered microbial composition, ameliorated cognitive deficits and AD pathological changes, and upregulated the expression levels of hippocampal synaptic proteins (Sun et al., 2019). Similar results were also detected in other species. When AD rats were treated with FOS from *Morinda officinalis*, the learning and memory abilities were significantly ameliorated, accompanied with maintenance of the diversity and stability of the gut microbial community (Chen et al., 2017). Interestingly, a recent study revealed that gut microbiota diversity and composition might also mediate the effects of chronic noise exposure on cognitive impairment and hippocampal Aβ deposition, and microbiota transplantation demonstrated that the host impairment of epithelial integrity and AD-like changes were driven by the noise exposure-altered microbiota (Cui et al., 2018).

Taken together, as reviewed by Sun et al. (Sun et al., 2020), the composition and diversity of gut microbiota may be regulated in many ways, such as antibiotics, probiotics, diet, plant extracts, and microbiota transplantation. These treatments were also shown to be deeply involved in AD pathology, especially the formation and deposition of Aβ, and behaviors. These results are summarized in **Figure 4** and **Table 5**.

CLINICAL APPLICATIONS OF PROBIOTICS AND ANTIBIOTIC ON BRAIN COGNITIVE FUNCTION

Limited clinical trials have addressed the effects of probiotics on brain function, including memory, depression, and stress. Steenbergen et al. reported that multispecies probiotic intervention could reduce negative thoughts associated with a sad mood in healthy volunteers (Steenbergen et al., 2015). Later, probiotic administration was shown to alter brain activities

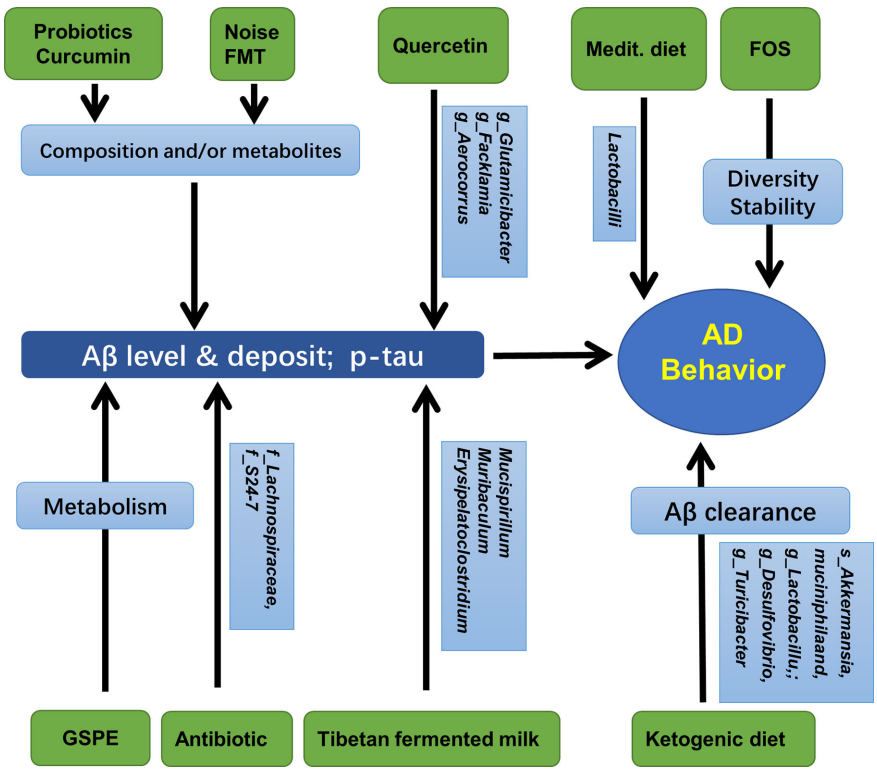

FIGURE 4 | Gut microbiota, hippocampal AD pathology, and AD behaviors. Medit, Mediterranean diet; GSPE, Grape seed polyphenol extract; FOS, fructooligosaccharides; FMT, fecal microbiota transplantation.

TABLE 5 | Gut microbiota affects AD pathology and behaviors.

Treatment	Gut microbiota	Hippocampal target	Pathology/Behavior	References
antibiotic	composition and diversity	microglia, Aβ		(Minter et al., 2016)
antibiotic	*Lachnospiraceae* and *S24-7*	microglia, astrocyte, Aβ		(Minter et al., 2017)
SLAB51 probiotic formulation	Composition and metabolites	Aβ deposit, ubiquitin proteasome system and autophagy	cognition (open field, novel object recognition, passive avoidance, elevated plus maze)	(Bonfili et al., 2017)
quercetin	*Glutamicibacter*, *Facklamia Aerocorrus*	BDNF, Aβ deposit, p-tau	learning and memory	(Lv et al., 2018)
curcumin	*Bacteroidaceae*, *Prevotellaceae*, *Lactobacillaceae*, *Rikenellaceae*	Aβ deposit	spatial learning and memory	(Sun et al., 2020)
TTK extract and Tibetan fermented milk	*Clostridiales*, *Erysipelotrichales*, *Desulfovibrionales*, *Lactobacilales, Bacteroides*, *Faecalibacterium* spp. Mucispirillum, *Ruminiclostridium*	Aβ deposit	spatial learning and memory	(Kim et al., 2020) (Liu et al., 2020) (Kim et al., 2020; Liu et al., 2020)
ketogenic diet	*Akkermansia* muciniphila, *Lactobacillus*; *Desulfovibrio*, *Turicibacter*	Aβ clearance		(Ma et al., 2018)
Mediterranean diet	*Lactobacilli*		AD -preventing	(Jin et al., 2018)
grape seed polyphenol extract	microbiota metabolism	Aβ deposit		(Wang et al., 2015)
noise/microbiota transplantation	composition and diversity	Aβ deposit	learning and memory	(Cui et al., 2018)
FOS	microbial composition	AD pathology, synaptic plasticity	cognition (open field, Morris water maze, object recognition)	(Sun et al., 2019)
FOS	diversity and stability		learning and memory	(Chen et al., 2017)

SLAB51, a formulation made of nine live bacterial strains [Streptococcus thermophilus, bifidobacteria (B. longum, B. breve, B. infantis), lactobacilli (L. acidophilus, L plantarum, L. paracasei, L. delbrueckii subsp. bulgaricus, L. brevis)]; TTK, Tetragonia tetragonioides Kuntze; FOS, prebiotic fructooligosaccharides.

related to emotional memory, decision-making tasks, anxiety, negative affect, and worry, which were also accompanied by subtle shifts in the gut microbiome profile (Bagga et al., 2018; Tran et al., 2019). *Bifidobacterium longum 1714*™ also modulated the resting neural activity in several brain regions including the hippocampus, fusiform, and temporal cortex, which correlated with enhanced vitality and reduced mental fatigue in healthy volunteers during social stress (Wang et al., 2019). Inoue et al. reported that probiotic *bifidobacteria* supplementation showed stronger effects on the improvement of mental condition compared to moderate resistance training (Inoue et al., 2018).

Probiotics have also been shown to be effective in patients with cognitive disorders. In patients with mild cognitive impairment, treatment with *Lactobacillus plantarum C29*-fermented soybean supplement (DW2009) resulted in significant improvement in cognitive function (Hwang et al., 2019). For major depressive patients, probiotics alone or in combination with antidepressants are effective and well tolerated (Miyaoka et al., 2018; Chahwan et al., 2019). Similarly, probiotic *Lactobacillus plantarum 299v* decreased kynurenine concentration and improved cognitive functions in patients with major depression (Rudzki et al., 2019). The probiotic *Lactobacillus plantarum P8* gender-dependently alleviated stress and enhanced memory and cognition, such as social emotional cognition, and verbal learning and memory (Lew et al., 2019).

In peripheral disorders, probiotics and antibiotics may affect brain function through regulation of microbiota. Probiotic *Bifidobacterium Longum NCC3001* administration has also been shown to reduce depression and alter brain activity in patients with irritable bowel syndrome (Pinto-Sanchez et al., 2017), improve impulsivity and decision-making in patients with fibromyalgia (Roman et al., 2018), and neurocognitive functions in human immunodeficiency virus transfected patients (Ceccarelli et al., 2017). Rifaximin is a gut-specific antibiotic. Several clinical trials demonstrated that in patients with minimal hepatic encephalopathy (MHE), rifaximin induced a significant improvement in cognition, including working memory that involved *Enterobacteriaceae*, *Porphyromonadaceae*, and *Bacteroidaceae*, endotoxemia, and several serum fatty acids. This treatment also decreased *Veillonellaceae* and increased *Eubacteriaceae*, inducing a shift from pathogenic to beneficial metabolite linkages (Bajaj et al., 2013; Ahluwalia et al., 2014). Additionally, in patients with MHE, oral capsular FMT (enriched in *Lachnospiraceae* and *Ruminococcaceae*) improved cognition. Inflammation was positively correlated with greater complexity of beneficial taxa, such as *Ruminococcaceae*, *Verrucomicrobiaceae*, and *Lachnospiraceae*; increased duodenal mucosal diversity with higher *Ruminococcaceae* and *Bifidobacteriaceae;* and lower *Streptococcaceae* and *Veillonellaceae*,

TABLE 6 | Clinical trials on gut microbiota and hippocampus-dependent behaviors.

Treatment	Gut microbiota	Pathology/Behavior	References
multispecies probiotics[1]		sad mood-related negative thoughts	(Steenbergen et al., 2015)
probiotic administration (Ecologic®825, etc.)	diversity and composition (*Bacteroides etc.*)	emotional memory, decision-making tasks, anxiety, negative affect and worry	(Bagga et al., 2018; Tran et al., 2019)
Bifidobacterium longum 1714™		social stress	(Wang et al., 2019)
bifidobacteria supplementation[2]		mental condition	(Inoue et al., 2018)
DW2009	*lactobacilli* population	cognitive functions	(Hwang et al., 2019)
Probiotics[3]		major depressive disorder	(Miyaoka et al., 2018; Chahwan et al., 2019)
Lactobacillus Plantarum 299v		major depression-related cognitive functions	(Rudzki et al., 2019)
Lactobacillus plantarum P8		stress, memory, and cognition (social emotional cognition and verbal learning and memory)	(Lew et al., 2019)
Bifidobacterium Longum NCC3001		depression and brain activity, impulsivity, and decision-making	(Ceccarelli et al., 2017; Pinto-Sanchez et al., 2017; Roman et al., 2018)
Rifaximin	*Enterobacteriaceae*, *Porphyromonadaceae*, *Bacteroidaceae*, *Veillonellaceae*, *Eubacteriaceae*	working memory	(Bajaj et al., 2013; Ahluwalia et al., 2014)
FMT (enrich in Lachnospiraceae and Ruminococcaceae)	*Ruminococcaceae*, *Verrucomicrobiaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Bifidobacteriaceae*, *Streptococcaceae*, *Veillonellaceae*	cognition and inflammation	(Bajaj et al., 2019a; Bajaj et al., 2019b).

1: Bifidobacterium bifidum W23, Bifidobacterium lactis W52, Lactobacillus acidophilus W37, Lactobacillus brevis W63, L. casei W56, Lactobacillus salivarius W24, Lactococcus lactis (W19 and W58)

2: B. longum BB536, B. infantis M-63, B. breve M-16V and B.breve B-3;

3: Clostridium butyricum MIYAIRI 588; Bifidobacterium bifidum W23, Bifidobacterium lactis W51, Bifidobacterium lactis W52, L. acidophilus W37, Lactobacillus brevis W63, Lactobacillus casei W56, Lactobacillus salivarius W24, Lactococcus lactis W19 and Lactococcus lactis W58;

Ecologic®825 contains nine bacterial strains: Lactobacillus casei W56, Lactobacillus acidophilus W22, Lactobacillus paracasei W20, Bifidobacterium lactis W51, Lactobacillus salivarius W24, Lactococcus lactis W19, Bifidobacterium lactis W52, Lactobacillus plantarum W62, and Bifidobacterium bifidum W23.

DW2009, Lactobacillus plantarum C29-fermented soybean supplement; FMT, fecal microbiota transplantation.

indicating the beneficial effects of capsular FMT on inflammation and cognition in patients with cirrhosis (Bajaj et al., 2019a; Bajaj et al., 2019). The above results were summarized in **Table 6**.

CONCLUSIONS

The gut microbiota is regarded as the second genome of the human body. Its composition and diversity changes frequently under different conditions. The hippocampus is the center for learning and memory, which is closely related to dementia and many other mental disorders. In this manuscript, we reviewed recent findings on the relationship between intestinal microbes and the plasticity, neurochemicals, and function of the hippocampus. We highlighted the advances in modulating hippocampal structure and behavior using probiotics, prebiotics, and diet through the gut microbiota-hippocampus axis, as summarized in **Figure 5**.

Evidence indicates that the gut microbiota is altered in AD. Therefore, modifying the gut microbiota may affect this disease (Agahi et al., 2018). An abundance of "good bacteria" such as *Bifidobacterium* or their products have generally been believed to be beneficial, while "bad bacteria" such as *Clostridium* are assumed to be detrimental (Park et al., 2017). *Escherichia coli* and *Salmonella enterica* are among the many bacterial strains that express and secrete Aβ and contribute to AD pathogenesis (Tse, 2017). Clinical studies have shown that, in cognitively impaired elderly patients with brain amyloidosis, the anti-inflammatory species *Eubacterium rectale* and *Bacteroides fragilis* were more abundant, while proinflammatory genera such as *Escherichia/Shigella* were higher. Supplementation with *Lactobacilli*- and *Bifidobacteria*-based probiotics was neuroprotective in AD subjects (Mancuso and Santangelo, 2018). However, the results of current studies are controversial. For example, Vogt et al. reported an increase in the abundance of *Bacteroidaceae*, *Rikenellaceae*, and *Gemellaceae*, but a decrease in that of *Ruminococcaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Mogibacteriaceae*, *Turicibacteraceae*, and *Peptostreptococcaceae* in AD patients when compared with the controls (Vogt et al., 2017); Zhuang et al. reported an increase in the abundance of *Ruminococcaceae*, *Enterococcaceae*, and *Lactobacillaceae*, but a decrease in that of *Lanchnospiraceae*, *Bacteroidaceae*, and *Veillonellaceae* compared with the control group (Zhuang et al., 2018).

The exact trigger of AD remains unknown. Current treatments for AD are limited, and great efforts have been made to target Aβ plaques, but these attempts have often ended in failure (Reiss et al., 2018; Salminen et al., 2018). Recent progress in the effects of gut microbiota on hippocampus-dependent learning and memory have opened a new window for understanding the onset and progression of AD. Thus, modulation of the gut microbiota has been regarded as a preventive and therapeutic target against this worldwide challenge. However, how the gut microbiota affects the structure and function of the hippocampus is far from clear. It has been shown that bacterial metabolites, such as LPS and Aβ, may act through the vagus nerve pathway, the systemic pathway (with the release of hormones, metabolites, and neurotransmitters), and the immune pathway (by the action of cytokines) to increase the permeability of the mucosa-intestinal barrier and blood-brain barrier, induce

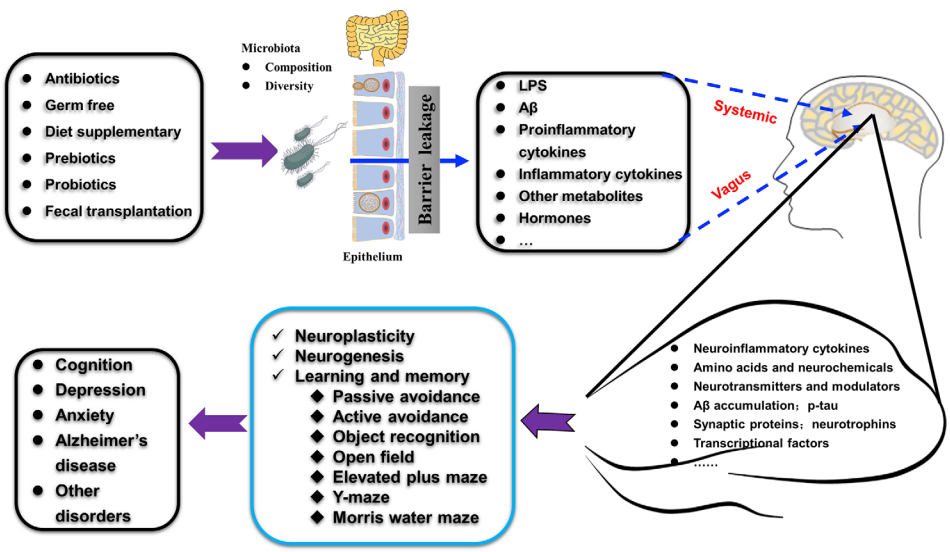

FIGURE 5 | Schematic illustration shows how gut microbiota affect hippocampal plasticity and behaviors through gut-hippocampus-axis. The composition and diversity, the levels of LPS and Aβ, proinflammatory and inflammatory factors, and other metabolites (such as hormones) of gut microbiota could be affected by many treatments (antibiotics, germ free administration, diet, pre- and pro-biotics, and fecal transplantation). The alterations occurred in the gut microbiota affect many aspects of the hippocampus (such as amino acids, expression of specific proteins, AD-related pathologies) through the vagus nerve pathway, the systemic pathway (with the release of hormones, metabolites, cytokines, and neurotransmitters) to increase the permeability of mucosa-intestinal barrier and blood-brain barrier and finally regulate hippocampus-dependent cognition and behaviors.

hippocampal inflammation, and ultimately affect hippocampus-dependent functions (Bostanciklioglu, 2019; Garcez et al., 2019; Gomez-Eguilaz et al., 2019). All these still require further experimental evidence, and we also lack human observational or interventional data to propose any clinical recommendations.

AUTHOR CONTRIBUTIONS

YY and DC conceived this article. WT and ZM performed the literature search, data analysis, and draft preparation. YY and DC critically revised the manuscript. NL, YL, and LL helped in the data analysis and draft preparation. All authors contributed to the article and approved the submitted version.

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Beneficial Effect of Alkaloids From *Sophora alopecuroides* L. on CUMS-Induced Depression Model Mice *via* Modulating Gut Microbiota

Ming Zhang[1], Aoqiang Li[2], Qifang Yang[1], Jingyi Li[1], Lihua Wang[1], Xiuxian Liu[3], Yanxin Huang[1] and Lei Liu[1]**

[1] National Engineering Laboratory for Druggable Gene and Protein Screening, Northeast Normal University, Changchun, China, [2] Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal University, Changchun, China, [3] School of Rehabilitation Science, Shanghai University of Traditional Chinese Medicine, Shanghai, China

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AgResearch Ltd, New Zealand

***Correspondence:**
Lei Liu
liul905@nenu.edu.cn
Yanxin Huang
huangyx356@nenu.edu.cn

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It was recently shown that the gut microbiota of both depression patients and depression model animals is significantly altered, suggesting that gut microbes are closely related to depression. Here, we investigated the effects of *Sophora alopecuroides* L.-derived alkaloids on the gut microbiota of mice with depression-like behaviors. We first established a mouse model of depression *via* chronic unpredictable mild stress (CUMS) and detected changes in depression-like behaviors and depression-related indicators. Simultaneously, 16S rRNA sequencing was performed to investigate gut microbiota changes. *Sophora alopecuroides* L.-derived alkaloids improved depression-like behaviors and depression-related indicators in mice. The alkaloids decreased the gut microbiota diversity of CUMS mice and depleted intestinal differentially abundant "harmful" microbiota genera. Spearman analysis showed that there is a certain correlation between the differential microbiota (*Lactobacillus*, *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, *Ruminococcus*), depression-like behaviors, and depression-related indicators. Combined with the predictive analysis of gut microbiota function, these results indicate that alkaloids improve depression in mice through modulating gut microbiota.

Keywords: depression, gut microbiota, function prediction, *Sophora alopecuroides* L., alkaloids, CUMS

INTRODUCTION

Depression is a common mental and emotional disorder that has become a major contributor to the global burden of disease (Yamagata et al., 2017). According to statistics from the World Health Organization in 2015, the incidence rate of depression exceeded 18%, and the disability rate was higher than that for any other disease (Winter et al., 2018). Depression is characterized by disorders of the neurotransmitter, neuroimmunity, neuroendocrine and metabolic systems; however, the pathogenesis of depression is still unclear (Zhang et al., 2015; Ma et al., 2016; Song et al., 2019).

Studies have shown that there is a complex network relationship between the brain and the gut microbiota (Bienenstock and Collins, 2010). The gut microbiota can have an important impact on

the host's stress response, depression, and cognitive function through the gut-brain axis, which is closely related to depression (Mayer, 2011). Clinical studies have found that the composition of the gut microbiota in patients with depression is altered compared with that of healthy people, and the diversity and abundance of the gut microbiota of patients have decreased significantly (Jiang et al., 2015; Aizawa et al., 2016; Liu et al., 2016). Gut microbiota disorders increase the susceptibility to depression (Frei et al., 2012; Bercik and Collins, 2014; Oriach et al., 2016; Köhler et al., 2017; Owen and Corfe, 2017). Improvement in the gut microbiota can relieve depression-like symptoms, while probiotic treatment can significantly reduce patients' depression and anxiety symptoms (Akkasheh et al., 2016; Pirbaglou et al., 2016; Abildgaard et al., 2017; Gibson et al., 2017). Traditional antidepressants, such as trans-cyclopropylamine and imipramine, have been found to have antibacterial effects while improving depression (Macedo et al., 2017). A new therapy consisting of a selective serotonin reuptake inhibitor (SSRI) combined with probiotics to improve intestinal microbes has a good antidepressant effect. These studies have confirmed that the treatment of depression can be achieved by regulation of the gut-brain axis (Schnorr and Bachner, 2016; Bambling et al., 2017).

Sophora alopecuroides L. (*S. alopecuroides*) is widely distributed in Xinjiang. Its main ingredient is alkaloids, which have antitumor, antibacterial, anti-inflammatory, and other biological activities (Zhang et al., 2017). Previous studies have shown that alkaloids from other plants have good antidepressant effects (Coetzee et al., 2016; Hamid et al., 2017; Jia et al., 2017; Wu et al., 2018; Arias et al., 2019; Chen et al., 2019; Khan et al., 2019), while the effect of alkaloids from *S. alopecuroides* on depression and gut microbes has not been investigated. In the current study, we used chronic unpredictable mild stress (CUMS) to establish a depression mouse model and assess the changes in depression-like behaviors and depression-related indicators to evaluate the beneficial effect of alkaloids on CUMS depression-like mice. We also used amplicon sequencing to study how the composition and structure of the gut microbial community in depression-like mice changed between different treatment conditions. The correlation between gut microbes and depression-related indicators was further analyzed. In this study, we aimed to provide experimental evidence for further research on how alkaloids from *S. alopecuroides* can improve depression in mice by regulating gut microbiota.

MATERIALS AND METHODS

Preparation and Analysis of Total Alkaloids

The seeds of *S. alopecuroides* were collected in October 2016 from Xinjiang Altai, China. The plant species was identified by the Xinjiang Food and Drug Administration. A voucher specimen (sa-201610-003) was deposited in the National Engineering Laboratory for Druggable Gene and Protein Screening, Northeast Normal University, Changchun. The specific process of extracting total alkaloids was as follows: briefly, the air-dried samples (10 kg) were ground into 60 mesh powder, ultrasonically extracted with 75% ethanol (5 × 2 h) at 30°C, and evaporated using a rotary evaporator (SENCO Technology Co., Ltd, China) to remove the ethanol. The ethanol extract was dissolved in 0.5% dilute hydrochloric acid solution, passed through a strong acid cation exchange resin column for full exchange, and then washed with water to neutrality. The resin was removed and dried and then wetted with 14% ammonia water. Finally, it was extracted with 95% ethanol, and the total alkaloids content was obtained after rotary evaporation. Identification of total alkaloids was performed by HPLC-MS (Prominence LC-20A, Japan) by using an SPD 20AV HPLC DAD coupled to an API 2000 quadrupole-MS (SCIEX, USA). Chromatographic separation was performed on a ZORBAX SB-C18 (150×2.1 mm, 5 μm) column (Agilent Technologies, USA). The flow rate was 1.0 mL/min, and mass spectra were recorded in positive ionization.

Animals and Groups

Male ICR mice (20-25 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Changchun, China), housed with a constant temperature (21 ± 1°C) and humidity (52 ± 2%) on a 12 h day/night cycle in a laboratory room, and given a standard diet and water ad libitum. After the mice had adapted to the new environment for one week, their body weight and sucrose preference were measured to remove outliers. The mice were divided into four groups (n = 10): the control group (Con, not exposed to CUMS), CUMS group, CUMS + imipramine group (CUMS + IMI) and CUMS + alkaloids group (CUMS + ALK). All procedures were performed in accordance with the guidelines for the National Institutes of Health guide for the care of laboratory animals and were approved by the Experimental Animal Care and Use Committee at Northeast Normal University. The specific experimental design is shown in **Figure 1**.

Chronic Unpredictable Mild Stress

The CUMS procedure was performed according to the literature with slight modifications (Huang et al., 2018; Liu et al., 2019). Mice were isolated and subjected to two different stressors every day, and the same stressors were not scheduled for two consecutive days to ensure that the animals could not predict the occurrence of the stress. These stressors were applied for seven weeks, and the details are shown in **Table 1**.

Treatments

The drug treatments began from the 4th week during the CUMS procedure and lasted four weeks. The four groups of mice were treated as follows: Con (0.9% saline), CUMS (0.9% saline), CUMS + IMI (30 mg/kg imipramine), and CUMS + ALK (30 mg/kg alkaloids). Except for the intraperitoneal injection of the imipramine group, all other groups underwent intragastric administration. Imipramine hydrochloride is a listed drug for treating depression and was used as a positive control in this experiment. Imipramine hydrochloride and alkaloids were

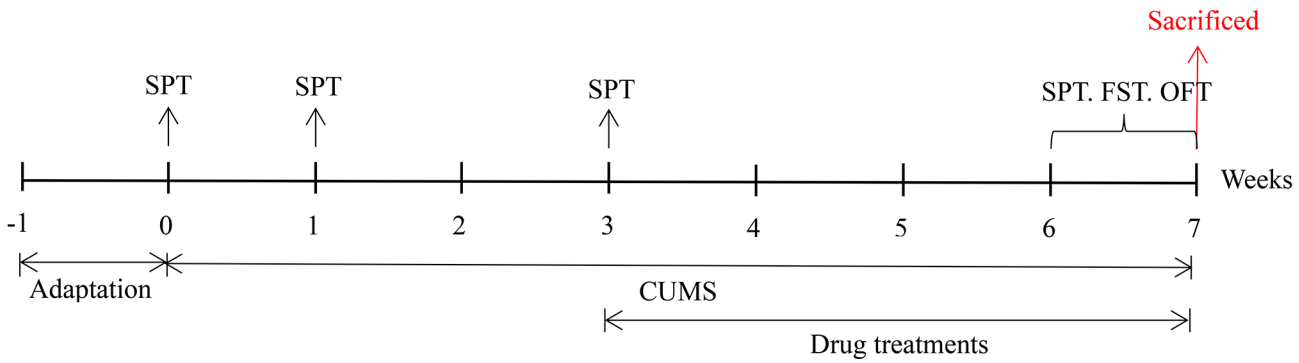

FIGURE 1 | The specific experimental design and implementation. SPT, sucrose preference test; FST, forced swimming test; OFT, open field test.

dissolved in 0.9% saline at a stock concentration of 3 mg/ml. Drug treatments were performed at the same time every day (9:00 a.m.-11:00 a.m.).

Sucrose Preference Test

A sucrose preference test (SPT) procedure was performed as described in the literature, with slight modifications (Liu et al., 2018). The SPT was performed at four time points: before CUMS (adaptation period, Monday to Thursday), before drug treatment (Monday to Thursday in the first and third weeks), and before the animals were sacrificed (Monday to Thursday in week 7). Mice were housed in single cages during the test. At the start of the test, mice were trained for 48 h to adapt to a 1% sucrose solution (w/v) and avoid the bottle place preference: two bottles of 1% sucrose solution were placed in each cage for 24 h, one of the bottles of 1% sucrose solution was replaced with a bottle of pure water for 12 h, and the positions of the two bottles were then exchanged for 12 h. Finally, the mice were deprived of food and water for 24 h. The SPT was then performed, with two bottles randomly placed, one bottle containing a sucrose solution and the other bottle containing pure water. The mice drank freely for 2 h, and the bottles were weighed before and after placement. The sucrose preference was calculated as a percentage of the consumed sucrose solution relative to the total amount of liquid consumed.

Forced Swimming Test

The forced swimming test (FST) has been used to evaluate the antidepressant effect of drugs. The shorter the immobility time is, the stronger the antidepressant effect of the drug (Sun et al., 2018). The FST was performed before the animals were sacrificed (Friday of week 7). The experimental device used for forced swimming was a cylindrical plastic container (25 cm height × 10 cm diameters) containing 25 ± 1 °C water, and the depth of the water was 20 cm. Each mouse was individually placed in the above device in a quiet environment and forced to swim for 6 min, which was recorded through a digital camera. After swimming, the mice were dried and quickly returned to their home cage. The device was washed with clean water before each FST. Throughout the entire swimming test, the first 2 min were the pre-swimming phase, and the remaining 4 min were used to calculate the immobility time. The time during which the mice gave up struggling to float on the water surface or only slightly swing their limbs to prevent immersion in the water was counted as immobility time.

Open Field Test

The open field test (OFT) has been widely used to study the behaviors associated with neuropsychological changes in experimental animals, and it can be used to evaluate the effects of antidepressant treatment (Shyong et al., 2017). The OFT was

TABLE 1 | The schedule of CUMS stressors.

Weeks	Days						
	Mon.	**Tue.**	**Wed.**	**Thur.**	**Fri.**	**Sat.**	**Sun.**
Week 1	E+C	D+G	N	L+B	M+J	A+F	K+H
Week 2	K+G	D+C	N	E+A	H+B	L+M	F+J
Week 3	E+K	M+J	N	A+I	F+C	L+G	H+N
Week 4	H+J	E+D	N	F+L	K+C	A+I	M+G
Week 5	M+G	H+C	N	A+L	F+B	E+J	K+D
Week 6	D+C	E+G	N	I+B	K+F	A+J	H+M
Week 7	E+G	D+J	N	A+L	F+K	B+C	M+H

A-food deprivation for 12 h; B-water deprivation for 12 h; C-lights on at night for 12 h; D-empty cage for 12 h; E-wet bedding for 12 h; F-confinement in a tube for 2 h; G-traffic noise (70-90 dB) for 2 h; H-tail suspension for 0.5 h; I-cage tilting for 12 h (45°); J-exposure to a stroboscope for 12 h; K-foreign body stimulation for 2 h; L-crowding for 12 h (ten mice within one cage); M-level shaking for 15 min; N-food and water deprivation for 24 h.

performed before the animals were sacrificed (Sunday of week 7). The device used was a black open box (30 cm×30 cm×30 cm), and the bottom of the apparatus was divided into 16 equally sized squares. Each mouse was placed in the center of the device in a quiet room for 6 min, and the first 1 min was the adaptation period. We used a digital camera to record the activities of the next 5 min, including the numbers of crossings in the horizontal direction (entry onto the square with all 4 limbs was counted as 1 time), numbers of rearings (the mouse's forelimbs were counted once when they left the bottom surface), and the numbers of modifications (modifying their heads or mouth with their forelimbs was counted as 1). After each test, the excrement in the box was cleaned, and the box was wiped with an alcohol pad to ensure that there was no smell or dirt from the test mouse.

Sample Collection

After the mice were decapitated, blood was collected immediately with a blood collection tube and then centrifuged at 3000 rpm for 10 min at 4°C to separate serum and blood cells. The hippocampus and prefrontal cortex were rapidly separated on ice plates, frozen immediately in liquid nitrogen and then stored in a refrigerator at −80°C. The cecum was rapidly removed for collection of intestinal contents (a set of sterile forceps and scissors was used for each mouse to avoid cross-contamination) and frozen in liquid nitrogen until DNA extraction.

Enzyme-Linked Immunosorbent Assay

The brain-derived neurotrophic factor (BDNF) protein concentration in the hippocampus and prefrontal cortex and the concentrations of serotonin (5-HT), norepinephrine (NE) and dopamine (DA) in serum were measured using the corresponding commercial enzyme-linked immunosorbent assay (ELISA) kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's instructions.

DNA Extraction

The DNA of intestinal content samples was extracted using a DNeasy PowerWater Kit (QIAGEN, Inc., Netherlands) following the manufacturer's instructions and stored at -20°C for further analysis. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis were used to measure the quantity and quality of DNA samples.

16S rRNA Gene Amplicon Sequencing

The V3–V4 region of bacterial 16S rRNA genes was amplified using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Xiao et al., 2019). The genes were amplified under an appropriate amplification system and parameters. Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and a PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA) were used to purify and quantify the PCR amplicons, respectively. Equimolar concentrations of the purified amplicons were pooled and sequenced on the Illumina NovaSeq-PE250 platform.

Sequence processing was performed using Quantitative Insights Into Microbial Ecology version 2 (QIIME2, 2019.4 release) (Bolyen et al., 2018). Briefly, we imported the demultiplexed paired-end fastq files into the QIIME2 pipeline. Cutadapt was used to remove primer sequences and discard the unmatched reads (Martin, 2011). Quality filtering, denoising and chimera removal were performed using the DADA2 plugin in QIIME2, with default settings (Callahan et al., 2016). After denoising, amplicon sequence variant (ASV) feature sequences and tables were generated, and singleton ASVs were removed. To generate taxonomy tables, ASV feature sequences were taxonomically assigned using the feature-classifier classify-sklearn plugin, with a naïve Bayes classifier trained on the Greengenes database 13.8 (DeSantis et al, 2006). Furthermore, to standardize sampling effort across samples, the ASV table was rarefied by the diversity alpha-rarefaction plugin according to 90% of the minimum sample frequency.

Statistical Analysis

All biological results were confirmed as the mean ± standard deviation (SD) of at least three independent experiments. SPSS (Abacus Concepts, Berkeley, CA, USA) and Prism 8.0 (GraphPad, San Diego, CA, USA) software were used. A t test was used to analyze the significant differences between treatments.

Alpha diversity measures (the Chao1 richness index and Shannon diversity index) were produced by the QIIME2 diversity alpha plugin to analyze the alpha diversity level for different treatment groups. The nonparametric Kruskal-Wallis test was used to compare the differences in alpha diversity between different groups since normal distributions of the data or homogeneity of variances were rejected according to the Shapiro and Bartlett test of the package *stats*, respectively. We then used the false discovery rate (FDR) correction to calculate pair comparisons between group levels (*post hoc* analyses) for multiple testing. Bray-Curtis distances were used to calculate the beta diversity and were visualized with principal coordinates analysis (PCoA) in the R package *vegan*. A nonparametric analysis of variance (PERMANOVA) based on 999 permutations was used to test for differences in community structure among groups, and *post hoc* pairwise testing (pairwise differences between groups) was assessed with the function "*pairwise.adonis*" from the package *pairwiseAdonis*. Homogeneity of dispersion among sample groups was also assessed using the *betadisper* function (Arbizu and PairwiseAdonis, 2019; Oksanen, 2019). We also performed analysis of similarity (ANOSIM) to determine whether the difference between groups was greater than the difference within the groups. To identify the ASVs most responsible for differences among groups, linear discriminant analysis (LDA) effect size (LEfSe) was performed with the default recommended settings (Segata et al., 2011). Taxon abundances at the phylum and genus levels were statistically compared between groups by Metastats (White et al., 2009) and visualized as column plots. A taxonomic tree was produced to display the composition of all taxonomic levels. In addition, to verify the correlation between the gut microbiota genera and depressive-like behaviors and depression-related indicators, the relative abundance of the top 10 ASVs was selected for redundancy analysis (RDA) and the Spearman correlation test. The RDA was generated by R software (v4.0.3, package vegan). Spearman correlations were calculated and plotted by R (v4.0.3, package corrplot). The metabolic potential of the gut microbiota was

predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) analysis using the KEGG Pathway Database.

RESULTS

Alkaloids

Total alkaloids from the seeds of *S. alopecuroides* were analyzed by HPLC-MS (positive ionization). The results showed the presence of six main alkaloids, which were identified as oxymatrine, oxysophoridine, sophoridine, sophocarpine, matrine, and lupanine (Liu et al., 2011; Wang et al., 2012) (**Supplementary Figure 1** and **Supplementary Table 1**).

Effect of Alkaloids on Depression-Like Behaviors in Mice

The SPT, FST and OFT were used to evaluate the effect of alkaloids on depression-like behaviors in CUMS mice. As shown in **Figure 2A**, the SPT was performed four times (at 0, 1, 3, and 7 weeks). Compared with that of the control group mice, the sucrose preference rate of mice under CUMS did not change significantly in the first week. However, a significant difference occurred by the third week ($P < 0.001$), indicating that the depression mouse model may have been successfully established. After 4 weeks of drug treatment, the mice were tested with the SPT on the 7th week. Compared with that of the CUMS group, the sucrose preference rate of the alkaloids ($P < 0.05$) and imipramine ($P < 0.001$) treatment groups was significantly increased. At the same time, the FST and OFT behavioral tests were also carried out in the 7th week. Compared with CUMS alone, alkaloids and imipramine significantly reduced the immobility time in the FST ($P < 0.001$, **Figure 2B**), and the beneficial effects of alkaloids and imipramine were almost the same. Alkaloids also increased the numbers of crossings ($P < 0.05$, **Figure 2C**), rearings ($P < 0.05$) and modifications in the OFT ($P < 0.05$, **Figure 2D**). These results suggest that alkaloids have a significant beneficial effect on the depression-like behaviors of CUMS mice.

Effect of Alkaloids on the Relative Contents of Depression-Related Indicators in Mice

Depression is often accompanied by a decrease in the relative content of BDNF and monoamine neurotransmitters (Nemeroff, 2007; Garcia et al., 2008; Racagni and Popoli, 2010;

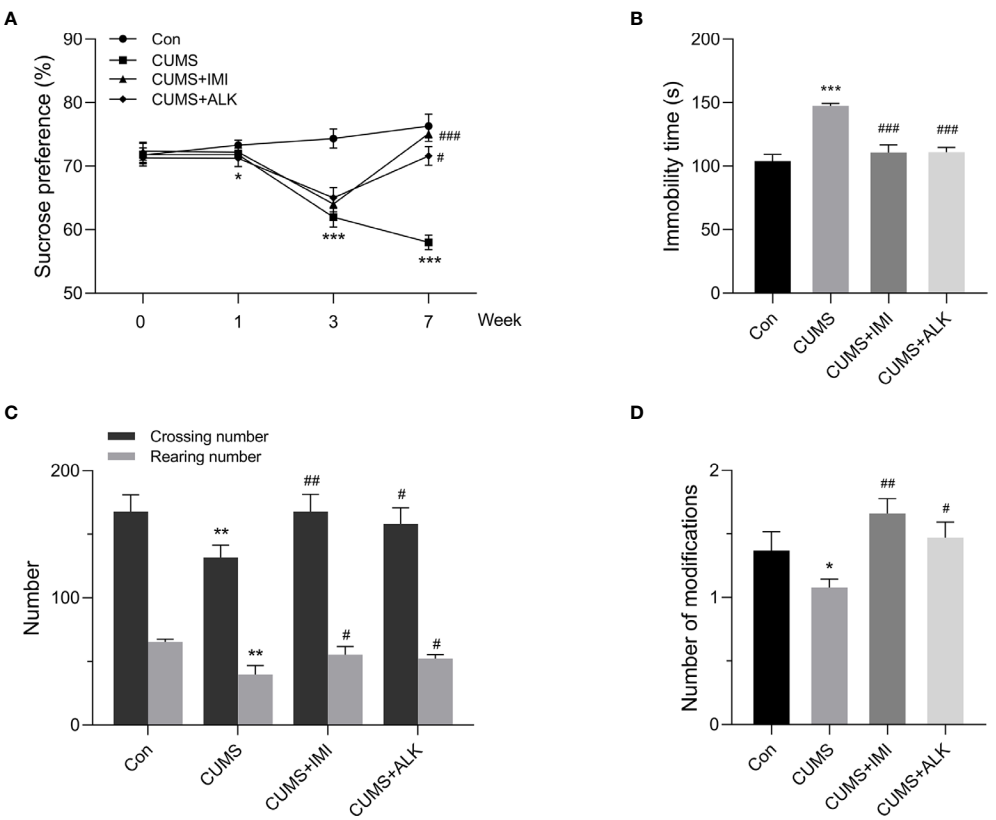

FIGURE 2 | Effect of alkaloids on depression-like behaviors. **(A)** Sucrose preference in the SPT. **(B)** Immobility time in the FST. **(C)** The number of crossings and rearings in the OFT. **(D)** The number of modifications in the OFT. Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group (Con); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the CUMS group.

Zhou et al., 2014; Nagy et al., 2020). As shown in **Figure 3**, compared with the control condition, CUMS significantly reduced the relative concentration of the BDNF protein in the hippocampus and prefrontal cortex ($P < 0.001$, **Figure 3A**) and the relative concentrations of 5-HT ($P < 0.001$), NE ($P < 0.001$), and DA ($P < 0.05$) in serum (**Figure 3B**). Moreover, compared with those of the CUMS group, the relative levels of BDNF in the prefrontal cortex and hippocampus and of the monoamine neurotransmitters (5-HT, NE, and DA) in serum were significantly increased after imipramine treatment ($P < 0.001$, **Figures 3A, B**). Alkaloids also increased the levels of BDNF in the hippocampus and prefrontal cortex of CUMS mice ($P < 0.05$, **Figure 3A**) and increased 5-HT ($P < 0.001$), NE ($P < 0.05$), and DA ($P < 0.05$) levels in serum (**Figure 3B**). These results further indicate that alkaloids can improve CUMS-induced depression in mice.

Microbiota Structure Changes in CUMS Mice Under Alkaloids Treatments

To explore the changes in the gut microbiome of CUMS mice under alkaloids treatment, we conducted an in-depth analysis of 16S rRNA sequencing. The alpha diversity analysis of intestinal content samples revealed differences within the composition of the microbial community of each group. As shown in **Figure 4**, compared with that of the CUMS group, the Chao1 index of the imipramine and alkaloids treatment groups decreased, but the diversity values between the two groups were not significantly different (**Figure 4A**, CUMS-CUMS+IMI: Padj < 0.05; CUMS-CUMS+ALK: Padj < 0.05; CUMS+IMI-CUMS+ALK: Padj = 0.58). Similar results were obtained for Shannon diversity (**Supplementary Figure 2**, CUMS-CUMS+IMI: Padj < 0.01; CUMS-CUMS+ALK: Padj < 0.001; CUMS+IMI-CUMS+ALK: Padj = 0.58). It is suggested that our drug treatment reduces the diversity of the gut microbiota of CUMS mice, and the effects of alkaloids and imipramine exhibited the same trend.

PCoA results showed that there was a significant clustering difference among the different treatment groups (PERMANOVA: CUMS-CUMS+IMI: F = 3.90, *Padj* = 0.003; CUMS-CUMS+ALK: F = 2.84, *Padj* = 0.006; CUMS+IMI-CUMS+ALK: F = 6.14, *Padj* = 0.003; **Figure 4B**). Furthermore, ANOSIM also confirmed shorter distances between intragroup samples than between intergroup samples (R = 0.506, $P = 0.001$, **Supplementary Figure 3**). In addition, there was no difference in the dispersion of variances among the different treatment groups (*betadisper*: $F_{(2,27)} = 3.25$, $P = 0.054$). The results showed that there were differences in microbes between different treatment groups, and the difference between the groups was greater than the difference within the groups.

Across all samples, the dominant taxa in the mouse gut microbiota were from three main phyla: Firmicutes, Bacteroidetes, and Proteobacteria (**Figures 4C, D**). After treatment with imipramine and alkaloids, compared with that of the CUMS group, the relative abundance of Firmicutes increased, while the relative abundance of Bacteroidetes and Proteobacteria decreased. However, although the most abundant taxa were similar in different treatment groups at the genus level, the relative abundance of some taxa was different. As shown in **Figure 4E**, compared with that in the CUMS group, the relative abundance of *Lactobacillus* significantly increased in the imipramine and alkaloids groups (CUMS-CUMS+IMI: *Padj* < 0.001; CUMS-CUMS+ALK: *Padj* < 0.05), while the relative abundance of *Oscillospira* significantly decreased (CUMS-CUMS+IMI: *Padj* < 0.01; CUMS-CUMS+ALK: *Padj* < 0.05). Moreover, the relative abundances of *Helicobacter* (CUMS-CUMS+IMI: *Padj* < 0.05), *Mucispirillum* (CUMS-CUMS+IMI: *Padj* < 0.05) and *Ruminococcus* (CUMS-CUMS+IMI: *Padj* < 0.05) in the imipramine group were significantly reduced, and the relative abundance of *Desulfovibrio* was significantly reduced in the alkaloids group (CUMS-CUMS+ALK: *Padj* < 0.05). In general, at the phylum and genus level, the relative

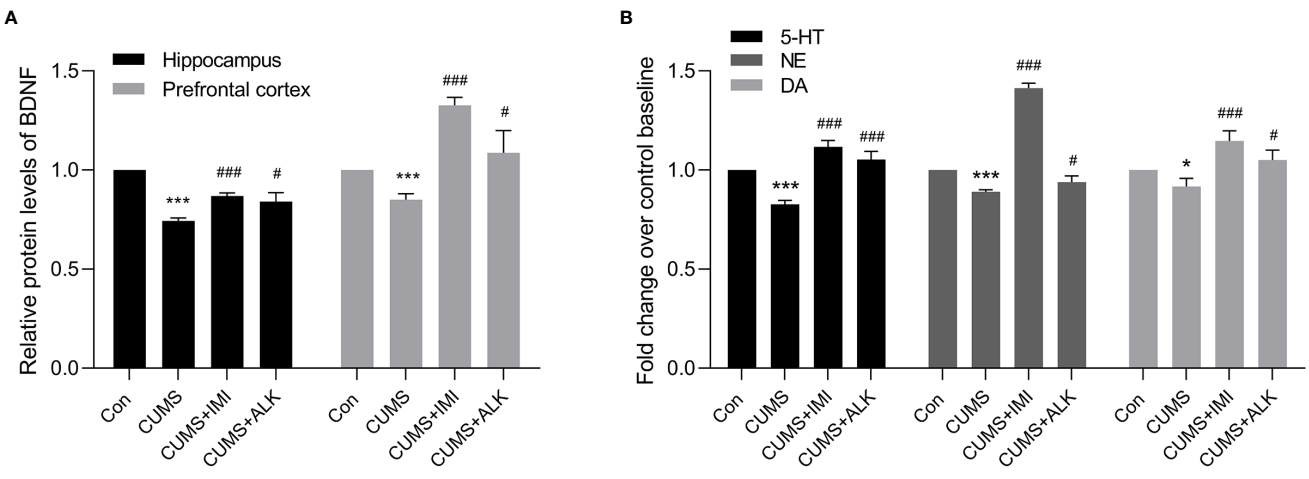

FIGURE 3 | The relative levels of BDNF, 5-HT, NE, and DA. **(A)** The relative protein levels of BDNF in the hippocampus and prefrontal cortex. **(B)** The relative monoamine neurotransmitter (5-HT, NE, and DA) levels in serum. Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$ versus the control group (Con); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the CUMS group.

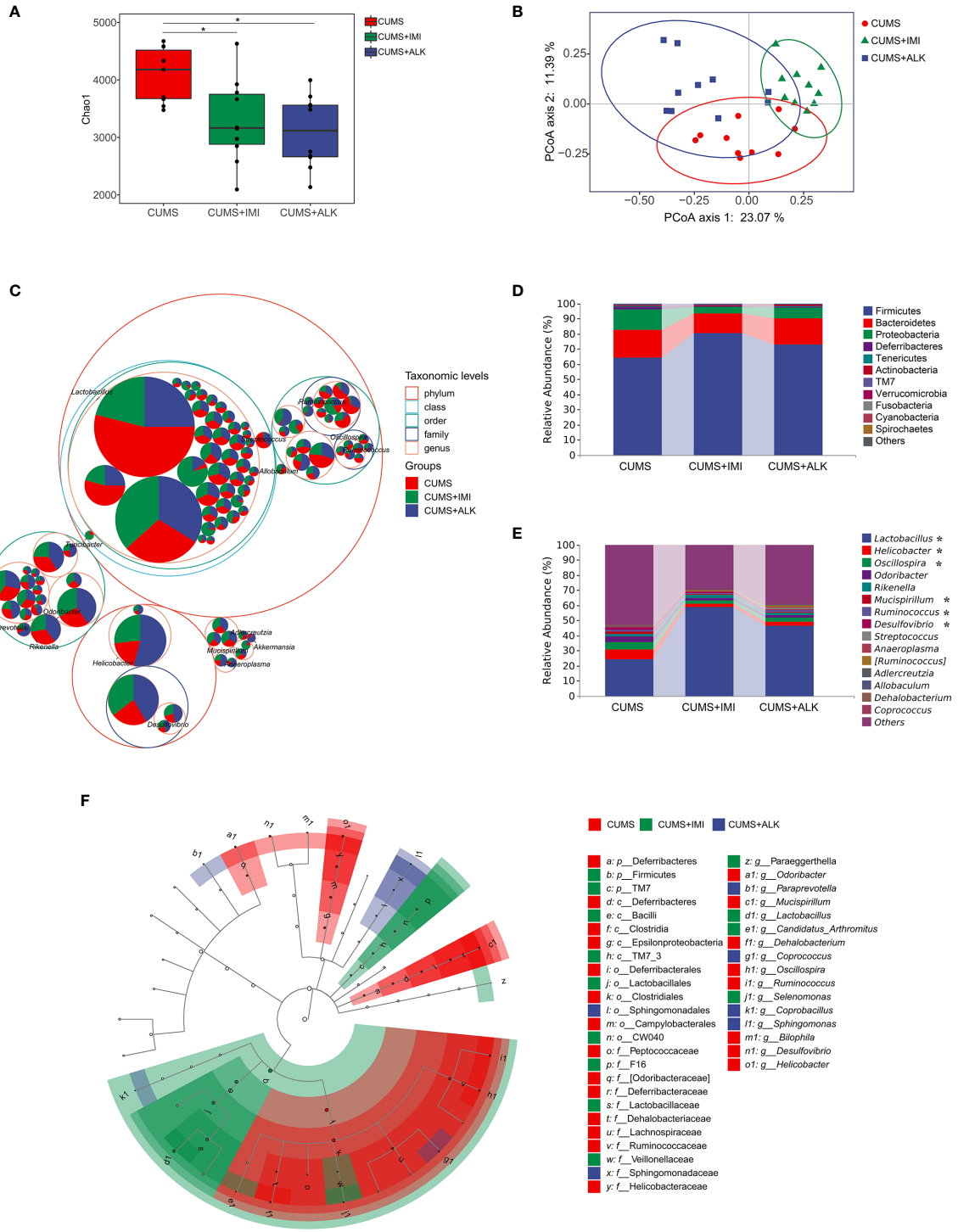

FIGURE 4 | Diversity variation and differential abundance in the gut microbiota composition of mice in different treatment groups. **(A)** The Chao1 index of different treatment groups. Data are presented as the mean ± SE. Mann-Whitney U test results are shown at the top of each paired comparison. *$P < 0.05$ versus the CUMS group. **(B)** PCoA based on the Bray-Curtis distance for gut microbiota (PCo1 vs. PCo2). Ellipses indicate 95% confidence intervals (CIs). **(C)** Taxonomic tree in packed circles. The largest circles represent the phylum level, and the inner circles represent class, order, family, and genus. The pie chart shows the proportion of each ASV in the different treatment groups. **(D)** Stacked bar chart of the most abundant phyla for CUMS, CUMS+IMI and CUMS+ALK mice. **(E)** Stacked bar chart of the mean relative abundances of bacterial taxa at the genus level for CUMS, CUMS+IMI and CUMS+ALK mice. Asterisks represent microbial taxa with significant differences. **(F)** Cladogram of ASVs with LDA scores greater than 2 based on LEfSe. Different colors represent different groups. Letters represent the position of ASVs in the cladogram.

abundance of microbiota between different treatment groups has a certain trend of change.

LEfSe was performed to identify ASVs driving the differences in beta diversity. A total of 41 ASVs were determined to explain the difference in the gut microbiota among different treatment groups (LDA > 2, $P < 0.05$). The results showed that the dominant species in the CUMS group at the genus level were *Odoribacter*, *Mucispirillum*, *Dehalobacterium*, *Oscillospira*, *Ruminococcus*, *Bilophila*, *Desulfovibrio*, and *Helicobacter*. The dominant species at the genus level in the imipramine treatment group were *Lactobacillus*, *Candidatus_Arthromitus*, *Paraeggerthella*, and *Selenomonas*. The dominant species at the genus level in the alkaloids treatment group were *Paraprevotella*, *Coprococcus*, *Coprobacillus*, and *Sphingomonas* (**Figure 4F**). Additionally, the relative abundance levels of each of the abovementioned microbiota genera were assessed in the different treatment groups (the top 50 ASVs at the genus level were selected) (**Supplementary Figure 4**).

Depression-Like Behaviors and Depression-Related Indicators Correlate With Gut Microbes

We performed RDA to study the effects of depression-like behaviors and depression-related indicators on changes in the microbiota genera. The RDA results showed that depression-like behaviors and depression-related indicators influenced the change in the gut microbiota based on the top 10 ASVs at the genus level (**Figure 5**). Among them, SPT ($P < 0.001$), OFT-numbers of crossings ($P < 0.05$), OFT-numbers of rearings ($P < 0.05$), BDNF-h ($P < 0.001$), BDNF-p (BDNF in the prefrontal cortex) ($P < 0.01$), 5-HT ($P < 0.05$), and DA ($P < 0.01$) had significant effects on the gut microbiota. In addition, OFT (numbers of crossings) had the greatest effect on the community structure, accounting for 21.8% of the variation, followed by DA (21.0%), BDNF-h (14.7%), 5-HT (11.1%), OFT-numbers of rearings (11.1%), SPT (10.9%), BDNF-p (9.4%) and DA (10.1%) (**Supplementary Table 2**).

To further investigate the correlations among the gut microbiota genera, depression-like behaviors, and depression-related indicators, we analyzed the Spearman correlations (**Figure 6**). We observed that there are 7 genera (*Lactobacillus*, *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, *Ruminococcus*, and *Desulfovibrio*) among the top 10 genera in relative abundance and differences in LEfSe analysis. Among them, *Lactobacillus*, *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, and *Ruminococcus* were associated with depression-like behaviors and depression-related indicators. The genera *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, and *Ruminococcus* were positively correlated with each other and with immobility time in the FST, which was negatively correlated with most of the other depression-like behaviors and depression-related indicators. However, *Lactobacillus* was negatively correlated with immobility time in the FST and with *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, and *Ruminococcus*. *Lactobacillus* was positively correlated with depression-related indicators, SPT, OFT-numbers of crossings, and OFT-numbers of rearings. These results led us to conclude that changes in the gut microbiota are associated with depression-like behaviors, and depression-related indicators.

Prediction of Gut Microbiota Function

To further clarify the correlation between changes in gut microbiota and metabolic pathways, we evaluated the differences in the function of gut microbiota in the CUMS group, imipramine group, and alkaloid group. PICRUSt 2 was used to predict gene content, and classify gene families through the KEGG database. The PCoA results of the functional unit show that there were differences between the groups (**Figure 7A**). The metabolic pathway statistics

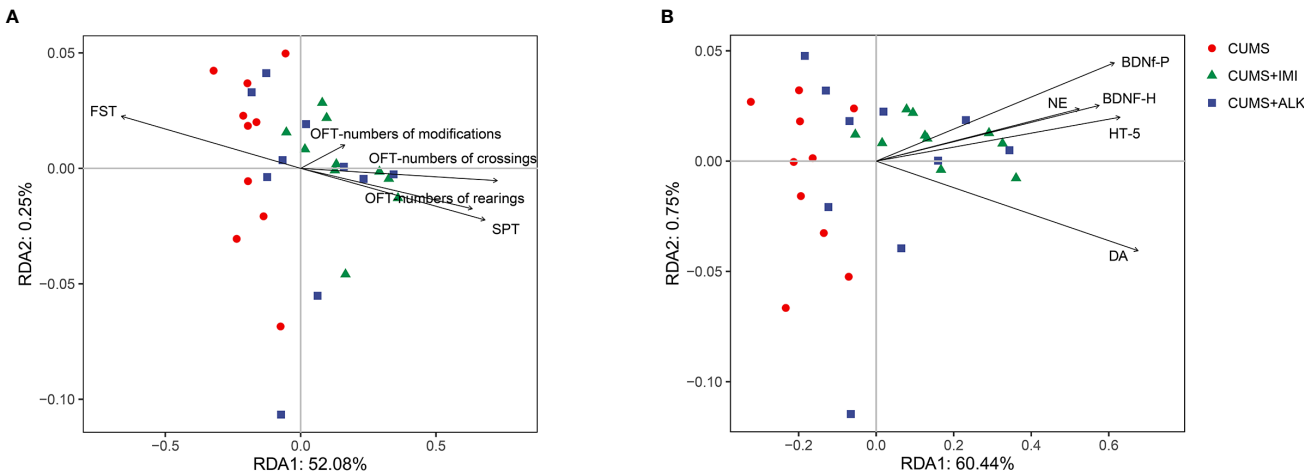

FIGURE 5 | Redundancy analysis (RDA) between gut microbiota, depression-like behaviors, and depression-related indicators. **(A)** RDA was performed to assess the contributions of the depression-like behaviors to the gut microbiota between different treatment groups. **(B)** RDA was performed to assess the contributions of the depression-related indicators to the gut microbiota between different treatment groups.

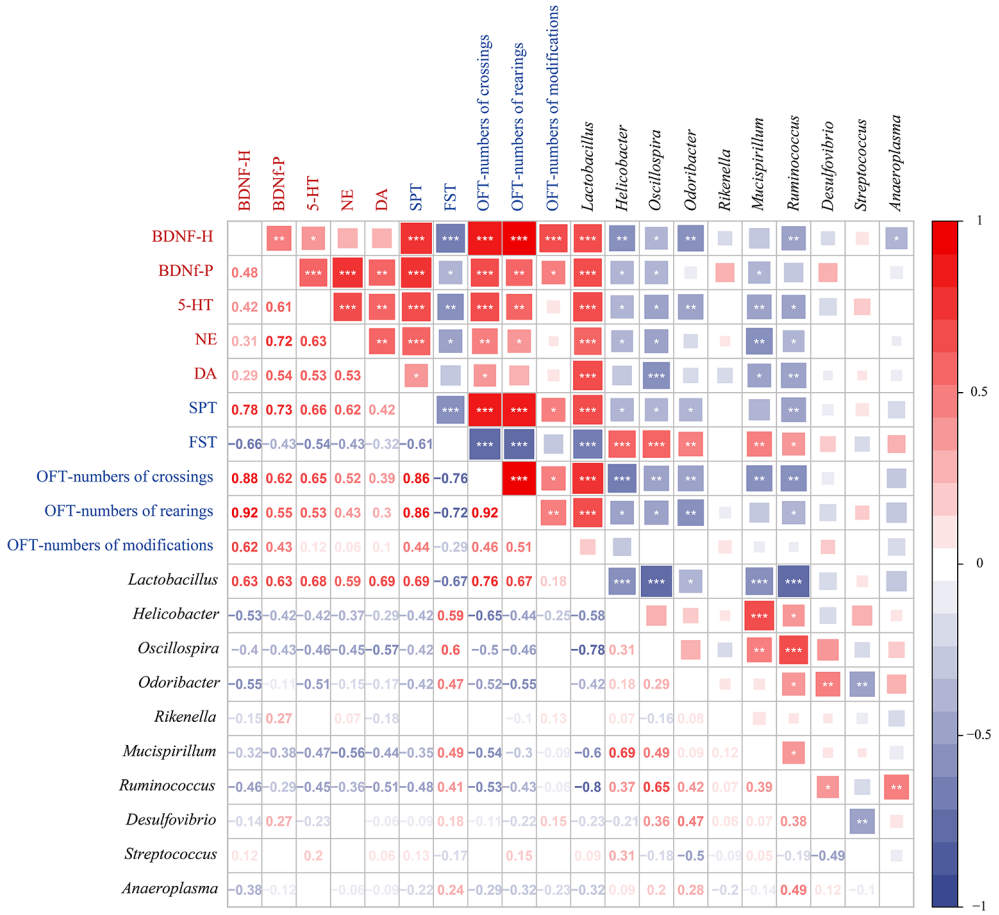

FIGURE 6 | Spearman correlations between gut microbiota, depression-like behaviors, and depression-related indicators. Spearman's rank correlation coefficient among 5 depression-related indicators, 5 depression-like behaviors, and 10 gut microbiota that differed significantly in abundance between different treatment groups. Axis label: red, depression-related indicators; blue, depression-like behaviors; black, gut microbiota. Numbers on the lower left area: value of correlation coefficient; symbols on the upper right area: results of correlation and significance test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

chart shows that its pathways mainly concentrated in the metabolism, followed by genetic information processing, cellular processes, environmental information processing, organic systems, and human diseases (**Supplementary Figure 5**). The results of LEfSe analysis showed that the differential pathways in the CUMS group were valine leucine and isoleucine biosynthesis, biotin metabolism, lipopolysaccharide biosynthesis, arginine and proline metabolism, phenylalanine metabolism, insulin signaling pathway, peroxisome, and lysine degradation; the differential pathways of the imipramine group were phosphotransferase system PTS, galactose metabolism, RNA transport, amino sugar and nucleotide sugar metabolism, pyrimidine metabolism, peptidoglycan biosynthesis, base excision repair, streptomycin biosynthesis, and benzoate degradation; the differential pathways of the alkaloids group were fatty acid biosynthesis, lipoic acid metabolism, vitamin B6 metabolism, lysine biosynthesis, pyruvate metabolism, ascorbate and aldarate metabolism, tyrosine metabolism, aminobenzoate metabolism, and selenocompound metabolism (**Figure 7B**). These results imply that the gut microbiota directly participates in the metabolic process of the host, which may further affect the changes in depression-related indicators and depression-like behaviors.

DISCUSSION

Studies have shown that CUMS is a reliable method for establishing depression models (Chen et al., 2015; Davis et al., 2016; Yu et al., 2017). It exposes experimental animals to a variety of unpredictable mild environmental stimuli within a period of time, thereby effectively simulating the onset of depression in real life. The environment has certain application value for studying the mechanism of antidepressants and the pathophysiology of depression. This article combines CUMS with the isolation model. In addition to establishing a model quickly and steadily, it can also prevent mice from influencing the structure of each other's gut microbiota through their feces.

To date, since the etiology of depression is still unclear, the pathogenesis is very complicated, and effective treatment methods

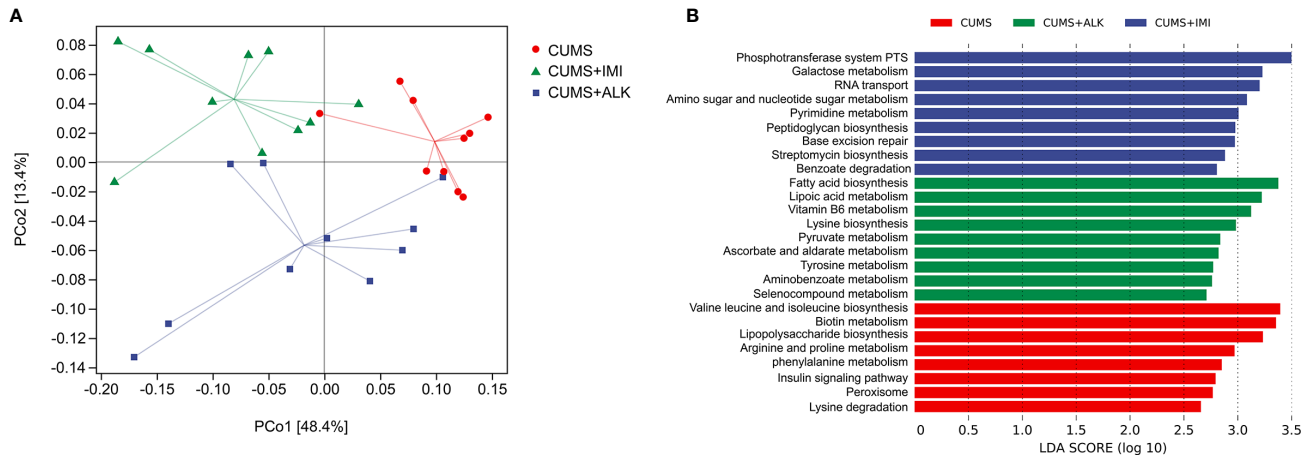

FIGURE 7 | Differences in functional units and metabolic pathways among different treatment groups. **(A)** Principal component analysis of functional units in different treatment groups. PCoA based on Bray-Curtis distance for gut microbiota (PCo1 vs. PCo2). Ellipses indicate 95% confidence intervals (CIs). **(B)** Differential metabolic pathways between different treatment groups, a histogram of linear discriminant analysis (LDA) scores calculated by LEfSe analysis.

are lacking. Generally, the neurotrophin and monoamine hypotheses are recognized. Previous studies demonstrated that a reduction in brain BDNF levels may predict major depressive disorder, while an increase in brain BDNF levels is associated with antidepressant effects (Garcia et al., 2008; Zhou et al., 2014). Depression is associated with decreased levels of monoamine neurotransmitters, such as 5-HT, NE, and DA (Nemeroff, 2007; Racagni and Popoli, 2010; Nagy et al., 2020). Therefore, in addition to behavioral testing, to investigate the potential correlation between the gut microbiota and brain metabolites, we also tested the content of brain-derived neurotrophic factors and monoamine neurotransmitters. In our results, BDNF and monoamine neurotransmitters showed the same trend results as those previously reported, which not only verified the success of the model and demonstrated the effect of alkaloids in improving depression but also laid a better foundation for future research on gut microbes in depressed mice.

The results of our alpha analysis show that the diversity of microbes in the gut of mice treated with drugs (imipramine and alkaloids) decreased. Consistent with the results of previous studies, chronic stress and depression can increase the diversity and richness of the gut microbiota (Naseribafrouei et al., 2014; Jiang et al., 2015; Li et al., 2018), while antibiotics and antidepressants reduce the diversity of microbes in the gut (Manichanh et al., 2010; Lukic et al., 2019). However, some studies have proven that the pressure of CUMS reduces alpha diversity and that antidepressants can improve the reduction in microbial diversity and make the community more stable (Kelly et al., 2016; Bharwani et al., 2017). According to the literature, many factors could affect the diversity of intestinal microbes, such as genetics, environment, age, and sex, which may be the reason for the differences in analysis results (Laukens et al., 2016).

The imbalance of the gut microbiota may be one of the important factors in the development or deterioration of depression. **Figures 4A–E** shows that different levels of microbiota changed under drug treatment. To explore the different microbes in each group, we used LEfSe to analyze the genus level and found that the CUMS group had more differential microbes than the other groups (**Figure 4F**). Among them, the different microbiota comprising the top ten with respect to abundance were *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, *Ruminococcus*, and *Desulfovibrio*. Previous studies have shown that *Helicobacter pylori* infection results in a significantly higher risk of depressive symptoms (Gu et al., 2019), and the incidence of peptic ulcer disease in patients with depression is twofold higher than that in the general population (Hsu et al., 2015). The relative abundances of *Oscillospira* in the intestines of adrenocorticotropic hormone-induced depression model mice were significantly increased compared with those in the control group, and the abundances were relatively decreased after drug treatment (Song et al., 2019). Compared with that in the control group, the relative abundance of *Odoribacter* in the CUMS group increased significantly (Li N. et al., 2019). Brain BDNF levels were significantly positively correlated with *Odoribacter* (Jiang et al., 2020), and *Mucispirillum* is associated with mood disorders (Getachew and Tizabi, 2019). Some species of *Mucispirillum* are related to intestinal inflammation caused by intestinal "leakage", and intestinal leakage is also a key factor in the comorbidity of depression and intestinal diseases (Kunugi, 2016; Romijn et al., 2017). *Ruminococcus* is a type of pathogenic bacteria. Studies have reported that traditional Chinese medicine treatments and marketed antidepressants can reduce the abundance of *Ruminococcus* in the intestine (Lukic et al., 2019; Qu et al., 2019). Consistent with the results of a CUMS rat study, the level of *Ruminococcus* in psychotic patients also increased (Clark and Mach, 2016; Zhuang et al., 2018). The *Desulfovibrio* genus produces lipopolysaccharides, and the reduction in *Desulfovibrio* content reduces the release of inflammatory factors, thereby improving depressive behaviors (Zhu et al., 2019). Through the above information on the different microbes in the CUMS group in this study, the gut microbiota and depression are inevitably linked. In addition, our study showed that the *Lactobacillus* genus

was differentially abundant in the imipramine treatment group (among the top 10 in overall abundance) and that the relative abundance of *Lactobacillus* also increased significantly after alkaloids treatment. *Lactobacillus* is a well-known beneficial bacterium that also has a positive effect on the central nervous system and plays a role in regulating depression, which has a positive effect on depression. Pressure application leads to a decrease in the content of *Lactobacillus* in the intestine, and oral administration of *Lactobacillus* can improve stress-induced behavior and mild depression (Marin et al., 2017). Moreover, we found that the abundances of these differentially abundant "harmful bacteria" (*Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, *Ruminococcus*, and *Desulfovibrio*) in the gut of CUMS mice were significantly reduced after imipramine and alkaloids treatment (**Supplementary Figure 4**). These changes remind us that one of the mechanisms by which alkaloids exert their antidepressant effects is likely improvement of the gut microbiota, and regulating the microbiota may be a viable treatment for depression.

The bidirectional communication and regulation of the brain and gut microbes play a vital role in depression (Zheng et al., 2016; Yang et al., 2017). When exploring the interaction between the gut microbiota and the brain in CUMS-induced depression in this study, we used RDA to establish the correlation between depression-like behaviors and depression-related indicators and the gut microbiota (top ten differentially abundant microbiota constituents). Excitingly, the results show that there was a certain correlation in general; specifically, the gut microbiota has a strong correlation with SPT, OFT-numbers of crossings, OFT-numbers of rearings, BDNF-h, BDNF-p, 5-HT, and DA. Next, Spearman correlation analyses further showed that the relative abundance of *Lactobacillus*, *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, and *Ruminococcus* generally appeared to be specifically correlated with depression-like behaviors and depression-related indicators. Previous studies reported that the expression of neurotransmitter (5-HT and DA) receptors is regulated by *Lactobacillus* in the intestine through the vagus nerve (Yu et al., 2017). As discussed earlier, *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, and *Ruminococcus* were closely related to depression. The prediction results of the gut microbiota metabolism pathway show that there are differences in abundance between the treatment groups. In addition, according to relevant literature reports, research on the combination of gut microbes and metabolomics may make the results of gut microbial metabolic pathways and functions more solid and convincing (Yu et al., 2017; Li J. et al., 2019). From these results, it is not difficult to conclude that gut microbes and related metabolic pathways had a direct or indirect effect on depression symptoms in CUMS mice in our study. *S. alopecuroides* alkaloids have the potential to improve depression by regulating the bidirectional signaling system of gut microbiota and brain.

CONCLUSION

In conclusion, the present study shows that alkaloids from *S. alopecuroides* can improve depression in mice and regulate the types of gut microbiota constituents in CUMS mice. The modulation of the relative abundances of key microbiota constituents at the genus level (e.g., *Lactobacillus*, *Helicobacter*, *Oscillospira*, *Odoribacter*, *Mucispirillum*, *Ruminococcus*, *Desulfovibrio*) by alkaloids is beneficial for the improvement of depression. The correlation between gut microbes and depression-like behaviors and depression-related indicators indicates that alkaloids can improve depression in mice by regulating gut microbes. Overall, our research initially revealed the mechanism of action of alkaloids in the treatment of depression, which in turn provides ideas for the study of depression pathogenesis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SRP281158.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Care and Use Committee at Northeast Normal University.

AUTHOR CONTRIBUTIONS

MZ contributed to the design, data acquisition, analysis, and drafting and critical revision of the manuscript. AL analyzed and wrote the 16S sequencing data. QY, JL, and LW contributed to the data acquisition. XL, YH, and LL designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.665159/full#supplementary-material

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Alteration of Gut Microbiome and Correlated Lipid Metabolism in Post-Stroke Depression

Wenxia Jiang, Lei Gong, Fang Liu, Yikun Ren and Jun Mu *

Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China

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Tingtao Chen,
Nanchang University, China

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Hai-yue Liu,
Southern Medical University, China
Shengjie Li,
Nanchang University, China
Shaoming Fang,
Fujian Agriculture and Forestry University, China

***Correspondence:**
Jun Mu
jmu@hospital.cqmu.edu.cn

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Background: The pathogenesis of post-stroke depression (PSD) remains largely unknown. There is growing evidence indicating that gut microbiota participates in the development of brain diseases through the gut-brain axis. Here, we aim to determine whether and how microbial composition and function altered among control, stroke and PSD rats.

Materials and Methods: After the PSD rat model was successfully established, gut microbiome combined with fecal metabolome approach were performed to identify potentially PSD-related gut microbes and their functional metabolites. Then, correlations between behavior indices and altered gut microbes, as well as correlations between altered gut microbial operational taxonomic units (OTUs) with differential metabolites in PSD rats were explored. Enrichment analysis was also conducted to uncover the crucial metabolic pathways related to PSD.

Results: Although there were some alterations in the microbiome and metabolism of the control and stroke rats, we found that the microbial and metabolic phenotypes of PSD rats were significantly different. The microbial composition of PSD showed a decreased species richness indices, characterized by 22 depleted OTUs mainly belonging to phylum *Firmicutes*, genus *Blautia* and *Streptococcus*. In addition, PSD was associated with disturbances of fecal metabolomics, among them Glutamate, Maleic acid, 5-Methyluridine, Gallocatechin, 1,5-Anhydroglucitol, L-Kynurenine, Daidzein, Cyanoalanine, Acetyl Alanine and 5-Methoxytryptamine were significantly related to disturbed gut microbiome ($P \leq 0.01$). Disordered fecal metabolomics in PSD rats mainly assigned to lipid, amino acid, carbohydrate and nucleotide metabolism. The steroid biosynthesis was particularly enriched in PSD.

Conclusions: Our findings suggest that gut microbiome may participate in the development of PSD, the mechanism may be related to the regulation of lipid metabolism.

Keywords: post-stroke depression, gut microbiome, metabolome, metabolic pathways, lipid metabolism

INTRODUCTION

Post-stroke depression (PSD) is a common mood disorder, which often indicates a poor prognosis (Kutlubaev and Hackett, 2014) and high mortality (Bartoli et al., 2013). At any point within five years of the stroke, approximately one-third of stroke survivors have PSD (Hackett and Pickles, 2014). The hypotheses regarding the pathogenesis of PSD, include psychosocial distress, alteration of monoamine neurotransmitter, activation of the hypothalamic-pituitary-adrenal axis and disturbed energy metabolism (Villa et al., 2018). However, the specific pathophysiology of PSD remains unknown.

Gut microbiome is the major microbial community that settles in the human body, and affects the host's nutrition, metabolism and immune function (Ghaisas et al., 2016). Previous studies found that altered gut microbiome has been implicated in stroke and depression (Zheng et al., 2016; Zhu et al., 2016; Chen et al., 2019; Lee et al., 2020; Zheng et al., 2020). The microbial metabolites are also associated with depression and stroke. A previous study showed that depressed mice were characterized by disturbances in carbohydrate and amino acid metabolism (Zheng et al., 2016). Another study demonstrated that stroke was related to disturbances in amino acid and lipid metabolism (Yamashiro et al., 2017; Chen et al., 2019). Accumulating data indicated that the gut microbiota communicates with the central nervous system, influencing brain function and behavior through the microbiota-gut-brain axis (Cryan and Dinan, 2012; Cryan et al., 2019). Zhu et al. revealed that gut microbes can promote thrombosis by producing trimethylamine N-oxide (TMAO), thereby increasing the incidence of stroke (Zhu et al., 2016). Another study found a reduced fecal level of short chain fatty acids (SCFAs) in aged stroke mice, as a result, transplantation of fecal microbiota rich in SCFAs promoted the stroke recovery (Lee et al., 2020). However, so far, the gut microbiome and microbial metabolism of PSD rats have not been explored.

In order to determine whether the microbial composition and function of PSD rats have changed, and how they may have changed, 16S ribosomal RNA (rRNA) gene sequencing technology was firstly applied to identify alteration of gut microbiome compositions. Then, the changes of metabolites in the fecal samples of PSD rats were captured by gas chromatography-mass spectrometry (GC-MS), before the correlation between gut microbes and bacterial metabolites was revealed.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley (SD) rats about 180-200g were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). All animal procedures were approved by the Ethics Committee of Chongqing Medical University (Permit No. 2020–459) and complied with the guidelines of Animal Use and Care of the National Institutes of Health. The workflow is displayed in **Figure 1A**. The rats were housed in groups of five per cage, ad libitum food and water. The room was maintained in 12-hour light/dark cycle (lights on at 8:00 a.m.), with constant temperature of 21–22°C and humidity of 50 ± 5%. They were allowed to acclimatize for 1 week before sucrose preference test (SPT) was performed at baseline. At the same time, they were trained to consume 1% sucrose solution for taste adaptation. 86 rats with similar baseline performance were included. There were 14 rats in the control group, and 72 rats receiving ischemia-reperfusion (IR) surgery. Animals that underwent IR surgery and survived 24 hours later were randomly divided into the stroke group (n = 21) and the PSD group (n = 30). Three days after surgery, rats in the PSD group were housed in a single cage and subjected to a chronic unpredictable mild stress (CUMS) regimen for 4 weeks.15 rats died within 24hrs after surgery. Six rats without neurological symptoms were excluded. In view of the protective effect of estrogen on ischemic injury, only male rats were used in this study (Xu et al., 2018).

Focal Cerebral Ischemia and Reperfusion Surgery

As described by Belayev, et al, the FCIR model was established by transient middle cerebral artery occlusion (MCAO) (Belayev et al., 1996). Briefly, rats were anesthetized *via* intraperitoneal injection of 3.5% chloral hydrate (350 mg/kg). A midline incision was made on the neck, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed. A nylon monofilament suture (diameter 0.26 mm, Beijing Xinong Technology Co. Ltd., China) with a slightly enlarged round tip (diameter 0.34–0.36 mm) was gently advanced from the ECA into the lumen of the ICA, until it reached and occluded the middle cerebral artery (MCA). The distance from bifurcation of CCA to the tip of the suture inserted to occlude MCA, averaged 18–20 mm. After 2 hrs of ischemia, the suture was carefully removed to establish reperfusion. The body temperature was maintained at 36.5-37.5°C by a heating pat after the surgery. Animals in the control group underwent the same surgical procedure without suture insertion.

Measurement of Cerebral Infarct Volume

Rats were euthanized on the 3rd day after reperfusion. The brains were removed and frozen for 30 min at −20°C. The forebrain was cut into consecutive 2 mm-thick coronal slices, stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, USA) at 37°C for 10 min. The slices were photographed and image analysis software (ImagePro Plus 6.0, Media Cybernetics Co. USA) was used. The percentage of infarct volume was calculated *via* the following formula: right hemisphere infarct volume/total volume ×100% (Ikeda-Matsuo et al., 2006).

Chronic Unpredictable Mild Stress

The CUMS regimen used in this study was adapted from Wang, et al. (2009), with minor modifications. Details were shown in **Supplementary Table S1**. Briefly, 1-2 of the following stressors were arranged in a random order daily: water deprivation for 24 hrs and followed by a sucrose preference test, food deprivation for 24 hrs, swimming in 4°C water for 5 min, tail pinch for 1 min, 45° cage tilt for 24 hrs, soiled cage (200mL water in 100g

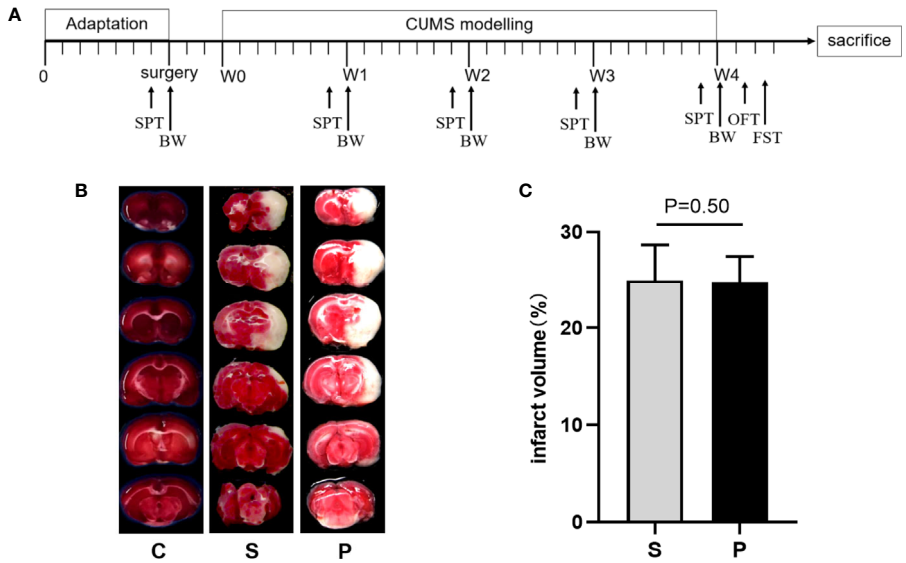

FIGURE 1 | Time schedule of experimental procedures and TTC-stained brain sections. **(A)** Time schedule of experimental procedures. CUMS, chronic unpredictable mild stress; W0, Beginning of CUMS; W1–W4, CUMS was performed as described in the materials and methods section for 4 weeks; BW, body weight; SPT, sucrose preference test; OFT, open field test; FST, forced swimming test. **(B)** The representative TTC-stained brain sections. **(C)** Quantification of infarction volumes was calculated based on TTC staining (n = 6 per group, P > 0.05). C, Control; S, Stroke; P, post-stroke depression.

padding) for 24 hrs, overnight illumination (lights on for a total of 36 hrs), restrained with steel wire tube for 4 hrs (well-ventilated, rats were not able to move forward or backward in tubes).

Behavioral Tests

All behavioral tests were performed by well-trained and experienced observers who were blind to the grouping of the animals.

Neurological Deficit Scoring Evaluation

The neurological deficits of the animals were evaluated at 24hrs after the IR surgery with the 5-point neurological scale according to Longa et al. (1989). Specifically, score 0, no neurologic deficit; score 1, failure to extend left forepaw fully when held by tails; score 2, circling to the left side; score 3, falling to the left side; score 4, no walk spontaneously with depressed level of consciousness. Rats with a score of 0 or 4 were removed from further study.

Sucrose Preference Test

SPT was used to assess anhedonia of rats (Guo et al., 2009). Before testing, animals were water deprived for 24 hrs. One bottle of purified water and another bottle of 1% sucrose solution were provided to each rat for 1 hr. The positions of two bottles will be switched after 30 min. SPT was performed before surgery and once a week during CUMS. Sucrose preference rate was calculated by sucrose intake (g)/[sucrose intake (g) + water intake (g)].

Open Field Test

OFT was used to evaluate the locomotor activity, overall exploratory and anxiety of rats in a new environment (Choleris et al., 2001; Niu et al., 2015). OFT was performed at the end of CUMS. The experimental device consists of a box (100 cm × 100 cm × 40 cm) and an automatic data acquisition and processing system (SMART 3.0, Panlab, Spain). Each rat was placed at the center of the box and monitored for 5 min. The total distance of spontaneous moves and percentage of duration time spent in the center square (duration = time spent in the center square (s)/total time (s) × 100%) were videotaped and quantified by a video-computerized tracking system. Each rat was tested individually and only once. The box was cleaned thoroughly before each animal was tested.

Forced Swimming Test

FST was used to assess behavioral despair of rodent (Cryan et al., 2005). FST was performed at the end of CUMS. Each rat was placed in a plastic cylinder (60 cm high, 20 cm diameter) with water (21–23°C, 30 cm in depth) for 6 min. The last 4 min of immobility time was recorded by a video-computerized tracking system (SMART 3.0, Panlab, Spain).

Body Weight Measurement and Fecal Samples Collection

Body weight measurement and fecal samples collection were performed before surgery and once a week during CUMS. Fecal samples were collected by lifting tail and immediately frozen in liquid nitrogen and stored at −80°C.

Deoxyribonucleic Acid Extraction, Polymerase Chain Reaction Amplification, and Illumina MiSeq Sequencing

Total bacterial DNA was extracted from fecal samples using the OMEGA-soil DNA Kit (Omega Bio-Tek, USA) according to the

manufacturer's protocols. The V3-V4 region of the bacteria 16S rRNA gene was targeted and PCR amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR cycling conditions were as follows: 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. PCR reactions were performed in triplicate 20μL mixture. Amplicons were extracted from a 2% agarose gel and further purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluo-ST (Promega, USA). Purified amplicons were paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols.

16S rRNA Gene Sequence Analysis

Raw fastq files were demultiplexed, and quality-filtered using QIIME (version 1.17, http://qiime.org/). Truncate the 250bp reads at any site of more than three sequential, the average quality score < 20 were accepted. Reads shorter than 50 bp containing barcode/primer errors or ambiguous base calls were discarded. Chimeric sequences were identified and removed using UCHIME (http://drive5.com/uchime). Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/). Species diversity indices (Shannon, Simpson) and species richness indices (Ace and Chao) were used to evaluate α-diversity. Principal co-ordinate analysis (PCoA) was used to visually evaluate the whole difference and similarity of bacterial communities among control, stroke and PSD group (n=6 per group) (Zhang et al., 2019). The key bacterial taxa responsible for discrimination among the three groups were identified with linear discriminant analysis effect size (LEfSe) analysis (Segata et al., 2011). LEfSe analysis was used to identify the different OTUs by calculating the effect of the abundance of each OTU (LDA > 2.0 and P value ≤ 0.05).

Fecal Metabolome Analysis

Fecal metabolome analysis was performed as previously described (Chi et al., 2017; Zheng et al., 2019). Briefly, fecal samples were processed under anaerobic conditions. Each fecal sample (100 mg) was transferred into 1.5 mL eppendorf tubes with reduced sterile phosphate buffered saline and an equal volume of donor suspension was used to prepare pool. Homogenized samples were extracted put in -20°C for 30 min, and then were centrifuged at 13000 rpm, 4°C for 15 min. Supernatant was dried with a centrifugal freeze dryer, then the dried extracts were used to derivatization. Gas chromatography-mass spectrometry (GC/MS; Agilent 7890A/5975C, CA, USA) was used to characterize the fecal metabolome analysis. The GC/MS three-dimensional matrices comprised of sample names (observations), peak indexes (RT-m/z pairs), and normalized peak area percentages were imported into a SIMCA (version14.0, Umetrics, Umeå, Sweden). Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA) was used to visually discriminate the PSD subjects from control and stroke (n=8 per group). By analysis of Principal Component Analysis (PCA) loadings, the differential metabolites responsible for discriminating among the three groups were identified (variable importance plot (VIP) > 1.0, and p-values < 0.05). Pathway analyses were carried out based on Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database and conducted by MetaboAnalyst 4.0.

Statistical Analysis

Statistical analyses were carried out using SPSS version 19 (SPSS, Chicago, IL, US). Continuous variables such as sucrose preference, total distance and center time of OFT, immobility time in FST and body weight were analyzed by one-way analysis of variance (ANOVA). The results were presented as mean ± standard error of the mean (SEM) unless otherwise indicated. The least significant difference (LSD) post-hoc test was used to find out which two groups differed significantly if a significant difference was observed in ANOVA. The α-diversity was analyzed by Kruskal-Wallis Test. Beta-diversity was analyzed by Adonis analysis. The cerebral infarct volume between stroke and PSD was analyzed by student t-test, statistical result was presented as mean ± SEM. The non-parametric test was used when appropriate. Spearman correlation analysis was used to determine the correlation between the behavior indices of PSD and the discriminative OTUs, as well as the correlation between changes in gut microbes and the main differential fecal metabolites in PSD. The multiple testing corrections were conducted using Benjamini and Hochberg False Discovery Rate. Statistical significance level was set at $p < 0.05$.

RESULTS

Behavioral Characteristics of the PSD Rats

Before the initiation of CUMS, the cerebral infarct volume showed no significant difference between the groups (**Figures 1B, C**; n = 6 per group, P > 0.05). Compared with control group, PSD rats were characterized by decreased sucrose preference and body weight on 4th week of CUMS (**Figures 2A, B**; n = 8 per group, P<0.01). There was no significant difference in the total distance of OFT among the three groups (**Figure 2C**; n = 8 per group, P > 0.05). But, compared to control and stroke group, PSD rats showed significantly decreased center time (**Figure 2D**; n = 8 per group). Meanwhile, compared to control and stroke rats, PSD rats showed significantly decreased immobility time in FST (**Figure 2E**; n = 8 per group). These results suggested that our PSD modeling was successful and depressive phenotype was independent of infarct volume.

Decreased Species Richness Indices (Ace and Chao) in PSD

The 16S rRNA gene sequencing method was used to compare the fecal microbial composition of PSD, control and stroke. In the discovery set, we obtained 2117702 high-quality reads across all

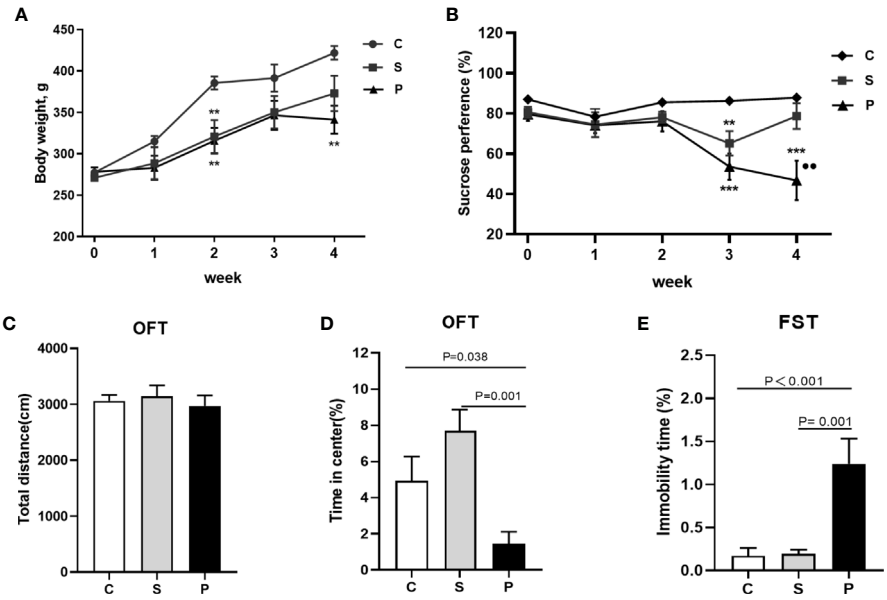

FIGURE 2 | Body weight and behavioral tests. **(A)** Body weight during CUMS of three group (n = 8 per group); **(B)** Sucrose preference during CUMS of three group (n = 8 per group); **(C)** The total distance of OFT was no significant difference among 3 groups after a 4-week CUMS exposure (n = 8 per group); **(D)** Percentage of duration time spent in the center square of OFT was compared among 3 groups after a 4-week CUMS exposure (n = 8 per group); **(E)** Immobility time comparison of FST among 3 groups after a 4-week CUMS exposure (n = 8 per group); C, Control; S, Stroke; P, PSD; PSD group vs. control group, **P < 0.01 and ***P < 0.001; PSD group vs. stroke group, ●● P < 0.01; SPT, sucrose preference test; OFT, Open field test; FST, Forced swimming test.

samples, with an average length of 418.13. These reads were clustered into 1410 OTUs at 97% sequence similarity. The α-diversity values including species diversity indices (Shannon and Simpson) and species richness indices (Ace and Chao) were compared among the PSD, control and stroke groups. We found that microbial richness indices were significantly different among three groups. Compared to control, the PSD rats were characterized by decreased Chao, Ace indices (**Figure 3A**, $p < 0.01$), suggesting decreased species richness indices in PSD.

Alternations of Microbial Composition in PSD Rats

To determine whether PSD was associated with altered microbial composition, β-diversity analysis was performed. PCoA revealed that the gut microbial composition of rats with PSD was significantly different from that in control and stroke (**Figure 3B**; n = 6 per group). The relative abundance of gut microbes at the OTU level was shown in **Figure 3C**. In order to identify the microbial characteristics that can distinguish PSD from control and stroke, the LEfSe analysis method was used to analyze the differential OTU among the three groups (**Figures 4A, B**). In total, 91 differentially abundant OTUs were identified. The stroke rats were characterized by 18 OTUs, mainly belonging to *Muribaculaceae* (3 OTUs), *Prevotellaceae* (2 OTUs), *Lachnospiraceae* (2 OTUs), *Lactobacillaceae* (2 OTUs), *Christensenellaceae* (1 OTUs), *Erysipelotrichaceae* (1 OTUs), *Ruminococcaceae* (1 OTUs), *Desulfovibrionaceae* (1 OTUs), *Akkermansiaceae* (1 OTUs), *Bacteroidaceae* (1 OTUs) and *Streptococcaceae* (1 OTUs) at the family level (**Figure 4B**). Compared with control and stroke, the PSD were characterized by 22 OTUs, mainly belonging to *Firmicutes* (14 OTUs), *Proteobacteria* (3 OTUs), *Bacteroidetes* (2 OTUs), *Tenericutes* (2 OTUs), and *Actinobacteria* (1 OTU) at the phylum level. 22 OTUs particularly overrepresented in PSD were assigned to the families of *Lachnospiraceae* (6 OTUs), *Lactobacillaceae* (1 OTU), *Streptococcaceae* (2 OTUs), *Erysipelotrichaceae* (1OTU), *Ruminococcaceae* (2 OTUs), *Veillonellaceae* (1 OTU), *Enterococcaceae* (1 OTU), *Muribaculaceae* (2 OTUs), *Burkholderiaceae* (1 OTU), *Enterobacteriaceae* (1 OTU), *Mycoplasmataceae* (1 OTU) and *Eggerthellaceae* (1 OTU). At the genus level, the 22 discriminative OTUs of PSD mainly belong to *Blautia* (2 OTU), *Streptococcus* (2 OTU), *Lactobacillus* (1 OTU), *Bacteroides* (1 OTU), *Veillonella* (1 OTU), *Klebsiella* (1 OTU), *Ralstonia* (1 OTU), *Marvinbryantia* (1 OTU), *Mycoplasma* (1 OTU) and *Enterococcus* (1 OTU) (**Supplementary Table S2**).

Correlations of Depressive-Like Behaviors With Altered Gut Microbes in PSD

We found that the differential bacterial OTUs were generally associated with behavior indices (**Figure 5**). Overall, the 22 discriminative OTUs of PSD were positively correlated with FST results and negatively correlated with SPT and OFT results. This is consistent with the behavioral test performance of PSD rats. 50% (11/22 OTUs) of altered bacterial OTUs showed significant correlations with FST, OFT and SPT results

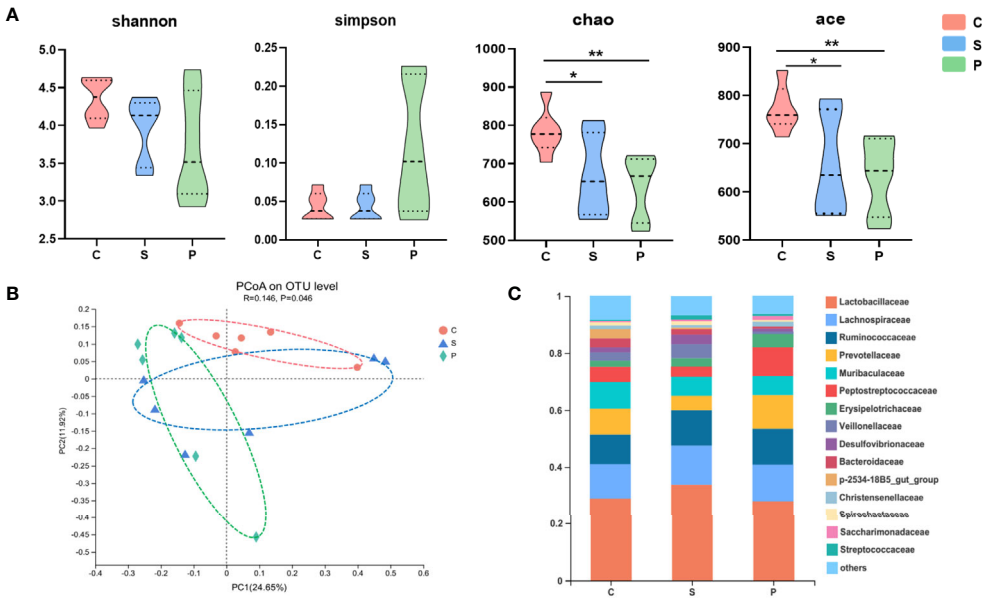

FIGURE 3 | Gut microbial characteristics of control, stroke and PSD. **(A)** α-phylogenetic diversity analysis showing that PSD subjects were characterized by lower microbial richness in two indexes (Ace, Chao) relative to controls (n=6 per group), *P < 0.05, **P < 0.01. **(B)** At the OTU level, principal co-ordinates analysis (PCoA) showed gut microbial composition of rats with PSD was significantly different from that in control and stroke (n = 6 per group). **(C)** Relative abundance of gut microbes at the family level.

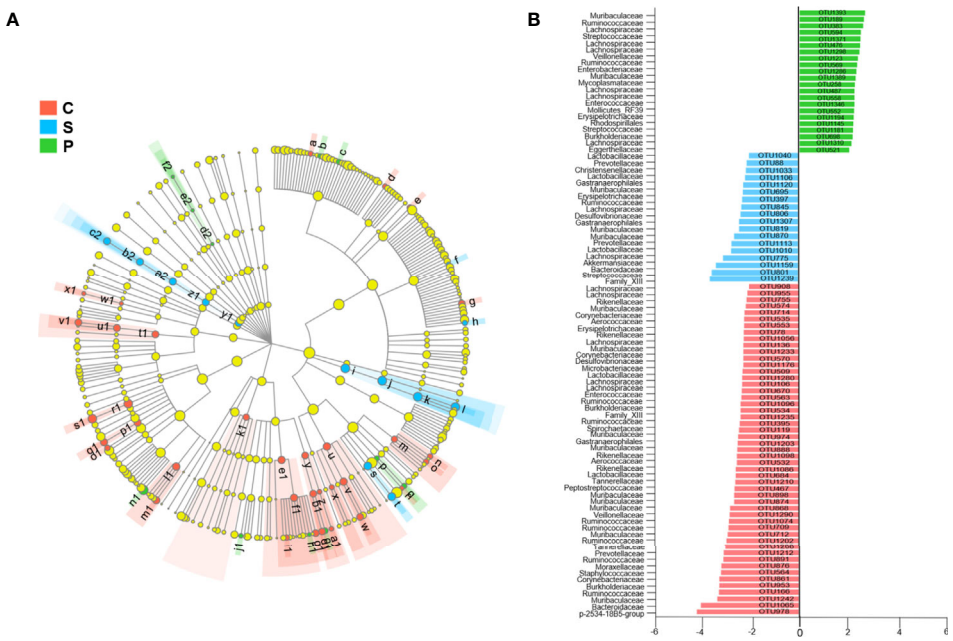

FIGURE 4 | Linear discriminant analysis effect size (LEfSe) analysis (LDA > 2.0). Cladogram **(A)** and histogram **(B)** illustrated 91 OTUs responsible for discriminating the PSD, stroke and control groups. Compared to stroke and control groups, the PSD rats were characterized by 22 discriminative OTUs (n = 6 per group).

(r > ± 0.45, p-value < 0.05). Those 11 OTUs were mainly belonging to *Firmicutes* (OTU1145, OTU552, OTU1371, OTU258, OTU383, OTU1298, OTU594), *Proteobacteria* (OTU1181, OTU569), *Bacteroidetes* (OTU1393) and *Tenericutes* (OTU1389). These results indicate that PSD was characterized by disturbed gut microbiome.

Disturbances of Fecal Metabolisms in PSD Rats

Considering that the gut microbiota is always involved in the regulation of host's metabolic pathways, the fecal metabolome is regarded as functional readout of gut microbiome. GC-MS based metabolomic method was used. The fecal metabolic phenotype of PSD was significantly different from that of control and stroke (**Figure 6A**; n = 8 per group). Compared with control and stroke, there were 25 differential fecal metabolites in PSD rats, of which 18 metabolites increased and 7 metabolites decreased (**Supplementary Table S3**, VIP > 1.0 and p-values < 0.05). These differential metabolites were further used for pathway analysis by MetaboAnalyst 4.0. Among 19 pathways revealed, the lipid-related metabolic pathway (steroid biosynthesis) was most significantly enriched (**Figure 6B**, p-values<0.05) (**Supplementary Table S4**). Specifically, these differentially expressed metabolites were related to lipid metabolism (Squalene, Lanosterol, Stigmasterol and Lignoceric Acid), amino acid metabolism (L-Kynurenine, 5-Methoxytryptamine, Tyramine, Glutamate, Maleic acid, Cyanoalanine and Phenylacetic acid), carbohydrate metabolism (Arbutin, N-Acetyl-D-Mannosamine, Glutamate, Acetyl Alanine and Sucrose-6-Phosphate) and nucleotide metabolism (Cytosine).

Correlations Between Gut Microbes and Fecal Metabolites

To further explore the potential correlations of the altered gut microbial OTUs with fecal metabolome, correlation analysis was performed (**Figure 6C**). 54.55% (12/22 OTUs) of altered bacterial OTUs had a significant correlation with a series of differential metabolites (r > ± 0.6, p-value < 0.05). These 12 OTUs mainly belonged to *Firmicutes* (OTU383, OTU258, OTU552, OTU1298, OTU189, OTU698), *Proteobacteria* (OTU1181, OTU569, OTU1194), *Bacteroidetes* (OTU1286, OTU1393) and *Actinobacteria* (OTU1310). The significantly correlated metabolites were assigned to lipid metabolism (Lanosterol, Stigmasterol and Lignoceric Acid), amino acid metabolism (L-Kynurenine, 5-Methoxytryptamine, Tyramine, Glutamate, Cyanoalanine and Maleic acid), carbohydrate metabolism (N-Acetyl-D-Mannosamine, Glutamate, Acetyl Alanine and Sucrose-6-Phosphate), nucleotide metabolism (Cytosine), biosynthesis of other secondary metabolites (Daidzein, Ferulic Acid and Gallocatechin) and metabolites without metabolic pathways (Lactobionic Acid, 5-Methyluridine and 1,5-Anhydroglucitol). Our findings demonstrated that PSD rats were characterized by both disturbed gut microbiome and fecal metabolome, and altered gut microbiota can affect the metabolism of PSD rats.

DISCUSSION

In this study, we compared the gut microbiome and fecal metabonomics among the PSD, control and stroke rats. We found that the microbial phenotype of PSD rats was significantly

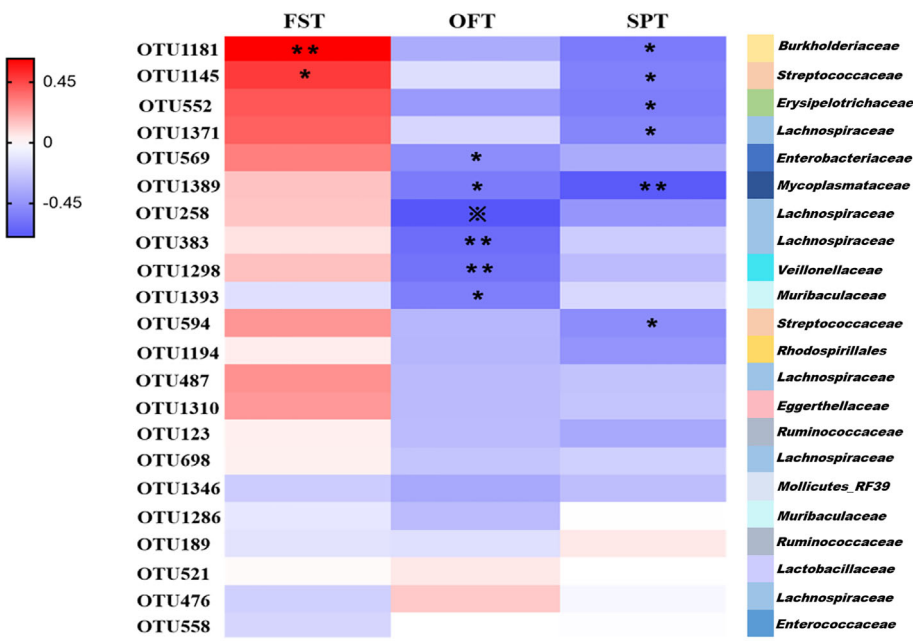

FIGURE 5 | Associations of altered gut microbes with behavior indices. Heat map of the Spearman's rank correlation coefficient of 22 discriminative OTUs for PSD and 3 behavior indices. Red rectangle indicates positive associations between these microbial species and behavior indices, blue rectangle indicates negative associations (n = 6 per group). Overall, 22 discriminative OTUs for PSD were positively associated with FST, and negatively associated with SPT and OFT. 11 of 22 differential microbial variances (50%) were significantly associated with 3 behavior indices (p value < 0.05) and correlation coefficient were ≥0.45 or ≤ –0.45, tested by spearman correlation. The statistical significance was denoted on the rectangle (*p < 0.05; **p < 0.01; ※p < 0.001).

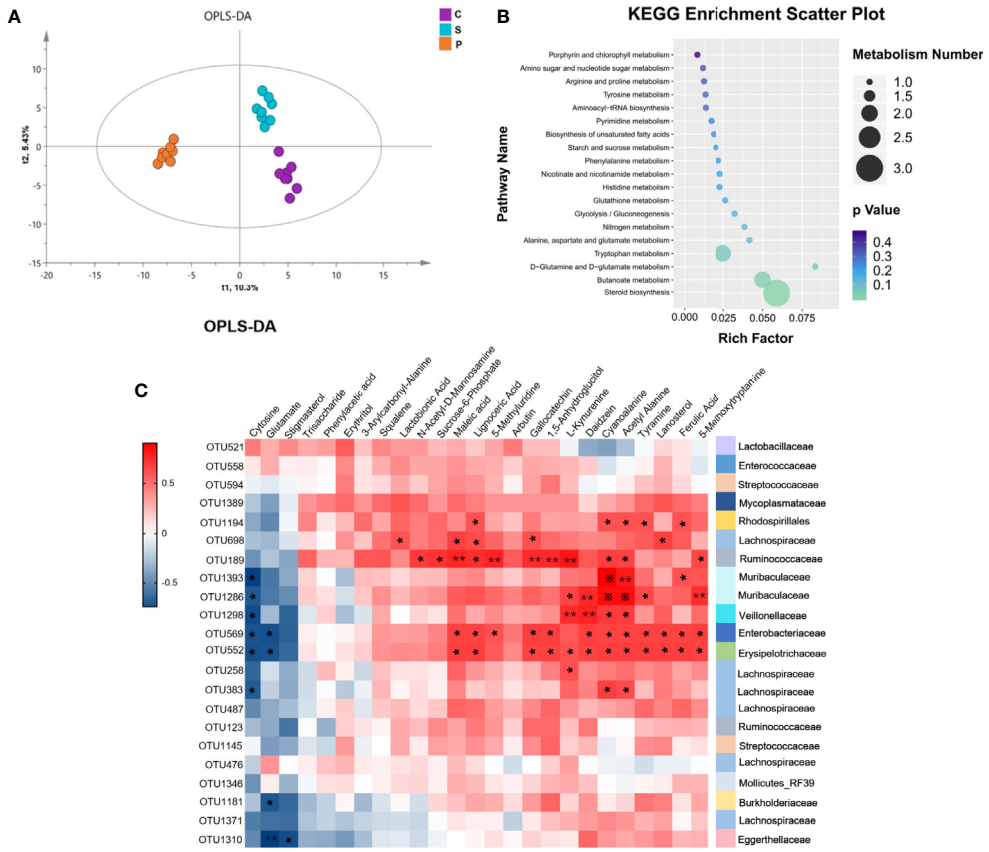

FIGURE 6 | Fecal metabolism characteristics of PSD and its related KEGG enrichment pathways. **(A)** Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA) showed fecal metabolism of PSD was significantly different from that in control and stroke (n = 8 per group). **(B)** The 25 differential metabolites for PSD rats compared with control and stroke rats were enriched in 19 KEGG pathways. **(C)** Associations of gut microbial OTUs with fecal metabolites. Heat map of the spearman's rank correlation coefficient of 22 discriminative OTUs and 25 differential metabolites for PSD. Red squares indicate positive associations between these microbial OTUs and metabolites, blue squares indicate negative associations (n = 8 per group). 54.55% (12/22 OTUs) of altered bacterial OTUs showing significant correlations with a range of differential metabolites ($p < 0.05$) and correlation coefficient were ≥ 0.6 or ≤ –0.6, tested by spearman correlation. The statistical significance was denoted on the squares (*$p < 0.05$; **$p < 0.01$; ※$p < 0.001$).

different from that of the control and stroke groups. Importantly, the altered gut microbial OTUs were highly correlated with a series of metabolites. Moreover, the altered gut microbes of PSD rats were highly consistent with their behavioral performance. Enrichment analysis further uncovered the crucial metabolic pathways related to PSD. Our findings suggest that gut microbiome may participate in the development of PSD, and the mechanism may be related to the regulation of lipid metabolism.

One of the problems faced by post-stroke depression research, is the lack of model that highly simulates the clinical disease. The PSD model in our study, is one of the most commonly used and widely accepted models in the world. Previous studies have found that it can partially reflect the pathophysiological mechanism of post-stroke depression (Pang et al., 2015; Zhang et al., 2017; Villa et al., 2018). It should be noted that all rats in our study were housed in a specific pathogen free (SPF) environment with unidirectional flow. The water was purified. The food and padding were sterilized. Therefore, the soiled cage was mainly served as a stress factor in our study, with no substantial impact on the experimental results.

Compared with the control, the microbial composition of PSD showed decreased species richness indices (**Figure 3A**). The diversity of the human gut microbiota is considered evidence of health (Eckburg et al., 2005). Herein, decreased species richness in PSD rats may suggest disordered physiological processes. The gut microbiome of PSD rats has not been explored before, however, previous study on depression showed disturbances of *Lachnospiraceae*, *Lactobacillaceae*, *Streptococcaceae*, *Erysipelotrichaceae* and *Ruminococcaceae* (Zheng et al., 2016). We found similar microbes, such as *Lachnospiraceae*, *Lactobacillaceae*, *Streptococcaceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, *Veillonellaceae*, *Enterococcaceae*, *Muribaculaceae*, *Burkholderiaceae*, *Enterobacteriaceae*, *Mycoplasmataceae* and *Eggerthellaceae* were altered in PSD rats. Unlike PSD and depression, Lee et al. reported that the

disturbance of gut microbiome during stroke related to *Bifidobacteriaceae* and *Clostridiaceae* (Lee et al., 2020). Furthermore, the altered bacterial OTUs were highly consistent with the behavioral test performance of PSD rats (**Figure 5**), suggesting the abnormal microbial state of PSD has been verified on the behavioral level. Specifically, our findings showed OTU569 which belongs to *Enterobacteriaceae*, had a significant correlation with both OFT and Lignoceric Acid (**Figures 5** and **6C**). Lignoceric Acid, one of the metabolites detected in our study, was involved in lipid metabolism and also significantly related to *Enterobacteriaceae*. Studies showed that lignoceric acid was associated with increased risk of cardioembolic stroke (Chung et al., 2015) and autoimmune diseases (Tsoukalas et al., 2019). As is known, neuroimmune was involved in central nervous system (CNS) disorders, including PSD (Cacabelos et al., 2016).

We also found that PSD was associated with disturbances of fecal metabolomics. These discriminating fecal metabolites in PSD rats were mainly involved in lipid metabolism, amino acid metabolism, carbohydrate metabolism and nucleotide metabolism. These overlap with the metabolic pathways of depression and stroke. A previous study showed that depressed mice were characterized by disturbances in carbohydrate and amino acid metabolism (Zheng et al., 2016). Another study in depressed cynomolgus macaque demonstrated disruption of carbohydrate and lipid metabolism (Qin et al., 2019). Stroke was also found to be related to disturbances in amino acid and lipid metabolism (Yamashiro et al., 2017; Chen et al., 2019).

Interestingly, a further analysis revealed two lipid-related metabolic pathways in PSD, mainly involved in biosynthesis of steroid and unsaturated fatty acids. Among them, the steroid biosynthetic pathway is significantly enriched, with the highest enrichment density. As is known, cholesterol is the source of steroid-related hormone biosynthesis and is closely related to brain development and neurological diseases (Hussain et al., 2019). Cholesterol can either be converted into steroid-related hormones (estrogens, androgens, glucocorticoids) or vitamin D. On one hand, clinical study found that PSD patients had significantly lower vitamin D levels than non-PSD patients (Han et al., 2015). For depressive patients, low high-density lipoprotein (HDL) cholesterol and high triglyceride levels were associated with lower likelihood of long-term symptom resolution (Virtanen et al., 2017). On the other hand, both clinical and animal studies showed that corticosteroids increased the risk of depression (Weina et al., 2018). Elevated cortisol after stroke was even associated with morbidity and mortality of the patients (Barugh et al., 2014). In our study, of the three metabolites enriched in the steroid pathway, Lanosterol and Squalene were significantly increased, while Stigmasterol was significantly decreased. Lanosterol and Squalene will eventually be converted into cholesterol. Stigmasterol reduces the level of low-density lipoprotein (LDL) cholesterol. Therefore, lipid metabolism and PSD may be closely related. Abnormal lipid metabolism may provide novel clues for investigating the pathogenesis of PSD.

Our research has some limitations. (i) Although we provide evidence that the gut microbiota imbalance may be related to PSD, fecal transplantation experiments can be used to confirm the causality. (ii) Our research was only carried out in male rats. Female rats may also be of interest in future experiments. (iii) Due to the relatively limited resolution of the 16S rRNA sequencing technique (Hillmann et al., 2018), shotgun metagenomic sequencing method will be used to identify specific bacterial strains of PSD. (iv) Based on the identified metabolic pathways related to PSD, it is necessary to further explore their key regulatory targets.

In summary, using multi-omics data, we outlined the landscapes of bacteria as well as fecal metabolites in PSD rats. We found gut microbiome may participate in the development of PSD, the mechanism may be related to the regulation of lipid metabolism. Our findings provide a new perspective for understanding the pathogenesis of PSD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Chongqing Medical University (Permit No. 2020–459).

AUTHOR CONTRIBUTIONS

Conception and design: JM. Performed the experiments: WJ, LG, FL and YR. Performed the microbiome and metabolomic analysis: WJ. Drafted the manuscript: WJ. Final Approval of the Completed Manuscript: JM. 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.2021.663967/full#supplementary-material

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

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Washed Microbiota Transplantation Accelerates the Recovery of Abnormal Changes by Light-Induced Stress in Tree Shrews

Jing Wang[1,2†], Qianqian Li[3,4†], Qi Huang[5†], Meng Lv[6], Pan Li[3,4], Jing Dai[1], Minjie Zhou[1], Jialu Xu[1], Faming Zhang[3,4] and Jun Gao[1,7*]*

[1] Department of Neurobiology, School of Basic Medical Sciences, Nanjing Medical University, Nanjing, China, [2] Visual Cognition Laboratory, Department of Medicine, University of Fribourg, Fribourg, Switzerland, [3] Medical Center for Digestive Diseases, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, China, [4] Key Lab of Holistic Integrative Enterology, Nanjing Medical University, Nanjing, China, [5] PET Center, Huashan Hospital, Fudan University, Shanghai, China, [6] Animal Core Facility of Nanjing Medical University, Nanjing Medical University, Nanjing, China, [7] Department of Rehabilitation Medicine, Jiangsu Shengze Hospital Affiliated to Nanjing Medical University, Nanjing Medical University, Nanjing, China

The gut and brain interact constantly in a complex fashion. Its intricacy and intrigue is progressively being revealed in the study of the "gut–brain axis". Among many factors, abnormal light exposure is a potential powerful stressor, which is becoming ever more pervasive in our modern society. However, little is known about how stress, induced by staying up late by light, affects the gut–brain axis. We addressed this question by extending the normal circadian light for four hours at night in fifteen male tree shrews to simulate the pattern of staying up late in humans. The behavior, biochemical tests, microbiota dynamics, and brain structure of tree shrews were evaluated. The simple prolongation of light in the environment resulted in substantial changes of body weight loss, behavioral differences, total sleep time reduction, and an increased level of urine cortisol. These alterations were rescued by the treatment of either ketamine or washed microbiota transplantation (WMT). Importantly, the sustainability of WMT effect was better than that of ketamine. Magnetic Resonance Imaging analysis indicated that ketamine acted on the hippocampus and thalamus, and WMT mainly affected the piriform cortex and lateral geniculate nucleus. In conclusion, long-term light stimulation could change the behaviors, composition of gut microbiota and brain structure in tree shrews. Targeting microbiota thus certainly holds promise as a treatment for neuropsychiatric disorders, including but not limited to stress-related diseases.

Keywords: gut–brain axis, staying up late, stress, tree shrew, magnetic resonance imaging, washed microbiota transplantation

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Tingtao Chen,
Nanchang University, China

Reviewed by:
Hailong Cao,
Tianjin Medical University General Hospital, China
Gang Wang,
Jiangnan University, China

****Correspondence:***
Jun Gao
gaojun@njmu.edu.cn
Faming Zhang
fzhang@njmu.edu.cn

†These authors have contributed equally to this work and share first authorship

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INTRODUCTION

The intricate interplay of gut and brain is quickly and surely revealing its implications to many facets of human health, including stress-related neural diseases (Gilbert et al., 2018; Cryan et al., 2019; Zhang et al., 2019; Pennisi, 2020). Any disturbance of this system could so lead to ill-fated effects. Like the spreading pervasive presence of artificial light. It has, on one side, drastically increased humans' productivity on a societal scale and distinctly enriched parts of their Umwelt in a stimulating manner from La Ville Lumière to Times Square. Otherwise, it's also a potential powerful stressor and disturbing factor for organisms and their well-being. Recent studies have demonstrated that exposure to light at night had negative influences on gut microbiota. Benedict et al. observed that two days of recurrent partial sleep deprivation, that is, only staying up late for 4 h daily, caused changes in gut microbiota of young adults (Benedict et al., 2016). Additionally, excessive light exposure affects mood and brain circuits, such as the suprachiasmatic nucleus of the hypothalamus, medial amygdala, lateral habenula and hippocampus (Bedrosian and Nelson, 2017). However, it is currently unclear how long-term staying up late affects the gut–brain axis. To answer this question, we need a suitable animal model to better mimic our modern predicament.

The omnivorous tree shrew (*Tupaia belangeri*) is a highly qualified candidate model organism for such aims. First and foremost the tree shrew has a close genetic relationship with primates and resembles the pattern of human sleep on a more fundamental level than rodents do, due to being day-active and having longer, more continuous bouts instead of the fractured, polyphasic, nocturnal nature of rodent sleep (Liu et al., 2001; Coolen et al., 2012; Fan et al., 2013). Second, tree shrews have a small size (150–170 g), a short gestation (43 days) and a rapid sexual maturity (3–6 months), which facilitates their ease of use as a laboratory species next to rats and mice. Third, tree shrews have established their place in the study of a wide variety of neurological diseases, including psychosocial stresses, visual cognition and depression as a new primate-like animal organism (Wang et al., 2013a; Schmelting et al., 2014; Khani et al., 2018). Meanwhile, tree shrew is highly vulnerable to stress which makes them a promising stress model (Fang et al., 2016). Therefore, we developed here a staying up late model in tree shrews. It was evaluated by measuring physiological and biochemical indicators, combined with characterization of gut microbiota and brain structure through magnetic resonance imaging (MRI).

To ascertain whether targeting gut microbiota could reverse the changes in brain structure and ill-health Washed Microbiota Transplantation (WMT) was used. Given that gut microbiota can have a major impact on brain function and behavior through the gut–brain axis (Vendrik et al., 2020). WMT is a modification of Fecal Microbiota Transplantation (FMT), where the manual manipulations are substituted by a more automated microbiota purification system and washing process. FMT has extremely high efficacy in recurrent or refractory *Clostridium difficile* infection with the ability to restore healthy microbial ecology, and thus holds promise as a therapy for other diseases influenced by dysbiosis of the intestinal environment (Zhang et al., 2020). Additionally, WMT is demonstrated to be safer, more precise and more quality-controllable than the crude FMT (Zhang et al., 2020). Converging evidence suggests that both germ-free and antibiotic treatment have an impact on animal stress (Sudo et al., 2004; Gareau et al., 2011; Peirce and Alvina, 2019; Wang et al., 2020). This could make results from germ-free animals more difficult to interpret and translate. Considering that this is a stress animal model, healthy tree shrews were chosen without any antibiotic treatment. Our study aimed to evaluate the alterations of behavioral and brain structures after gut microbiota reconstruction using WMT. We hypothesize that WMT could ameliorate the physiological changes and accelerate the recovery of abnormal structural changes in the brain induced by extended light exposure to tree shrews.

MATERIALS AND METHODS

Animals

Adult male and female Chinese tree shrews (*T. belangeri Chinensis*, N = 18) weighing 110–160 g were obtained from the Kunming Institute of Zoology, Chinese Academy of Sciences. We used fifteen male tree shrews for all behavioral experiments and three females, who were isolated from males to avoid ovulation, as the negative controls in MRI. All animals were bred at the Animal Core Facility of Nanjing Medical University. They were given *ad libitum* access to food and water. Each tree shrew was housed individually in climate-controlled rooms (ambient temperature: 25–27°C, air humidity: 55–70%) under a 12 h light/12 h dark cycle (light, 08:00–20:00; dark, 20:00–08:00) in the baseline phase. All animal experiments were performed in accordance with the recommendations of the Experimental Animal Ethics Committee at the Nanjing Medical University. The related fecal donors' samples were approved by institutional committees.

Experimental Procedures

As shown in **Figure 1A**, the whole experiment included; a baseline phase (7 days), a light-induced stress phase (21 days) and a recovery phase (10 days). In the first phase, the baseline phase (T-Bas) we measured a range of physiological and biochemical indicators, including body weight, locomotion, morning urine cortisol, and total sleep time. Fecal samples of the tree shrews were collected for 16S ribosomal RNA (rRNA) screening. Nine male and three female animals were randomized and scanned with MRI to study brain structure changes. Then in the second phase, we prolonged environmental light until later in the evening by 4 h, that is, the 12 h light/12 h dark cycle was changed to a 16 h light/8 h dark cycle (light, 08:00–24:00; dark, 00:00–08:00). We did not force the animals to keep awake till 24:00 but simply left the light on. During this light-induced stress phase, all physiological and biochemical indicators were measured in the first and third weeks (T-S1wk and T-S3wk). In the last recovery phase, we maintained the 16 h light/8 h dark

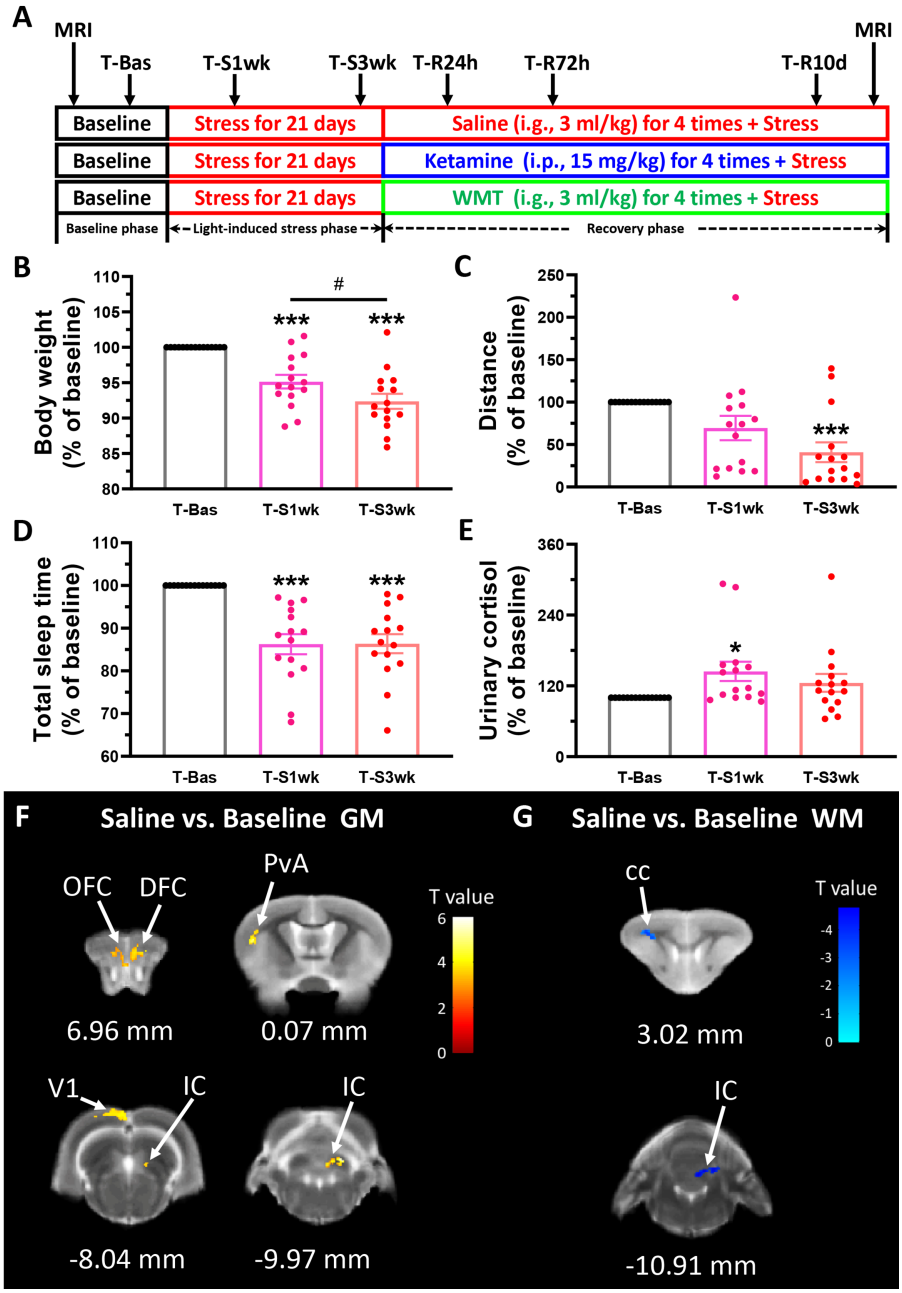

FIGURE 1 | Physiological responses and brain structure changes in the tree shrew after staying up late for 21 days. **(A)** Experimental design. Basic data was collected in the baseline phase (T-Bas), including MRI scanning, weight, urinary and stool collection, locomotor activity and total sleep time measurements. In the light-induced stress phase, the facility light cycle was changed (light on at 8:00, light off at 24:00). All of the above indicators, except MRI scanning, were tested extended light exposure for one week (T-S1wk) and three weeks (T-S3wk). Ketamine (15 mg/kg), WMT (3 ml/kg) or vehicle (0.9% saline, 3 ml/kg) were administrated at the beginning of the recovery phase for a total of four times. We tested all physiological and biochemical indicators in the ketamine, WMT or vehicle (saline) conditions 24 h (T-R24h), 72 h (T-R72h) and 10 days (T-R10d) after administration. At the end of the experiment, MRI scans were performed on the same animals. **(B–E)** All of the physiological and biochemical indicators after light-induced stress for 1 week (T-S1wk) and 3 weeks (T-S3wk) were normalized with their values in the baseline phase (T-Bas) to explore the effects of extended and prolonged light exposure. Body weight (**B**, N = 15), distance travelled in an open field box (**C**, N = 15) and total sleep time (**D**, N = 15) were all decreased, and morning urinary cortisol (**E**, N = 15, [F(42,2) = 3.025, p = 0.059], LSD Post Hoc: T-S1wk compared with T-Bas, p = 0.018) was increased after staying up late for 21 days. **(F)** The GM density signals from OFC (orbital frontal cortex), DFC (dorsal frontal cortex), PvA (parietal ventral area), V1 (primary visual cortex) and IC (inferior colliculus) were all increased (N = 3/group). **(G)** The WM density signals from cc (corpus callosum) and IC were decreased (N = 3/group). Error bars show the SEM. *means compared with control in baseline phase, *p <0.05, ***p <0.001. # means compared between each other in stress phase, #p <0.05.

cycle for the tree shrews and divided all animals into three groups of five: Saline, Ketamine and WMT. The microbiota for WMT was from one healthy donor in China Microbiota Transplantation System for the treatment condition. The methodology WMT based on an automatic microbiota purification system (GenFMTer, Nanjing, China) followed by centrifugation plus suspension for three times in a specifically designed laboratory at good manufacture practice (GMP) level (Zhang et al., 2020). All tree shrews were treated with WMT (~1.0×101^3 bacteria/ml colony-forming units, intragastric administration, i.g., 3 ml/kg) or ketamine (intraperitoneal injection, i.p., 15 mg/kg) or vehicle (0.9% sterile saline, i.g., 3 ml/kg). To reinforce the donor microbiota efficacy, drug or vehicle was administered four times: at 08:00 and 20:00 of the first day and 20:00 of the second and third days. We measured all physiological and biochemical indicators at 24 h, 72 h and 10 days (T-R24h, T-R72h and T-R10d) after the first administration. Finally, the brain structures of the animals scanned in the baseline phase were scanned again with MRI at the end of our experimental timeline. Following the experiments, tree shrews were put back, and sedated with diethyl ether to then be euthanized by rapid decapitation. The brain was quickly removed to be prepared and stored for future experiments.

Measurements

Body Weight and Analysis of Urinary Cortisol

Tree shrews were weighted between 7:45 and 08:00 before breakfast. Simultaneously urine samples were collected. Urine samples were stored at −20°C until analysis and free cortisol was measured by Access Immunoassay System (Unicel DxI 800, Beckman Coulter, Inc., USA).

16S rRNA Gene Sequencing and Processing

Fresh fecal samples of tree shrews were collected between 8:45 and 09:15 after breakfast. All of the fecal samples that were used for WMT, originated from healthy humans, who donated to the Chinese fecal microbiota bank (fmtBank). All donors provided written informed consents prior to participation in this study. This study was reviewed and approved by the Second Affiliated Hospital of Nanjing Medical University Institutional Review Board. Samples were stored at −80°C before the eventual analysis. Microbial DNA was extracted from stool samples. Bacterial 16S rRNA gene sequences were PCR amplified using bar-coded primers for the V4–V5 hypervariable region by the Phusion High-Fidelity PCR Master Mix with HF buffer (New England Biolabs, England). Products from each sample were mixed at equal molar ratios and then sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA), following standard Illumina sequencing protocols. Operational taxonomic units (OTUs) were clustered at 97% similarity and filtered using the UPARSE pipeline. Unweighted UniFrac distances were visualized with principal coordinate analysis (PCoA) using Python.

Locomotor Behavior Analysis

The open field box (length × width × height = 50 cm × 50 cm × 80 cm) was made of polymethyl methacrylate sheets (plexiglass), with one transparent side that faces the camera and the other three white and opaque. After all samples are collected, tree shrews were put into the open field box for 15 min. An Any-maze Animal Behavior Video Analysis System (ST-60000, Sterling, USA) was set to analyze the distance travelled in the box for evaluation of locomotor behavior.

Total Sleep Time

To monitor the sleep of the tree shrews, an infrared night-vision pinhole camera in the nest box was installed before the whole procedure. The monitoring started at 20:00 and ended at 08:30 the next morning. The time of the animals fell asleep and woke up was manually calculated by persons not involved in the experimental design nor drug administration for avoiding bias.

Magnetic Resonance Imaging

In Vivo MRI Scanning

All *in vivo* MRI scans were carried out on a 7.0T animal MRI scanner (Biospec 7T/20 USR, Bruker BioSpin GmbH, Germany). Nine of fifteen animals involved in locomotor analysis and three naïve animals were selected for MRI. Tree shrews were fasted overnight before each scanning session. Before placing them in a prone position on the scanning bed, we anesthetized them with isoflurane (5% for induction and 1.5–2.0% for maintenance) (Huang et al., 2018). T2-weighted anatomical images were acquired with Rapid Acquisition with Relaxation Enhancement (RARE) sequence (RARE factor = 8, repetition time = 2838.2 ms, echo time = 33 ms, matrix size 256 × 256 × 27, voxel size 0.06 × 0.04 × 1.00 mm^3, no slice gap). All the Bruker original images were converted to the DICOM format with software programs (Paravision 4.0) in the scanner.

MRI Data Processing

Voxel-based morphometry analysis was performed using MATLAB R2014a and SPM12 (Ashburner and Friston, 2000). First, the voxel size of all images was scaled by a factor of six to better approximate the human size. Then the images were spatially normalized into the stereotaxic space (Ni et al., 2016; Huang et al., 2018) and segmented into grey matter (GM) and white matter (WM) probability maps using the unified segmentation approach (Ashburner and Friston, 2005). After that, the maps were smoothed by a Gaussian kernel of three times of its voxel size. Finally, a two-sample t test was conducted to compare the difference between saline/ketamine/WMT and baseline group, as well as ketamine and WMT group, for GM and WM density respectively. In the statistical analysis, voxels with a value of <0.2 were excluded to avoid possible edge effects around the borders between the tissue classes and to include only voxels with sufficient tissue class proportions. The statistical significance was set at $p < 0.01$ and a cluster extent threshold of 155 voxels, where no significant difference survived between the negative controls.

Statistical Analysis

All data were analysed using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) or GraphPad (version 5; GraphPad Software, San Diego, CA, USA). Wilcoxon rank-sum test, independent t test and paired-samples t test were used to analyze differences between

two groups. More than two groups were analyzed by one-way ANOVA test followed by LSD Post Hoc test. A probability level less than 0.05 was considered as statistical significance except for MRI analysis.

RESULTS

Physiology, Behaviors and Brain Structure of Tree Shrews Were Altered After Staying Up Late by Light Exposure

An overview of the experimental procedure timeline can be seen in **Figure 1A**. Increased light exposure until midnight for 21 days alters physiological parameters of tree shrews. One-way ANOVA analysis showed a remarkable decrease in body weight [$F(42,2) = 21.572$, $p < 0.001$] and distance travelled in 15 min [$F(42,2) = 7.638$, $p = 0.001$] after three weeks of extended light exposure (**Figures 1B, C**). A LSD Post Hoc analysis revealed that body weight was more negatively affected by light-induced stress than locomotor behavior. It has already a significant decrease after 1 week ($p < 0.001$), and this decrease continued downwards into the third week ($p < 0.001$ compared to baseline, $p < 0.05$ compared to T-S1wk). The timing of sleep onset and waking up was monitored by an infrared night-vision pinhole camera in their next box. During the baseline phase, the animal usually woke up around 7:44 before the light turned on and fell asleep around 20:06 after the light turned off (**Table S1**). However, after extended light exposure, the total sleep duration of the tree shrews decreased significantly as seen in infrared monitoring [$F(42,2) = 17.910$, $p < 0.001$, **Figure 1D**]. This was mostly due to a delayed sleep onset (**Table S1**). Cortisol, a major stress hormone released by the adrenal gland, has been implicated in stress-related diseases when levels are chronically elevated (Pariante and Lightman, 2008). In tree shrews, like in humans, cortisol but not corticosterone is the main stress-related hormone (Wang et al., 2013b). Compared to their baseline, they exhibited a higher level of morning urinary cortisol in the first week ($p < 0.05$, **Figure 1E**).

For each group we performed two MRI scans: one at the beginning of the experiment when the tree shrews are still housed in a normally lighted environment (T-Bas), and another after the recovery phase (the day after T-R10d). The alterations in brain structure of the tree shrews in the saline group were considered to be caused solely by light-induced stress. Comparing between the saline group and the baseline, results demonstrated that there was a significant increase in the density of gray matter (GM) in the frontal cortex, somatosensory cortex, visual cortex and thalamus after staying up late, and a robust decrease in white matter (WM) density in the corpus callosum and thalamus (**Figures 1F, G** and **Table 1**). The behavioral and cortisol tests indicated that stress was successfully induced across groups, and that tree shrews that stay up longer at night due to light have alterations in the structure of their brain.

Gut Microbiota of Tree Shrews Were Changed After Extended Light Exposure

Previous studies indicated that stress could perturb the composition of the gut microbiota and affect host behavior (Foster et al., 2017). Thus, we collected the fecal samples and performed gut microbiota 16S rRNA gene sequencing in tree shrews before and after introducing extended environmental lighting. As shown in **Figure 2**, the α-diversity of the gut microbiota increased significantly after 21 days of extended light exposure [Chao 1 index: $F(42,2) = 6.619$, $p = 0.003$; Sobs index: $F(42,2) = 8.245$, $p = 0.001$]. Especially in the third week, both of them had a significant increase compared with the baseline (T-Bas) or the first week test (T-S1wk) (**Figures 2A, B**). We observed a trend where the *Firmicutes*-to-*Bacteroidetes* ratio went up and down (**Figure 2C**). The median relative abundances of the *Proteobacteria* and *Actinobacteria* increased along a decrease in *Firmicutes* and *Bacteroidetes* (**Figure 2D**). The β-diversity of the gut microbiota, described by principal components analysis (PCA) and represented in the heat map, revealed a remarkable alteration after extended environmental illumination (**Figures 2E, F**). Further correlation analysis between changes of microbiota and alterations of physiological responses (**Table S2**) demonstrated that targeting microbiota could be a potential method of treatment for neuropsychiatric disorders.

TABLE 1 | Brain regions of GM and WM density change after staying up late in saline group compared with baseline phase.

Brain region	Cluster location	Coordinate			Cluster size (mm^3)	T value	P
		x	y	z			
Increase							
Frontal cortex	**DFC**	−3	4	7	7.97	4.67	0.000***
Somatosensory cortex	**PvA**	6	5	0	8.91	5.06	0.000***
Visual cortex	**V1**	1	1	−8	2.64	4.16	0.001**
Thalamus	**IC**	−2	7	−10	2.67	5.96	0.000***
Decrease							
Corpus callosum	**cc**	4	3	3	3.47	3.99	0.001**
Thalamus	**IC**	−2	7	−11	3.23	4.64	0.000***

*Cluster location represented for the subregion of maximum t-value in the brain region according to the fine stereotaxic brain atlas of the tree shrew (Zhou and Ni, 2016). Coordinates (x, y, z): the coordinates in 3D stereotaxic coordinate system accordant with the histological atlas. Cluster size: the volume of a cluster. T value: the maximum t-value in each cluster. P, the maximum confidence level in each cluster. **p <0.01, ***p <0.001.*

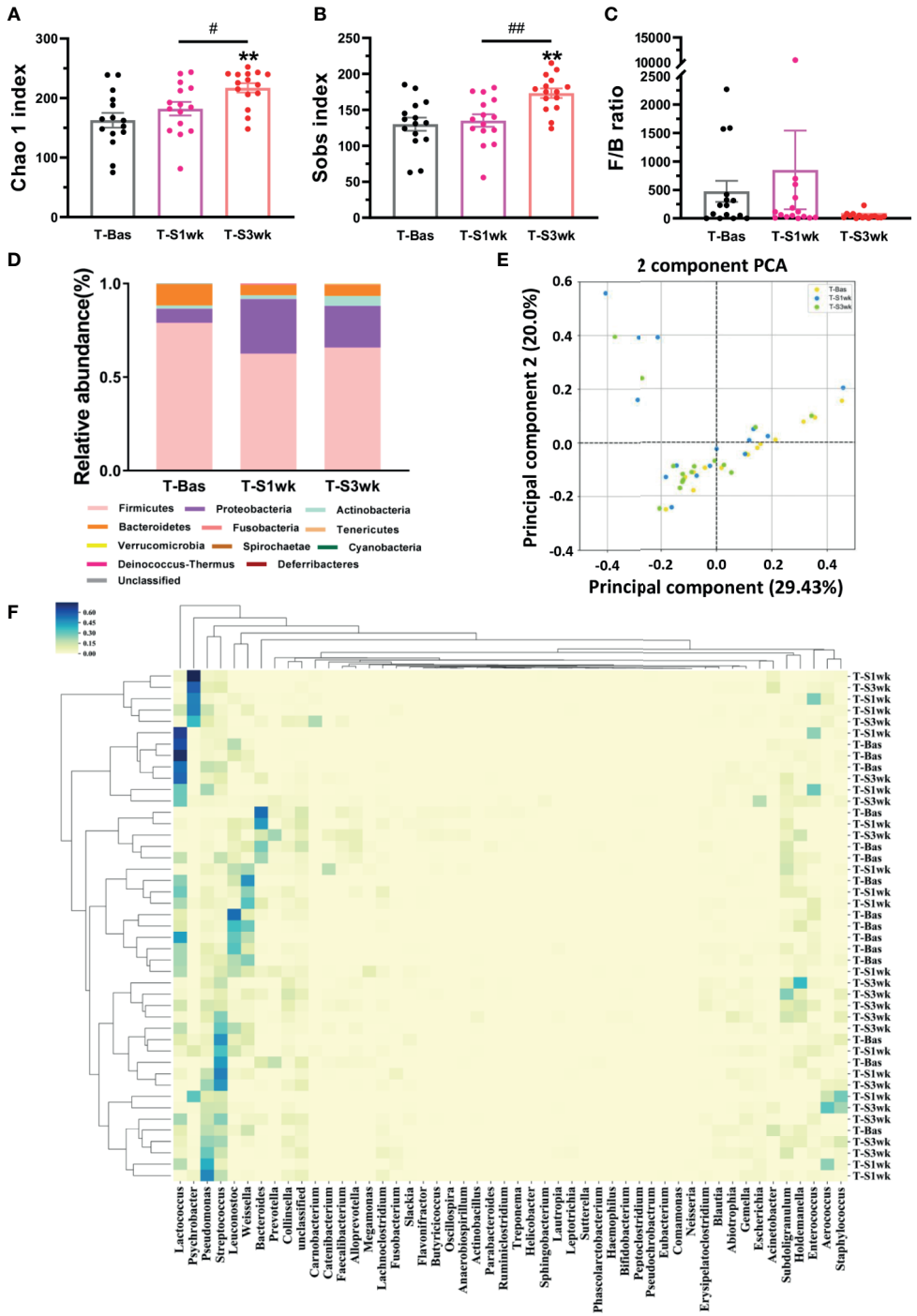

FIGURE 2 | The variations of gut microbiota composition and biodiversity after staying up late for 21 days in tree shrews. **(A)** The Chao 1 index significantly increased at T-S3wk in light-induced stress phase (LSD Post Hoc: compared with T-Bas, p = 0.001, compared with T-S1wk, p = 0.026). **(B)** The Sobs index showed an increased level at T-S3wk in light-induced stress phase (LSD Post Hoc: compared with T-Bas, p = 0.001, compared with T-S1wk, p = 0.002). **(C)** The ratio of *Firmicutes*-to-*Bacteroidetes* (F/B ratio) increased at T-S1wk but decreased at T-S3wk. **(D)** The median relative abundance of the *Firmicutes* and *Bacteroidetes* decreased but that of the *Proteobacteria* and *Actinobacteria* increased. **(E)** The PCA on gut microbiota community of the tree shrews at baseline was different from that in the third week after stress. Each dot stands for one sample. **(F)** The heat map presented the microbial distribution of the tree shrews at baseline was different from that in the first week and the third week after stress. N = 15. Error bars show the SEM. **means compared with control in baseline phase, **p <0.01. # means compared between each other in stress phase, #p <0.05, ##p <0.01.

Basic Gut Microbiota Structure Showed Similarities and Differences Between Healthy Humans and Tree Shrews

To preliminarily characterize the differences of gut microbiota composition and bio-diversity between healthy tree shrews and humans, we enrolled 15 healthy people from fmtBank and collected their fecal samples for 16S rRNA and did the same for 15 tree shrews. The results showed that humans had thirteen phyla and tree shrews had ten phyla. There were seven common phyla, including *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Tenericutes* and *Verrucomicrobia*. Similarities and differences were observed between humans and tree shrews with probiotic implications. Humans had a higher relative abundance of *Bacteroidetes*, while tree shrews seemed to have a lower and higher relative abundance of *Bacteroidetes* and *Proteobacteria* respectively (**Figures 3A, B**). The basic structure of microbiome in healthy humans is more anti-stress than healthy tree shrews. The α-diversity of tree shrew gut microbiota demonstrated that they have a poorer microbiota biodiversity compared to humans (**Figures 3C, D**).

WMT Reversed the Effects of Staying Up Late on Physiological Parameters Partially and the Changes on Brain Structure Effectively

We investigated whether WMT can remediate the alterations brought on by extended environmental lighting. Because healthy tree shrews are already prone to stress and have a higher baseline of *Proteobacteria*, the fecal microbiota from one human donor was administrated to light-stressed tree shrews. The enriched microbiota by washing process was prepared from one defecation of one healthy donor which was primarily screened for clinical medicine (Fecal Microbiota Transplantation-standardization Study, 2020; Zhang et al., 2020). To compare the therapeutic effect of WMT, ketamine, a rapid antidepressant (Berman et al., 2000), was used as the positive control and saline was used as the negative control. Body weight and distance travelled continuously decreased in the saline group (**Figures 4A, B**). Considering that the change of body weight is a long-term process, we only measured body weight at the end of 3 weeks of extended light exposure and 72 h after treatment administration. The ketamine group had a fast recovery of body weight 72 h after administration [T-R72h compared with T-Bas: $t(4) = 1.115$, $p = 0.327$, **Figure 4A**] and the distance travelled improved, then dipped, then improved again 24 h, 72 h and 10 days after administration respectively [T-R24h compared with T-Bas: $t(4) = 1.913$, $p = 0.128$; T-R10d compared with T-Bas: $t(4) = 1.572$, $p = 0.19$, **Figure 4B**]. Moreover, the WMT group showed a similar recovery efficacy as ketamine, with a quick recovery of body weight 72 h after treatment [T-R72h compared with T-Bas: $t(4) = 0.746$, $p = 0.497$, **Figure 4A**]. The improvement of locomotion of tree shrews after WMT took longer and returned to baseline after 10 days [T-R10d compared with T-Bas: $t(4) = 0.333$, $p = 0.756$, **Figure 4B**]. The total sleep time and urine cortisol levels in both ketamine and WMT groups statistically did not differ from the saline group (**Figures 4C, D**). However, the sleep onset time in WMT group was 1 h earlier than that in saline and ketamine

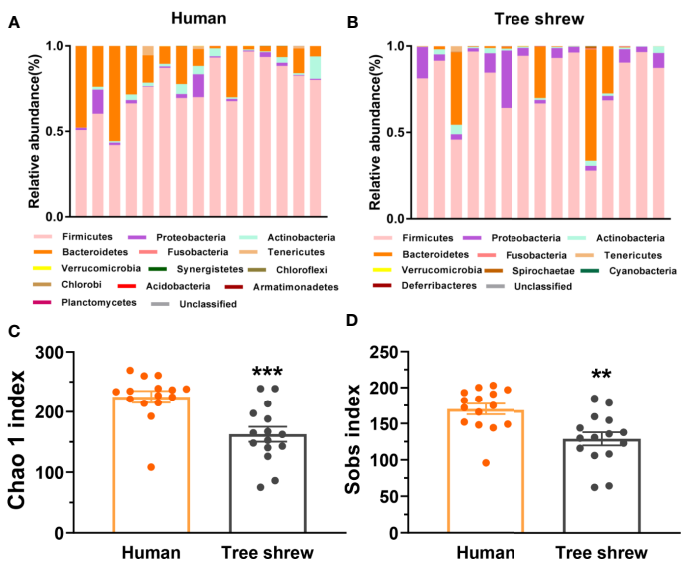

FIGURE 3 | Differential profiles of the gut microbiota composition and α-diversity between healthy humans and tree shrews. **(A, B)** The bar charts of gut microbiota abundance of healthy humans (**A**, N = 15) and tree shrews (**B**, N = 15). **(C, D)** Analysis of the α-diversity by independent t test showed that the Chao 1 index [**C**, $t(28) = -3.948$, $p = 0.000$] and Sobs index [**D**, $t(28) = -3.416$, $p = 0.002$] was lower in tree shrews than humans. Error bars show the SEM. **p <0.01, ***p <0.001.

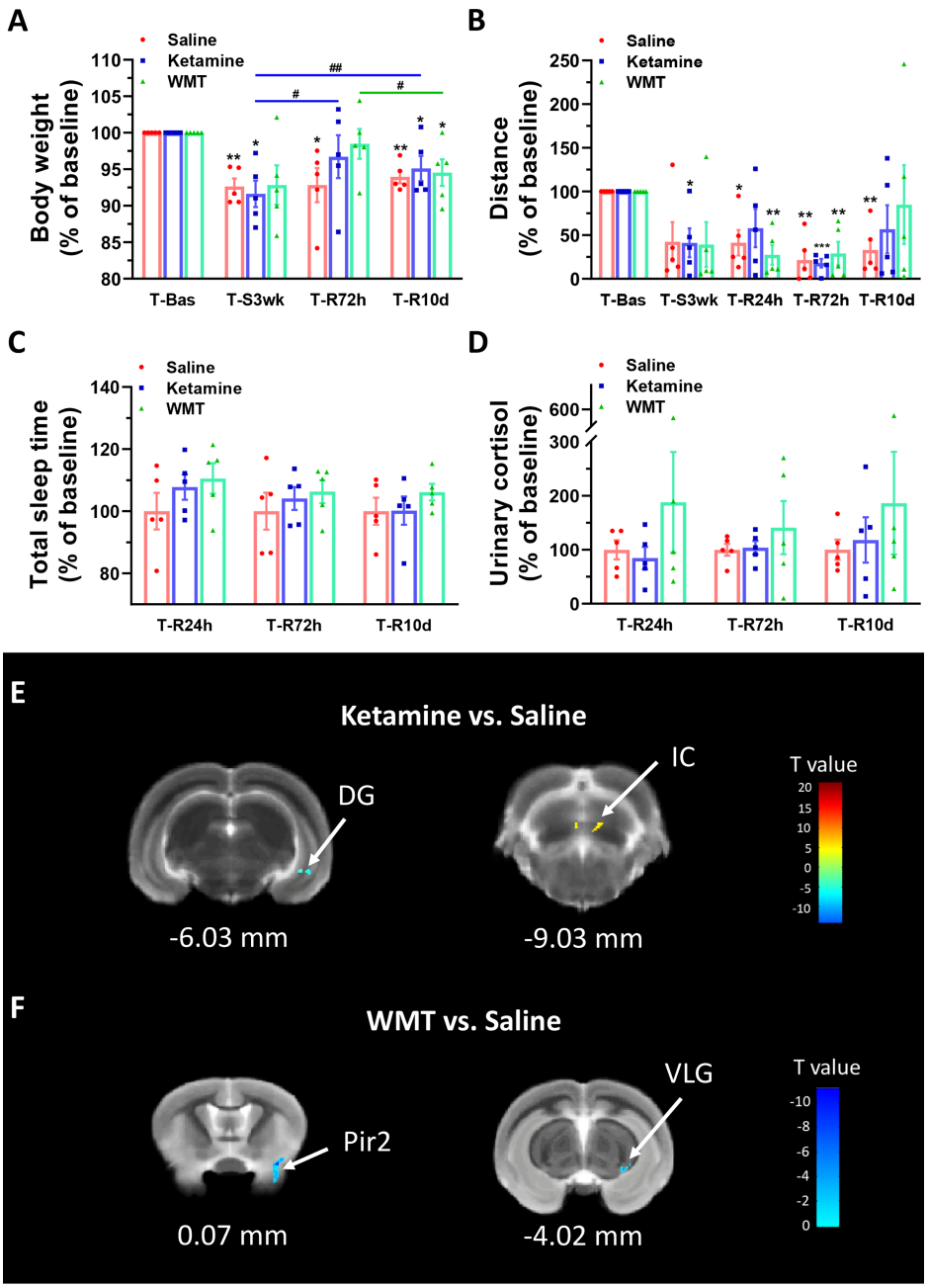

FIGURE 4 | Physiological responses and changes in grey matter density of brain structure in tree shrews under different treatments during the recovery phase. **(A, B)** Body weight and distance travelled in the open field box under saline, ketamine and WMT. They were normalized with the values in the baseline (T-Bas) by using paired-samples t test. **(A)** In the saline group (the red column, N = 5), the body weight was decreased in T-S3wk [t(4) = 6.735, p = 0.003], T-R72h [t(4) = 3.075, p = 0.037] and T-R10d [t(4) = 7.093, p = 0.002]. In the ketamine group (the blue column, N = 5), the body weight was temporarily recovered at T-R72h to a baseline level and was improved compared to T-S3wk [t(4) = 3.509, p = 0.025], then dropped a bit yet was still higher at T-R10d [t(4) = 6.945, p = 0.002] compared to T-S3wk. In the WMT group (the green column, N = 5), tree shrews gained more weight in T-R72h than T-R10d [t(4) = 4.916, p = 0.008]. **(B)** In the saline group (the red column, N = 5), distance travelled in the open field box was decreased in T-R24h [t(4) = 4.013, p = 0.016], T-R72h [t(4) = 6.585, p = 0.003] and T-R10d [t(4) = 5.216, p = 0.006]. In the ketamine group (the blue column, N = 5), the distance was decreased in T-S3wk [t(4) = 3.532, p = 0.024] and T-R72h [t(4) = 17.117, p = 0.000]. In the WMT group (the green column, N = 5), the distance showed a decrease in T-R24h [t(4) = 6.457, p = 0.003] and T-R72h [t(4) = 5.293, p = 0.006]. **(C, D)** Total sleep time (C, N = 5/group) and morning urinary cortisol (D, N = 5/group) of tree shrews under saline, ketamine and WMT treatments. They were normalized by comparing with the saline group to avoid errors from humans and instruments. **(E)** The signal from DG (dentate gyrus of the hippocampus) decreased but that from IC (inferior colliculus) increased after ketamine administration (N = 3/group). **(F)** The signals from Pir2 (piriform cortex, layer 2) and VLG (ventral lateral geniculate nucleus) decreased after WMT (N = 3/group). Error bars show the SEM. *means compared with control in baseline phase, *p <0.05, **p <0.01, ***p <0.001. # means compared between the stress and recovery phase, #p <0.05, ##p <0.01.

TABLE 2 | The change of grey matter density in ketamine and WMT groups compared with saline group.

A

Brain region	Cluster location	Coordinate			Cluster size (mm^3)	T value	P
		x	y	z			
Increase							
Thalamus	**IC**	−1	7	−9	2.90	8.73	0.000***
Decrease							
Hippocampus	**DG**	−6	10	−6	3.18	14.00	0.000***

B

Brain region	Cluster location	Coordinate			Cluster size (mm^3)	T value	P
		x	y	z			
Decrease							
Piriform cortex	**Pir2**	−5	10	0	3.20	11.09	0.000***
LGN	**VLG**	−4	9	−4		8.65	0.000***

*(A) Changed GM density of ketamine group compared with saline group. (B) Changed GM density of WMT group compared with saline group. ***p <0.001.*

groups (**Table S3**). Our analyses showed that ketamine and WMT had partial positive effects on the unfavorable influences of staying up late.

In order to characterize the therapeutic and recovery effects of ketamine and WMT, we performed two kinds of MRI comparisons. One pair is between the ketamine or WMT group and saline group, the other is between the ketamine or WMT group and baseline. Firstly, by comparing with the saline group, it was exhibited that the GM density of the ketamine group had a decrease in the hippocampus and an increase in the thalamus (**Figure 4E** and **Table 2A**). The signals of GM in the piriform cortex and lateral geniculate nucleus (LGN) decreased significantly in the WMT group (**Figure 4F** and **Table 2B**). Secondly, by comparing with the baseline, it was found that the GM and WM density changes by the light-induced stress in the frontal cortex, thalamus and corpus callosum still existed in the ketamine group, but disappeared in the WMT group (**Figure 5** and **Table 3**). New changes in the motor cortex (**Figure 5A** and **Table 3A**), anterior olfactory nucleus and cerebellum (**Figure 5B** and **Table 3A**) were observed in the ketamine group. Although the low dose of ketamine did not induce a phenotypic addictive response, the structural changes in the brain suggest that there might still be a slight risk of addiction. Note, that by comparing between the WMT group and baseline, we found that the trend of the whole brain density change was opposite to that after light-induced stress (**Figures 1F, G** and **Table 1**). The GM density in insular cortex and piriform cortex showed a significant decrease after WMT (**Figure 5C** and **Table 3B**). The WM density in corpus callosum, internal capsule and hippocampus were significantly increased after WMT (**Figure 5D** and **Table 3B**). The results indicated that WMT could contribute to the recovery-like effects for staying up late in tree shrews.

DISCUSSION

The present study successfully established a staying up late model in tree shrew by prolonging the light at night for 4 h. The behaviors, microbiota composition and brain signals were changed by the light-induced stress. Part of these alterations could be rescued by WMT. The present finding highlighted that tree shrews are light-stress sensitive animal model with close microbiota composition between the healthy tree shrews and humans.

Staying Up Late Model

With the advent of the incandescent bulb, unnaturally timed artificial light from the environment can and has disrupted our biological rhythms (Bedrosian and Nelson, 2013). To study this, we developed a staying up late model. From the point of view of sleep deprivation, it expands on the various ways of sleep depriving to accurately mimic the situation progressively more persons find themselves in (Colavito et al., 2013). The animal was not forced per se to stay awake. Light was just kept on longer and by consequence total sleep time decreased and was thus sleep deprived (Bandyopadhyay and Sigua, 2019). The extended light exposure at night resulted in a body weight loss, reduced locomotor activities, a loss of total sleep time and an elevated cortisol level, and differences in microbiota composition illustrating the drastic effects of an otherwise simple environmental change. This model can assist further explorations on how our modern lifestyle affects our health. It could aid studies of sleep, rhythm disturbances and stress as both dimensions reside in this model. We speculate that to a (limited) extent it might even be informative for depression research as aforementioned factors intersect with depression. Some similar symptoms of the model have been described in a chronic psychosocial stress model of tree shrews (Fuchs et al., 2001; Wang et al., 2013a; Fang et al., 2016). There, male tree shrews are used and show depressive-like symptoms due to social defeat (Meyer et al., 2001). It requires tree shrews to fight with each other, and is at times variable and unpredictable. The staying up late model is based on controllable light exposure and straightforward.

Light-Induced Stress and Microbiota

The Earth's rotation brings about a cyclical light-dark phase change, on which almost all living organisms have adapted their

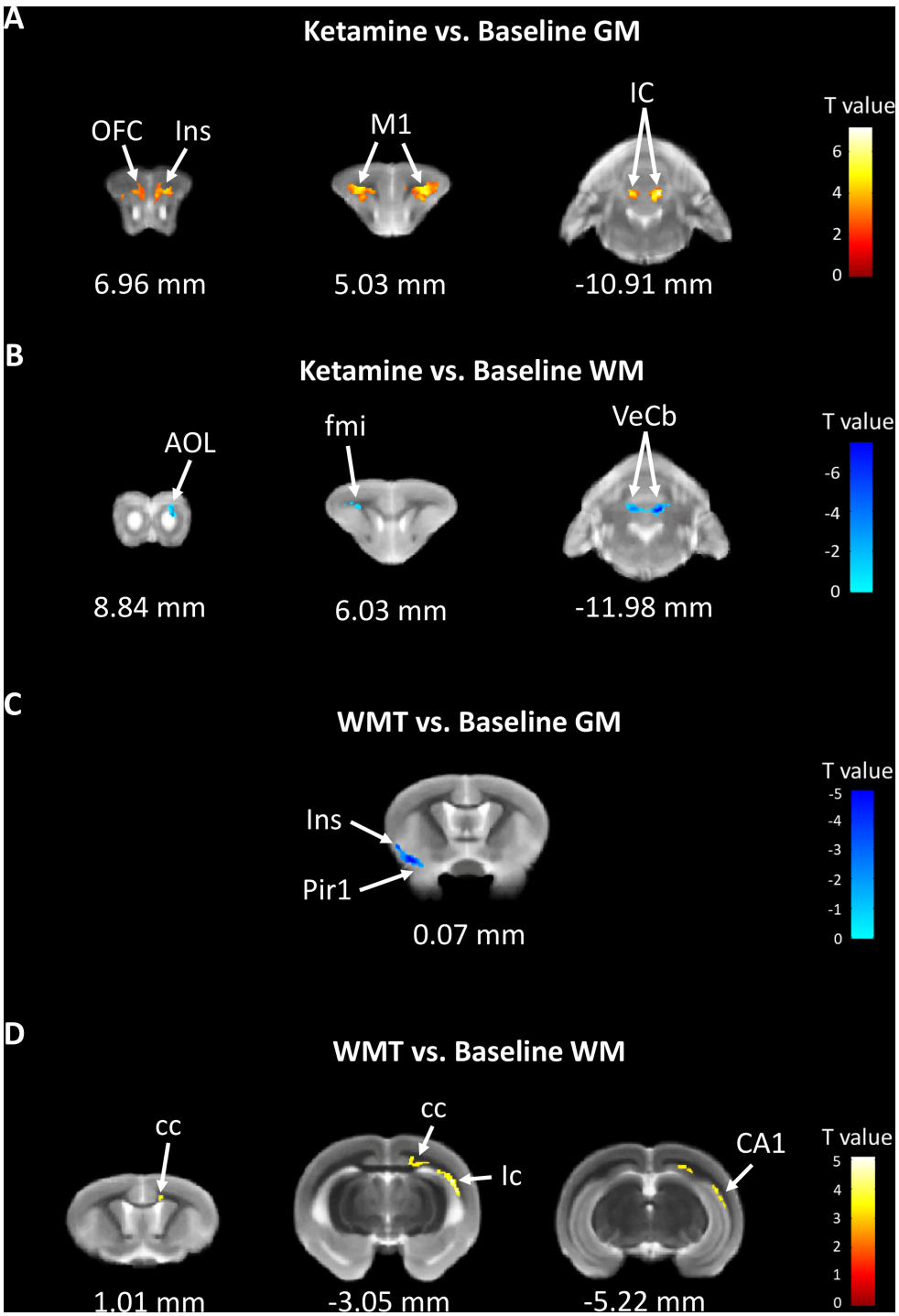

FIGURE 5 | The changes in brain structure showed the recovery effect of WMT but potential addiction effect of ketamine. **(A)** The GM density signals from OFC (orbital frontal cortex), Ins (insular cortex), M1 (primary motor cortex) and IC (inferior colliculus) were increased, but **(B)** the WM density signals from AOL (anterior olfactory nucleus, lateral part), fmi (forceps minor of the corpus callosum) and VeCb (vestibulocerebellar nucleus) were decreased after ketamine administration. **(C)** The GM density signals from Ins and Pir1 (piriform cortex, layer 1) were decreased, but **(D)** the WM density signals from cc (corpus callosum), ic (internal capsule) and CA1 (field CA1 of the hippocampus) were increased after WMT. N = 3/group.

TABLE 3 | Brain regions changes in tree shrews with the treatment of ketamine and WMT by compared with baseline phase.

A

Brain region	Cluster location	Coordinate			Cluster size (mm^3)	T value	P
		x	y	z			
Increase							
Insular cortex	**Ins**	3	5	7	8.16	3.92	0.001**
Motor cortex	**M1**	–4	4	5	9.44	3.55	0.002**
Thalamus	**IC**	–1	7	–11	3.96	7.14	0.000***
Decrease							
Anterior olfactory nucleus	**AOL**	–2	6	9	2.46	7.07	0.000***
Corpus callosum	**fmi**	3	4	6	2.93	5.31	0.000***
Cerebellum	**VeCb**	–1	8	–12	4.49	7.44	0.000***

B

Brain region	Cluster location	Coordinate			Cluster size (mm^3)	T value	P
		x	y	z			
Decrease							
Insular cortex	**Ins**	7	8	0	3.42	3.82	0.001**
Piriform cortex	**Pir1**	6	9	–0		36.9	0.002**
Increase							
Corpus callosum	**cc**	–2	3	1	4.97	4.07	0.001**
Internal capsule	**ic**	–7	6	–3	3.30	5.08	0.000***
Hippocampus	**CA1**	–7	6	–5		3.67	0.002**

*(A) Changed GM and WM density of ketamine group compared with baseline. (B) Changed GM and WM density of FMT WMT group compared with baseline. **p < 0.01, ***p < 0.001.*

physiology in sophisticated fashions. Fiddling with it and extended light exposure at night can then disrupts circadian rhythms leading to, in humans, the prevalence of psychiatric and behavioral disorders (Lambert et al., 2015). Along the gut-brain axis a recent investigation revealed that 24 h of continuous light- or dark-induced stress for 12 weeks influenced the memory and the composition of gut microbiota in mice (Kim et al., 2019). The observations of Cui et al. showed that prolonging the time of exposure to light could shape the gut microbiota composition in mice (Cui et al., 2016). Meanwhile, the gut microbiota, in turn, regulated the circadian rhythm of host metabolism (Kuang et al., 2019). Consistent with this, our observation showed that significant changes in the gut microbiota composition were found after only 4 h extended light exposure in tree shrews.

Firmicutes and *Bacteroidetes* present the main groups in gut microbiota (Kelly et al., 2016). Studies revealed that there was a decrease in *Firmicutes* but an increase in *Proteobacteria* in irritable bowel syndrome (IBS) and major depressed patients. The ratio of the *Firmicutes*-to-*Bacteroidetes* was also increased in IBS patients compared with healthy controls (Krogius-Kurikka et al., 2009; Inserra et al., 2018). Similarly, tree shrews under light-induced stress for one week showed a decrease of *Firmicutes*, an increase of *Proteobacteria*, and a higher ratio of *Firmicutes*-to-*Bacteroidetes*. Recent evidences showed that microbiota played a key role in circulating metabolites by altering the production of short-chain fatty acids (Komaroff, 2017; Vojinovic et al., 2019). A study comparing the microbiota of obese children with that of lean children provided evidence that the *Firmicutes*-to-*Bacteroidetes* ratio of obese children was much higher (Riva et al., 2017). Therefore, the reduction of the ratio of the *Firmicutes*-to-*Bacteroidetes* might be related to the body weight loss at the end of light-induced stress phase in tree shrews.

Gut Microbiota Changed the Structure of the Brain

Lines of evidence in both clinical and animal research suggest that the gut–brain axis can be a new therapeutic angle for central nervous system disorders (Long-Smith et al., 2020). Though few pilot studies observed the role of WMT on typical brain diseases (Xu et al., 2021), such as epilepsy (He et al., 2017), little is known on how WMT could alter brain structure. By comparing the GM density before and after the light-induced stress in our model, the MRI results revealed an increase in environmental light affected the frontal cortex and sensory system, particularly the visual system. Interestingly, these changes disappeared after WMT. This was accompanied by a robust decrease in the GM density from the insular and piriform cortex. The insular cortex is involved in consciousness in humans, and plays a role in emotion regulation. Evidence from rhesus monkeys revealed that the insular cortex was associated with the amygdala (Mufson et al., 1981). There is evidence that the posterior insula connects with the somatosensory cortex and involves in audio–visual integration tasks (Bushara et al., 2001). The piriform cortex, mainly contributing to ensemble coding of odor, was located between the insular cortex and the anteriorly and laterally of the amygdala in humans (Howard et al., 2009). Surprisingly, the anatomy of this location in tree shrews was strikingly similar to the humans, evincing of the close relationship of tree shrews to primates in regard of brain structure.

The WM density of corpus callosum, internal capsule and hippocampus increased significantly between the WMT

treatment and the baseline, which is consilient with previous engrossing explorations. The study from Cryan's lab indicated that the microbiota was necessary for appropriate cortical myelination at an ultrastructural level (Hoban et al., 2016). Gut dysbiosis in neonatal C57BL/6 mice can potentially alter myelination and thus impair cognition (Keogh et al., 2021). This provides direct evidence that gut microbiota plays an essential role in basic neurogenerative processes.

Additionally, this study compared the therapeutic effect of different treatments on the brain structure, and proved that ketamine treatment mainly affects the hippocampus (Price and Duman, 2020). The present findings showed that WMT mainly affected the piriform cortex and LGN. Therefore, ketamine acted different way from WMT in brain, at least in light-induced stress model.

In conclusion, we successfully established a staying up late model in tree shrews to explore the effects of extended light exposure on physiology, behavior and brain structure. Our findings indicated that this model could be informative for researching stress-sensitive diseases and that the strategy of targeting the gut-brain axis could perhaps one day bare therapeutic fruit.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Second Affiliated Hospital of Nanjing Medical University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Experimental Animal Ethics Committee at the Nanjing Medical University. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JW, JG and FZ designed the research. JW and ML participated in behavior and MRI experiments. PL and QL performed microbiota study and chemical tests. MZ prepared for the special open field box for tree shrews. JD and JX recorded the sleep video. JW, QL and QH analyzed data. JW, QL, FZ and JG drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.685019/full#supplementary-material

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Conflict of Interest: FZ invented the concept of GenFMTer and devices related to it.

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

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Lactulose Improves Neurological Outcomes by Repressing Harmful Bacteria and Regulating Inflammatory Reactions in Mice After Stroke

Quan Yuan, Ling Xin, Song Han, Yue Su, Ruixia Wu, Xiaoxuan Liu, Jimusi Wuri, Ran Li and Tao Yan *

Department of Neurology, Tianjin Medical University General Hospital, Tianjin Neurological Institute, Key Laboratory of Post-Neurotrauma, Neurorepair, and Regeneration in Central Nervous System, Ministry of Education and Tianjin City, Tianjin, China

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***Correspondence:**
Tao Yan
taoyan@tmu.edu.cn

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Background and Objective: Gut microbiota dysbiosis following stroke affects the recovery of neurological function. Administration of prebiotics to counteract post-stroke dysbiosis may be a potential therapeutic strategy to improve neurological function. We aim to observe the effect of lactulose on neurological function outcomes, gut microbiota composition, and plasma metabolites in mice after stroke.

Methods: Male C57BL/6 mice (20–25 g) were randomly divided into three groups: healthy control, photothrombotic stroke + triple-distilled water, and photothrombotic stroke + lactulose. After 14 consecutive days of lactulose administration, feces, plasma, and organs were collected. 16S rDNA sequencing, plasma untargeted metabolomics, qPCR, flow cytometry and Elisa were performed.

Results: Lactulose supplementation significantly improved the functional outcome of stroke, downregulated inflammatory reaction, and increased anti-inflammatory factors in both the brain and gut. In addition, lactulose supplementation repaired intestinal barrier injury, improved gut microbiota dysbiosis, and partially amended metabolic disorder after stroke.

Conclusion: Lactulose promotes functional outcomes after stroke in mice, which may be attributable to repressing harmful bacteria, and metabolic disorder, repairing gut barrier disruption, and reducing inflammatory reactions after stroke.

Keywords: stroke, gut microbiota, lactulose, dysbiosis, microbiota-gut-brain-axis

INTRODUCTION

As technology and science advance, a complex interaction among the brain, gut, and microbiota residing in the gut, which comprises the concept of a microbiota-gut-brain axis, has been gradually accepted (Cryan et al., 2019). Five pathways (Wang and Wang, 2016) related to neuroanatomy, neuroendocrine function, gut immune responses, metabolism, and barriers communicate in the microbiota-gut-brain axis. Stroke causes gut dysfunction, which involves increased intestinal permeability and dysmotility, leading to gut dysbiosis. Increased intestinal permeability might lead to bacterial translocation, which may result in post-stroke complications, such as pneumonia (Stanley et al., 2016). However, the results of dysbiosis after stroke differ in various studies. For example, altered short-chain fatty acid (SCFA)-producing bacteria were observed in several studies. Chuhong Tan et al. (2020) found decreased SCFA-producing bacteria and fecal SCFA levels in acute ischemic stroke patients, while Na Li et al. (2019) found enriched SCFA-producing genera, including *Odoribacter* and *Akkermansia*. Targeting the microbiota-gut-brain axis provides important new directions to treat or prevent stroke and its complications. In fact, therapies involving antibiotics (Benakis et al., 2016; Winek et al., 2016), fecal microbiota transplantation (Spychala et al., 2018; Chen et al., 2019), and prebiotic intervention (Anderson et al., 2009) have been used to treat stroke.

Lactulose, a common prebiotic composed of fructose and galactose, has many potential applications in the food and pharmaceutical industries (Nooshkam et al., 2018). It promotes probiotic bacteria growth, suppresses pathogenic bacteria, and has been widely used to treat hepatic encephalopathy and chronic constipation due to its ability to decrease blood ammonia levels, acidify gut contents, soften stool, and promote bowel movement (Schumann, 2002). Recently, an *in vitro* study explored the prebiotic effect of lactulose under various dosages, and a dose-dependent effect of lactulose on gut microbiota and SCFAs was found (Bothe et al., 2017). Clinically, lactulose is often used to treat post-stroke constipation, with a concentration of 66.7%. Several previous studies (Zhai et al., 2013; Mao et al., 2016; Zhai et al., 2018; Zhang et al., 2019a) showed that a lower concentration of lactulose had beneficial effects on normal or diseased mice. Zheng Zhang et al. (2019a) found that *Bifidobacterium* and *Bacteroides* and many metabolites including SCFAs were significantly increased in pregnant mice after 2 weeks of 15% lactulose intervention. Another study (Zhang et al., 2019b) found that 4 weeks of 15% lactulose intervention significantly lowered blood pressure increased by high-salt diets, decreased inflammatory factor expression, increased the abundance of *Bifidobacterium* and *Alloprevotella*, and altered fecal metabolic profiles. Furthermore, Shixiang Zhai et al.(2018) found that 3 weeks of lactulose intervention promoted hydrogen-producing bacteria (*Prevotellaceae* and *Rikenellaceae*), probiotics (*Bifidobacteriaceae* and *Lactobacillaceae*), and mucin-degrading bacteria (*Akkermansia* and *Helicobacter*) and decreased the abundance of *Desulfovibrionaceae* and harmful metabolites in normal mice. Recently, Xiao Chen et al. (2020) found that 6 weeks of lactulose intervention altered gut microbiota, increased SCFA levels, and ameliorated bone loss induced by lack of estrogen.

However, the effect of a low concentration of lactulose on stroke, including whether it can modulate gut dysbiosis and metabolic disorders and help improve outcomes after stroke, is unknown. Although several studies have associated lactulose with microbiota or metabolites, the direct effects of lactulose on stroke outcomes have not been determined. An understanding of how lactulose contributes to stroke outcomes may enable its use as a therapeutic target. In this study, we tested the hypothesis that 15% lactulose could improve stroke outcomes by examining inflammatory factor expression and fecal flora and plasma metabolite composition using omics technologies. Overall, we found that lactulose had an anti-inflammatory effect on both the brain and gut and partially corrected metabolic disorders and dysbiosis. The current data support a positive effect of lactulose on stroke outcomes.

MATERIALS AND METHODS

Experimental Design

Six to eight-week-old male C57BL/6 mice (20–25 g) were purchased from HFK Bioscience Corporation (Beijing, China). In the experimental period, mice were allowed to eat and drink freely in a room with a natural light cycle. All mice were randomly divided into three groups: healthy control (HC), photothrombotic stroke + triple-distilled water (PTS_TDW), and photothrombotic stroke + lactulose (PTS_LAC, Yuanye Bio-Technology Co., Ltd, Shanghai, China, with concentration of 15%, 150 μL per day.). A concentration of 15% lactulose was used because previous studies (Mao et al., 2016; Zhang et al., 2019b) indicated that this concentration was the most suitable for exhibiting prebiotic and anti-inflammatory effects. All experiments in this study were approved by the Tianjin Medical University General Hospital Animal Care and Use Committee. After one week of adaptation to the new environment, mice in the PTS_TDW and PTS_LAC groups were subjected to photothrombotic stroke modeling. Twenty-four hours after stroke, mice in these groups were treated with triple distilled water or lactulose, respectively, by oral gavage for 14 consecutive days. Eleven mice per group were used for neurological function tests and weight recording, 6 mice per group were used for flow cytometry and omics-related analysis, and 5 mice per group were used for qPCR and Elisa test.

Photothrombotic Stroke Model

This method has been described previously (Yan et al., 2020). Briefly, after anesthetization with 5% chloral hydrate by intraperitoneal injection, the scalp area was shaved and disinfected with iodophor, and then bregma was exposed. Ten minutes after intraperitoneal injection with Rose Bengal dye (10 mg/mL), mice were subjected to 20 minutes of illumination with a fiber-optic bundle of a cold light source. Finally, the incision was sutured and disinfected again.

Neurological Function Tests and Infarct Volume Measurement

A series of neurological functional tests (Yan et al., 2020) including determination of the Modified Neurological Severity Score (mNSS) and the foot-fault test were performed prior to stroke and on days 1, 3, 7, and 14 after stroke by an experimenter who was blinded to the study groups. The mNSS test, ranging from 0 to 18 points, mainly includes motor, sensory, balance, and reflex tests (6 points for motor function, 2 points for sensory function, 6 points for the balance beam test, and 4 points for reflex activities). The foot-fault test was used to evaluate the contralateral motor function deficits. Mice were photographed walking on an irregular grid for a period of time in a quiet environment. The contralateral limb foot faults percentage was determined. A higher score on these two tests indicated a more severe neurological deficit.

Paraffin block from brain was prepared and cut into 8-μm sections. Five coronal sections obtained from the lesion underwent HE staining. The percentage of lesion compared with the contralateral hemisphere was calculated for infarct volume measurement.

Body weight, which reflects the general physical condition of mice and stroke outcome and recovery, was recorded on days 0, 1, 3, 5, 7, and 14.

Quantitative Real-Time PCR

Briefly, total RNA was isolated from the brain and cecum with TRIzol reagent (Invitrogen, CA, USA) at 14 days after stroke. Then, RNA was quantified and reverse transcribed to cDNA using a cDNA Synthesis Kit (Transgen, Beijing, China). PCR reactions were performed with a CFX96 real-time PCR system (BioRad, Hercules, CA, USA). Relative gene expression was calculated using the 2−ΔΔCT method. All primer sequences used are shown below:

GAPDH (Gene ID: 14433):
Forward: GCCAAGGCTGTGGGCAAGGT; Reverse: TCTCCAGGCGGCACGTCAGA

MCP-1 (Gene ID: 20296):
Forward: CTGCTACTCATTCACCAGCAAG; Reverse: CTCTCTCTTGAGCTTGGTGACA

IL-17a (Gene ID: 16171):
Forward: TTTAACTCCCTTGGCGCAAAA; Reverse: CTTTCCCTCCGCATTGACAC

TNFα (Gene ID: 21926):
Forward: TACTCCCAGGTTCTCTTCAAGG; Reverse: GGAGGTTGACTTTCTCCTGGTA

IL-1β (Gene ID: 16176):
Forward: TCCAGGATGAGGACATGAGCAC; Reverse: GAACGTCACACACCAGCAGGTTA

Muc2 (Gene ID: 17831):
Forward: ACGTGTCATATTTGCACCTCT; Reverse: TCAACATTGAGAGTGCCAACT

TLR4 (Gene ID: 21898):
Forward: AGTCAGAATGAGGACTGGGTGAG; Reverse: GTAGTGAAGGCAGAGGTGAAAGC

TGFβ (Gene ID: 21803):
Forward: TGCGCTTGCAGAGATTAAAA; Reverse: CGTCAAAAGACAGCCACTCA

Nrf2 (Gene ID: 18024):
Forward: GGACATGGAGCAAGTTTGGC; Reverse: CCAGCGAGGAGATCGATGAG

Claudin1 (Gene ID: 12737):
Forward: TGTGTCCACCATTGGCATGA; Reverse: CTGGCATTGATGGGGGTCAA

Occludin (Gene ID: 18260):
Forward: TGGCAAAGTGAATGGCAAGC; Reverse: TCATAGTGGTCAGGGTCCGT

Flow Cytometry Analysis

Mice were sacrificed at 14 days after stroke and brain harvested and single-cell suspension prepared. In short, each brain was minced with eye scissors then digested in collagenase IV for 60 minutes. Next, after resuspended in 30% Percoll and centrifugation, the single-cell suspension of the brain was tested by flow cytometry (BD FACSAria, BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software. Antibodies specific to mouse CD45, CD11b, Ly6G, and F4/80 were used (BioLegend, Inc., San Diego, CA, USA).

Elisa

Plasma was obtained by extracting eyeballs, centrifuging (3000rpm, 10 min). 10μL/well was used in three replicates wells to run IL-17a and LPS Elisa (Beijing Gersion Bio-Technology Co., Ltd., Beijing, China) following standard protocol.

16S rDNA Amplicon Sequencing

Fecal genomic DNA was extracted according to the cetyltrimethylammonium bromide/sodium dodecyl sulfate method. DNA was diluted after the concentration and purity were determined. Then, using specific primers, the V3 and V4 variable regions of 16S rDNA were amplified. After PCR reactions were performed, the PCR products were quantified, qualified, mixed, and purified. Then, sequencing libraries were constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA). Finally, the constructed libraries were sequenced using an Illumina HiSeq 2500 instrument. The raw data were deposited in the NCBI-SRA database with the accession number SRP298849 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA686830).

Determination of SCFAs in Feces

Fecal samples were carefully thawed on ice. Then, 30 mg of each sample was added to a centrifuge tube, and 0.5% phosphoric acid, ethyl acetate, and 4-methyl valeric acid were sequentially added and homogenized while supernatants were extracted. An Agilent Model 7890A/5975C gas chromatography-mass spectrometry system (Agilent, Santa Clara, CA, USA) was used for gas chromatography-mass spectrometry analysis, and an MSD ChemStation (Santa Clara, CA, USA) was used to process data to quantify SCFAs.

Untargeted Metabolomics Analysis

Prior to sacrificing the mice, plasma was collected for metabolomics analysis. An ultra-high-performance liquid

chromatography device (Agilent 1290 Infinity LC) coupled with an AB Triple TOF 6600 was used for liquid chromatography tandem mass spectrometry analysis. Multi-dimensional and univariate statistical analyses were performed after raw data were processed.

Correlations

Differentially abundant genera and metabolites were scaled according to the Z-score and concatenated into one matrix. The Pearson algorithm in R Version 3.5.1 was used to calculate the correlation coefficients among all molecules in a matrix because raw data were non-normally distributed.

Statistical Analysis

Normal data were analyzed with GraphPad Prism 8.0 and are presented as the mean ± SEM. Two-way repeated measures ANOVA with Sidak's multiple comparisons test and one-way ANOVA were performed.

RESULTS

Lactulose Supplementation Significantly Improved Long-Term Functional Outcomes and Did Not Affect Body Weight After Stroke in Mice

To evaluate the therapeutic effects of lactulose supplementation in mice with stroke, modified neurological severity score and foot-fault tests were used to evaluate neurological function at 24 hours after stroke. **Figure 1A** shows that lactulose supplementation in mice with stroke significantly decreased the modified neurological severity score at 14 days after stroke. Furthermore, lactulose significantly decreased foot-fault test scores as early as 7 days after stroke as shown in **Figure 1B**. We further calculated infarct volume, and the results showed that lactulose supplementation significantly decreased lesion volumn (**Figure 1C**).

The body weight, which was used to determine the general physical condition of mice, was recorded on days 0, 1, 3, 5, 7, and 14. Mice in the PTS_TDW and PTS_LAC groups showed significant loss of body weight after the operation on day 1. No significant differences were found between these two groups at any time points (**Figure 1D**).

Lactulose Supplementation Significantly Decreased Inflammatory Reaction in the Brain

Localized inflammation, or even global brain inflammation (Shi et al., 2019), occurs after stroke onset. Cascades of inflammatory mediators such as cytokines and chemokines are produced, accompanied by leukocyte invasion. Peripheral leukocytes including monocytes/macrophages and neutrophils, infiltrate into the ischemic peripheral area, further aggravating stroke damage. Among the inflammatory mediators, interleukin (IL)-1β, tumor necrosis factor α (TNFα) and monocyte chemoattractant protein-1 (MCP-1) are classic factors that

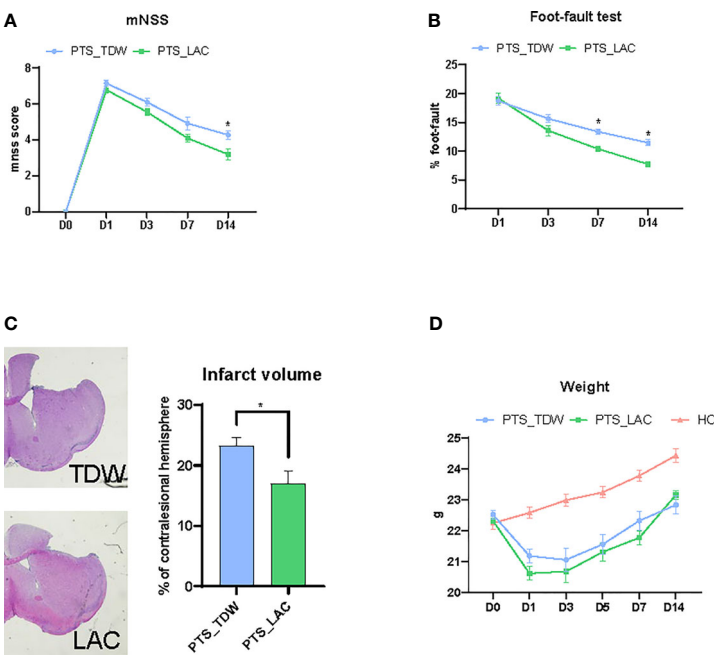

FIGURE 1 | **(A–C)** Lactulose supplementation significantly improved functional outcomes (n = 11/group, two-way repeated measures ANOVA with Sidak's multiple comparisons test, *p < 0.05) and decreased infarct volume (n = 6/group, unpaired 2-tailed Student's t-test., *p < 0.05) **(D)** Lactulose supplementation did not affect the body weight of mice (n = 11/group, two-way repeated measures ANOVA with Sidak's multiple comparisons test, *p < 0.05).

have been found in both experimental models and human stroke. Brain damage or inflammation caused by ischemic injury can activate TLR4, an important member of the TLR protein family (Caso et al., 2007). Transforming growth factor-β (TGFβ) is an anti-inflammatory and neuroprotective cytokine (Cekanaviciute et al., 2014). Nuclear factor erythroid 2-related factor 2 (Nrf2) also plays an important role in oxidative stress resistance and anti-inflammation responses, and it is a potential therapeutic target for central nervous system diseases, especially for ischemic stroke (Liu et al., 2019).

In this study, we found that the brain expression levels of IL-1β, TNFα, MCP-1, and TLR4 in the PTS_TDW group were significantly elevated, even at 14 days after stroke, compared with the HC group; while lactulose supplementation significantly decreased the expression of these factors, as shown in **Figures 2A, B**. Furthermore, lactulose supplementation remarkably increased TGFβ and Nrf2 expression.

Flow cytometry was then used to analyze the numbers of inflammatory cells in the brain among the three groups (the gate strategy is shown in **Supplemental Figure 1**). CD45 is expressed on leukocyte and microglia, CD45, CD11b and Ly6G are co-expressed on neutrophil, and CD45, CD11b and F4/80 are co-expressed on macrophage. We found that the number of $CD45^+$cell, $CD45^+CD11b^+Ly6G^+$ neutrophil, and $CD45^+CD11b^+F4/80^+$macrophage were significantly higher in the PTS_TDW group compared with the HC group, and the number of these three types of cells significantly decreased in the PTS_LAC group (**Figures 2C–E**). These data suggest that lactulose administration could reduce the inflammatory reaction by prohibiting inflammatory factors production and inflammatory cell infiltration.

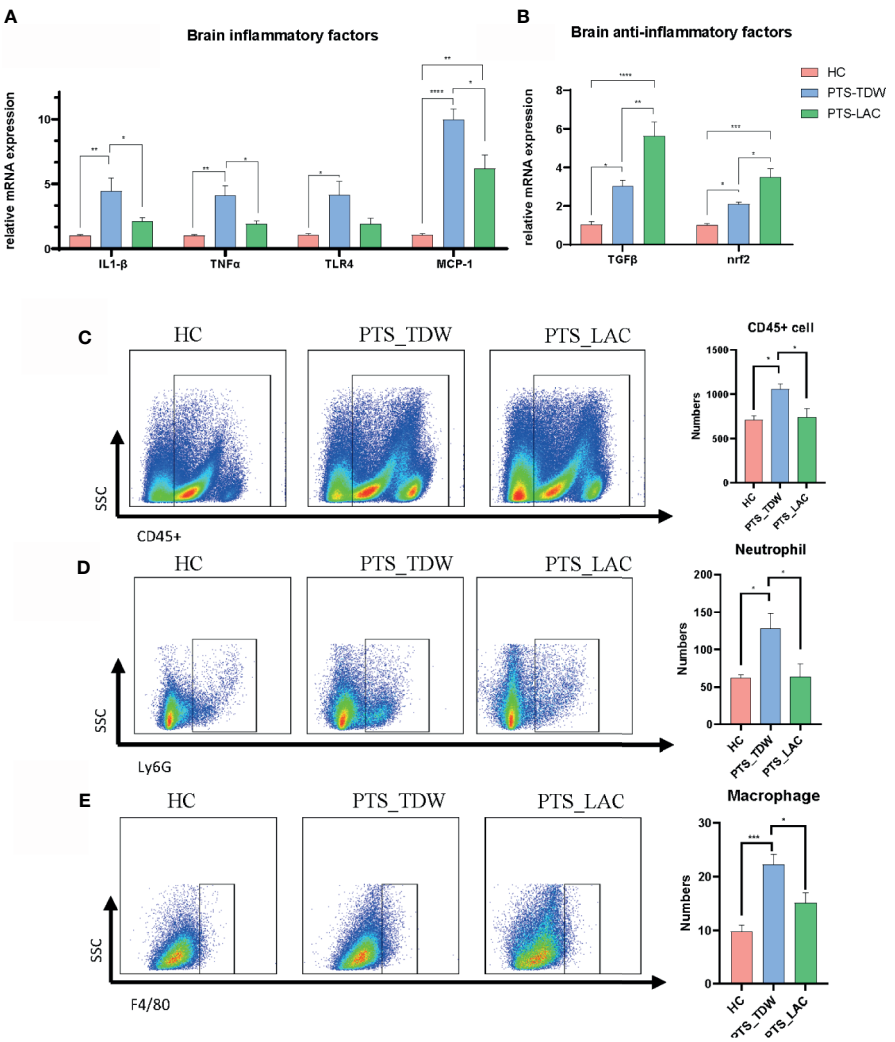

FIGURE 2 | **(A, B)** Lactulose supplementation significantly decreased inflammatory factor expression and increased anti-inflammatory factor expression and antioxidative regulators in the brain (n=5/group, one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05) at 14 days after stroke. **(C–E)** Lactulose supplementation significantly decreased the number of $CD45^+$cell, $CD45^+CD11b^+Ly6G^+$ neutrophil and $CD45^+CD11b^+F4/80^+$macrophage in the brain (n = 6/group, one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05) at 14 days after stroke. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Lactulose Supplementation Decreased the Inflammatory Reaction in the Gut and Restored Intestinal Barrier Injury After Stroke

In addition to the brain, stroke can also cause inflammation in other locations, such as the gut and blood (Spychala et al., 2018; Blasco et al., 2020). Many studies have found that inflammatory mediators are elevated after stroke, including IL-17a (Waisman et al., 2015).

In contrast to the brain, stroke markedly increased expression of inflammatory-related factors, including IL-17a, TNFα, and TLR4, in the gut (**Figure 3A**); however, IL-1β and MCP-1 levels were not increased as in the brain. After lactulose intervention, IL-17a, TNFα, and TLR4 were suppressed, in addition, TGFβ and Nrf2 were also activated (**Figure 3B**). We then examined the IL-17a level in the blood. The results showed that the level of IL-17a in the PTS_TDW group was significantly higher than that in the HC group, while the IL-17a level in the PTS_LAC group was significantly lower than that in the PTS_TDW group (**Figure 3C**).

The gut barrier is composed of a mucus layer, epithelial cells, intercellular junctions, and immune cells. The decreased expression levels of claudin-1, muc2, and occludin and the increased level of LPS indicated disruption of the intestinal barrier. Claudin-1, muc2, and occludin were significantly increased and LPS was significantly decreased after lactulose supplementation (**Figures 3D, E**), indicating that lactulose might help to restore and repair the intestinal barrier.

Lactulose Partially Restored Gut Microbiota Dysbiosis After Stroke

Feces were collected to investigate the gut microbiota in the three groups, and 16S rDNA sequencing was performed. Shared and distinct operational taxonomic units among the three groups are shown in a Venn diagram (**Figure 4A**). In total, 724 operational taxonomic units were shared among all groups. The α-diversity, including the observed species and ACE, Chao1, and Shannon indices, was used to analyze microbial diversity within the community, while the β-diversity was used to analyze diversity among different communities. In this study, lactulose significantly altered α-diversity because the diversity of the PTS_LAC group was lower than that of the PTS_TDW group (**Figure 4B**); however, after 14 days, there were no significant differences between the PTS_TDW and HC groups in this study. Principal coordinates analysis using the weighted UniFrac distances (**Figure 4C**) showed that all three groups can be clustered. A cladogram of the linear discriminant analysis effect size showed significantly different taxa among the three groups (**Figure 4D**).

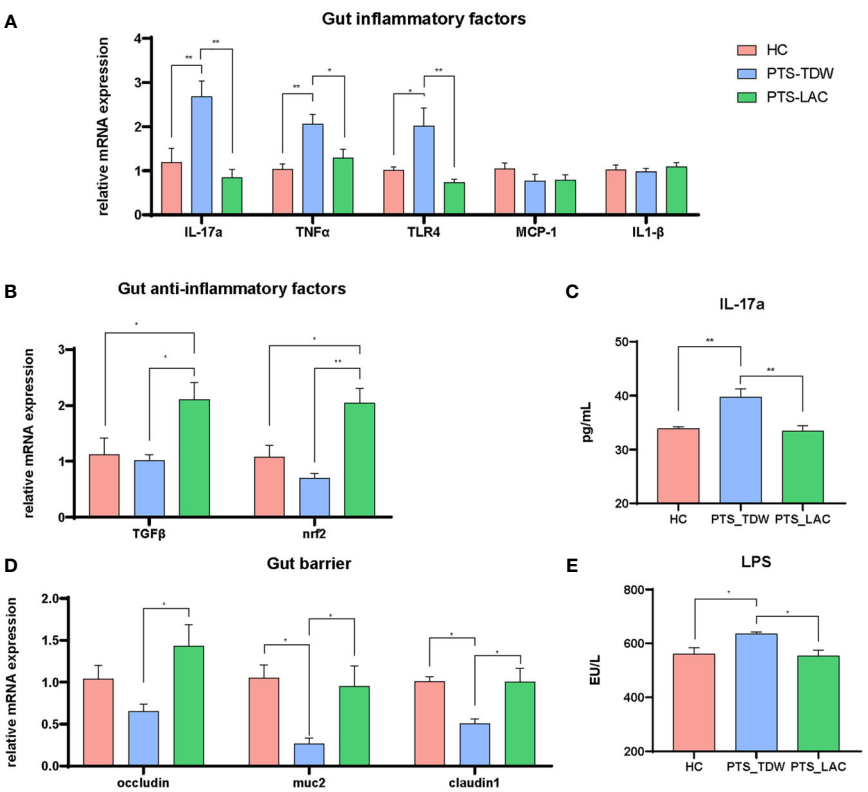

FIGURE 3 | **(A, B)** Lactulose supplementation significantly decreased inflammatory factor expression and increased anti-inflammatory factor expression and antioxidative regulators in the gut **(C)** Lactulose supplementation significantly decreased IL-17a level in the plasma. **(D, E)** Lactulose supplementation affected the intestinal barrier and muc2 expression (n = 5/group, one-way ANOVA with Tukey's multiple comparisons test, *p < 0.05) at 14 days after stroke. **p < 0.01.

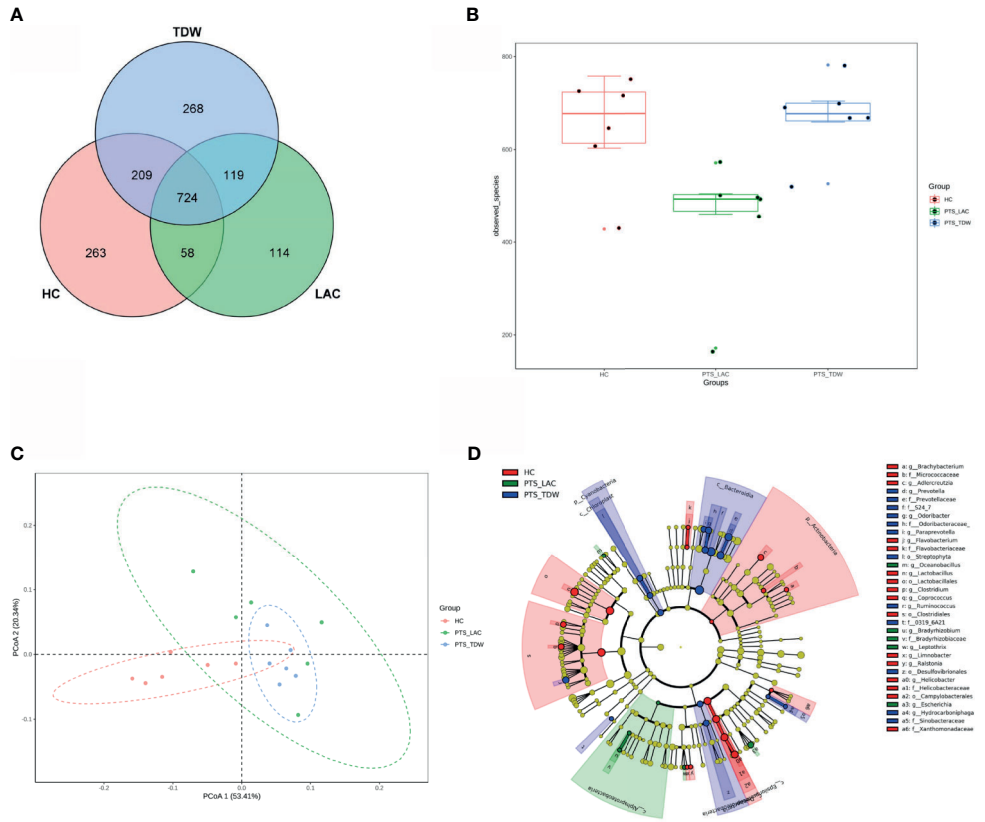

FIGURE 4 | Variations in microbiota among the three groups. **(A)** Venn diagram of the operational taxonomic units among the three groups. **(B)** Boxplots of α-diversity as measured by observed species (n = 6). **(C)** Variations in microbiota among the three groups according to principal coordinates analysis. **(D)** A cladogram of the linear discriminant analysis effect size shows significantly different taxa among the three groups from the phylum to family level.

The linear discriminant analysis effect size was used to analyze bacterial genera specific to each group, and biomarker taxa with a linear discriminant analysis value ≥2 are shown in **Figure 5**. The results showed that, at the phylum level, *Firmicutes* and *Actinobacteria* were more abundant in the HC group, while *Bacteroidetes* and *Cyanobacteria* were more abundant in the PTS_TDW group. At the family level, *Lactobacillaceae, Clostridiaceae, Helicobacteraceae, Micrococcaceae*, and *Flavobacteriaceae* were more abundant in the HC group; *Desulfovibrionaceae, Odoribacteraceae, Prevotellaceae*, and *Sinobacteraceae* were more abundant in the PTS_TDW group; and *Bradyrhizobiaceae* were more abundant in the PTS_LAC group. At the genus level, *Lactobacillus*, *Clostridium, Flavobacterium, Brachybacterium*, and *Helicobacter* were more abundant in the HC group; *Ruminococcus*, *Prevotella*, *Paraprevotella*, and *Odoribacter* were more abundant in the PTS_TDW group; and *Bradyrhizobium*, *Oceanobacillus*, *Escherichia*, and *Leptothrix* were more abundant in the PTS_LAC group.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to predict functions according to the species composition. Compared with the HC group, pathways including Endocrine System, Glycan Biosynthesis and Metabolism, Excretory System, Biosynthesis of Other Secondary Metabolites, Transport and Catabolism, Amino Acid Metabolism, Metabolism of Cofactors and Vitamins, Metabolism of Other Amino Acids, and Digestive System were highly expressed in the PTS_TDW group. Compared with the PTS_TDW group, pathways including Cardiovascular Diseases, Poorly Characterized, and Nervous System were highly expressed in the PTS_LAC group.

Lactulose Regulated Metabolomic Changes in the Plasma of Mice With Photothrombotic Stroke

The plasma metabolome may play an important role in the link among the gut, gut microbiota, and brain. Therefore, we performed an untargeted metabolome profiling analysis by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry to explore the impact of lactulose on metabolic changes in the plasma. We also examined the fecal SCFA level among three groups, and the results are shown in the **Supplemental Materials**.

A total of 64 significant differential metabolites and 46 altered KEGG pathways were identified between the PTS_TDW and HC group, 36 significant differential metabolites and 17 altered

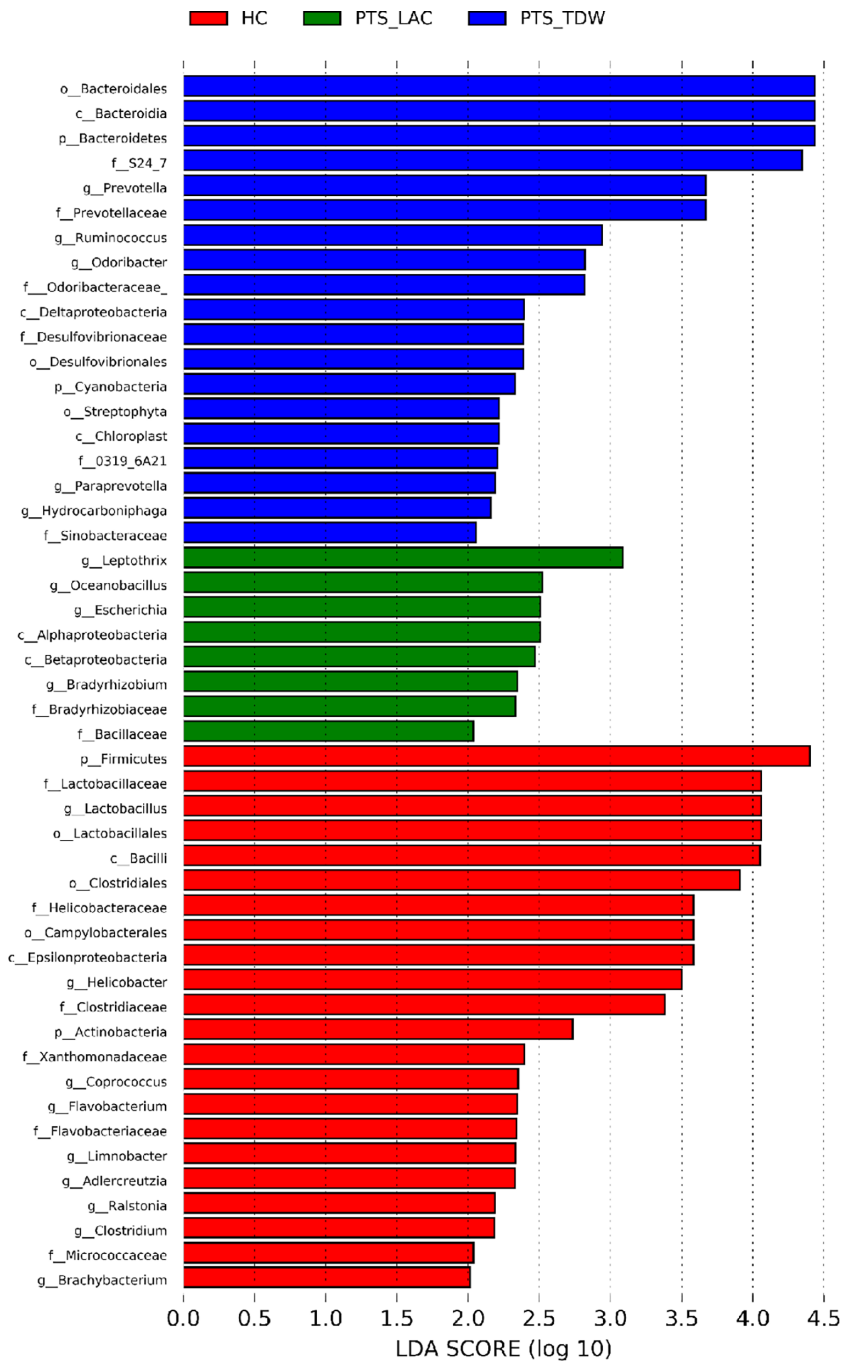

FIGURE 5 | Linear discriminant analysis (LDA) was used to analyze biomarker taxa (LDA scores≥2) in the three groups.

KEGG pathways were identified between the PTS_LAC and PTS_TDW groups, and 35 significant differential metabolites and 45 altered KEGG pathways were identified between the PTS_LAC and HC groups.

In order to explore specific differences after lactulose intervention, a volcano plot (**Figure 6A**) based on a univariate analysis and a hierarchical clustering graph (**Figure 6B**) were generated. The orthogonal partial least squares discrimination analysis score plots (**Figure 6C**) of positive modes showed significant dispersion of the two groups.

In addition, KEGG pathway enrichment analysis of differentially expressed metabolites between the PTS_TDW

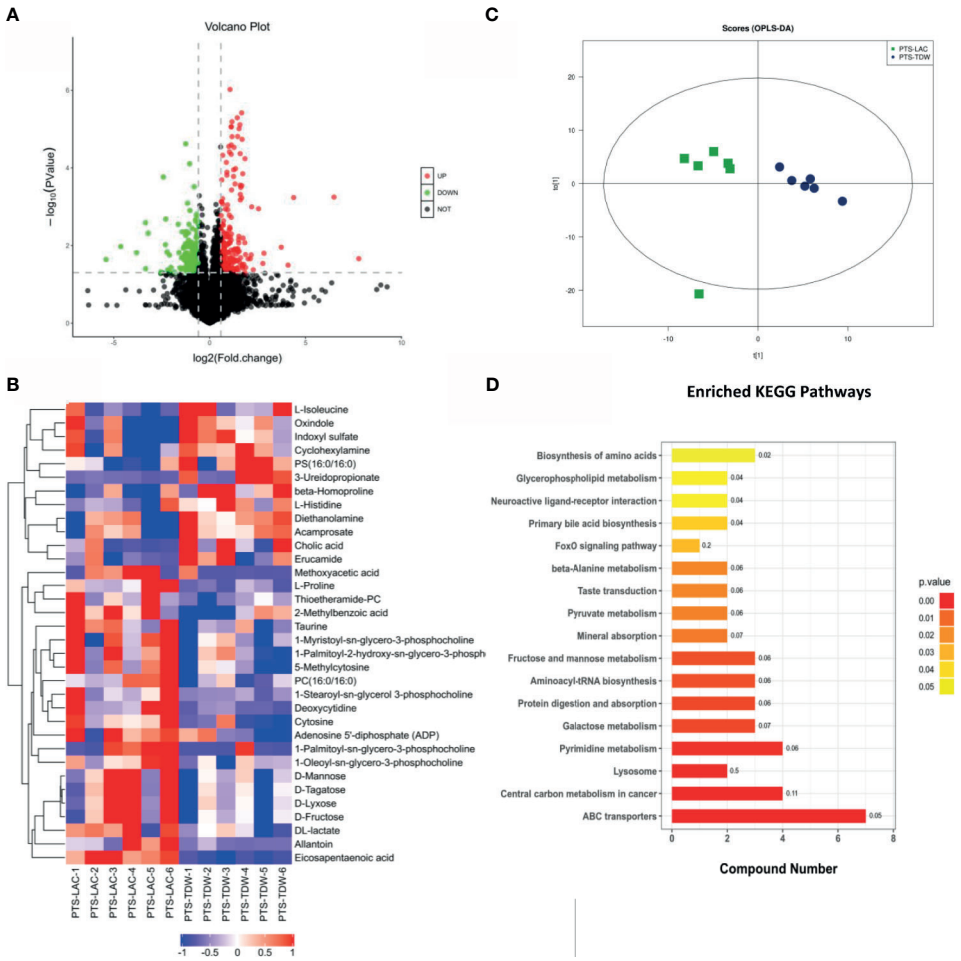

FIGURE 6 | **(A)** Volcano plot for the PTS_LAC and PTS_TDW groups in the positive mode. Red and green dots indicate significant differential metabolites with fold changes >1.5 and p values <0.05. **(B)** Heatmap showing normalized values of 34 metabolites that were differentially abundant among the PTS_LAC and PTS_TDW groups. **(C)** Orthogonal partial least squares discrimination analysis: score plots of the PTS_LAC and PTS_TDW groups in the positive ion mode. **(D)** Kyoto Encyclopedia of Genes and Genomes pathways enriched in the PTS_LAC group compared with the PTS_TDW group.

and PTS_LAC groups was performed using the Fisher exact test (**Figure 6D**). The results showed that a total of 17 pathways, including ABC transporters, Central carbon metabolism in cancer, Lysosome, Pyrimidine metabolism, Galactose metabolism, Protein digestion and absorption, Aminoacyl-tRNA biosynthesis, Fructose and mannose metabolism, Mineral absorption, Pyruvate metabolism, Taste transduction, β-Alanine metabolism, FoxO signaling pathway, Primary bile acid biosynthesis, Neuroactive ligand-receptor interaction, Glycerophospholipid metabolism, and Biosynthesis of amino acids, were significantly altered, and ABC transporters were highly altered.

Spearman correlation analysis was performed to further understand the correlation between different metabolites in the PTS_LAC and PTS_TDW groups. D-mannose was positively correlated with DL-lactate (+0.85), D-lyxose (+0.99), D-tagatose (+0.99), and D-fructose (+0.99). Allantoin was negatively correlated with indoxyl sulfate (IS) (−0.69). L-proline was positively correlated with deoxycytidine (0.84) and negatively correlated with diethanolamine (−0.77) and L-histidine (−0.76).

Correlation Analysis Between Plasma Metabolites and Fecal Microbiota Composition

Next, a correlation analysis was performed to investigate the association between plasma metabolites and the fecal microbiota composition, and the results are illustrated in a heatmap (**Figure 7**). There were several findings of note. First, the levels of IS and nicotinamide N-oxide were negatively correlated with Lactobacillus (−0.78 and −0.82), and IS was positively correlated with oxindole (+0.9). Second, the levels of eicosapentaenoic acid (EPA) and taurine were positively correlated with Oceanobacillus (+0.74 and +0.8), and EPA was also positively correlated with Bradyrhizobium (+0.8), Leptothrix (+0.83), and Wolbachia (+0.86) and negatively correlated with Desulfovibrio (−0.8), Ruminococcus (−0.79), and Helicobacter

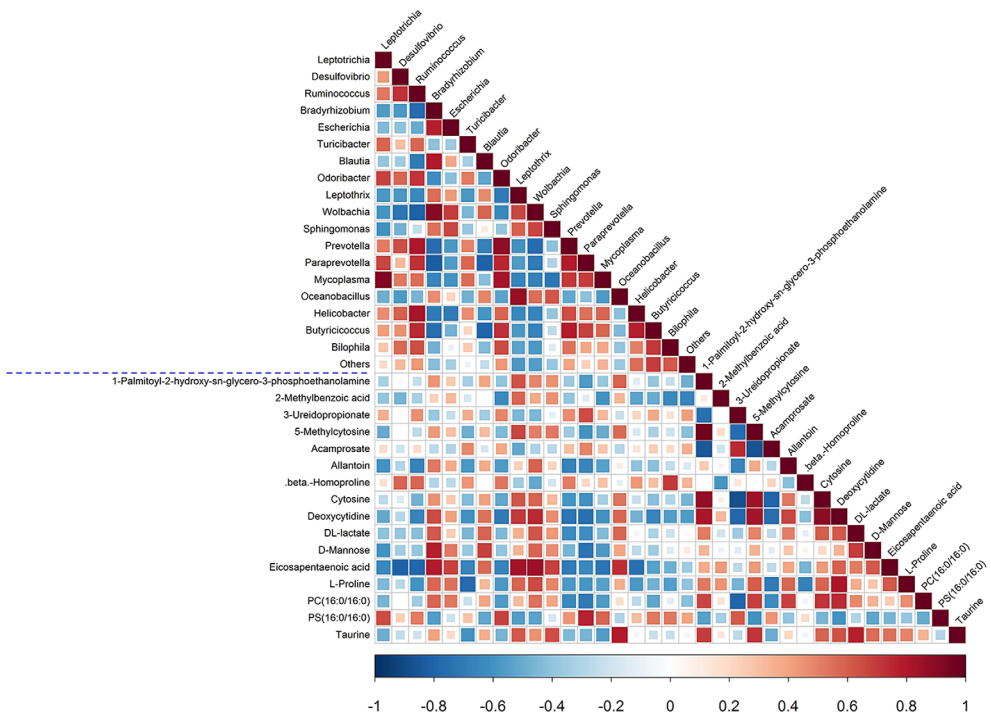

FIGURE 7 | Spearman correlation analysis of fecal microbiota and plasma metabolites. The p-values are depicted in blue and red, where red represents a positive correlation and blue represents a negative correlation.

(−0.81). Third, the allantoin level was positively correlated with Candidatus Phlomobacter (+0.96) and Leptothrix (+0.76) and negatively correlated with Leptotrichia (−0.75) and Fusobacterium (−0.77). Fourth, D-mannose was positively correlated with Bradyrhizobium (+0.77) and negatively correlated with Acamprosate (−0.77) and Benzylazanium (−0.78).

Hierarchical clustering was performed to investigate the varying trends of metabolites among the three groups. Four expression patterns (profile 0–3) were obtained in both positive and negative modes (**Supplemental Figure 3**). The x-axis indicates the different groups (HC, PTS_TDW, and PTS_LAC groups), and the y-axis indicates the standardized levels of metabolites. Among all patterns, patterns 1 and 2, which reflect metabolites fluctuating up and down, attracted our attention.

In the positive mode, pattern 1 included D-mannose, L-proline, citrate, uracil, 2'-deoxyuridine, L-leucine, thiamine, triethanolamine, and pantothenate, and pattern 2 included IS, ADP, L-glutamine, nicotinamide N-oxide, 2-methylbutyroylcarnitine, and 3-ureidopropionate. In the negative mode, pattern 1 included allantoin, taurine, DL-lactate, L-galactono-1,4-lactone, 3-hydroxydodecanoic acid, and 2-methyl-3-hydroxybutyric acid, and pattern 2 included acamprosate.

DISCUSSION

Our data showed that lactulose improved neurological function, suppressed inflammation in the brain and gut, regulated metabolic disturbance, and inhibited harmful bacteria in mice after stroke.

Lactulose downregulated inflammatory mediators and upregulated the expression of anti-inflammatory factors such as TGFβ and Nrf2 in both the brain and gut. Initiation of inflammation after stroke worsens the functional prognosis, while treatments that target inflammatory cytokines, such as TNFα and IL-1, have been shown to be effective (Lambertsen et al., 2019) in restoring neurological outcomes after stroke. In this study, administration of lactulose not only suppressed inflammation in the brain and gut but also promoted anti-inflammatory factors such as TGFβ and Nrf2, which is consistent with a previous study (Zhai et al., 2013). The effects of lactulose on different organs were mediated by various inflammatory pathways/mechanisms.

Furthermore, we found that lactulose significantly upregulated gut barrier markers including claudin-1, muc2, and occludin. These results provided evidence that lactulose might restore gut barrier damage after stroke. In addition, stroke leads to gut dysbiosis and activates the immune system, which aggravates the neurological outcome after stroke (Benakis et al., 2016; Durgan et al., 2019).

In this study, no significant difference in α-diversity was observed between the HC group and the PTS_TDW group. However, there is no uniform conclusion in published studies regarding the change in α-diversity after stroke (Singh et al., 2016; Li et al., 2019). Compared with the HC group, the abundance of probiotics such as *Lactobacillus* decreased, and

pathogens such as *Neisseria* and *Fusobacterium* increased in the PTS_TDW group. Compared with the PTS_TDW group, lactulose supplementation decreased the abundance of pro-inflammatory taxa (Gao et al., 2018; Ma et al., 2018) such as *Desulfovibrio, Helicobacter*, and *Turicibacter*, which might partially explain the decrease in α-diversity after drug administration [The relative abundance genus csv file and the heatmap of clustering for genus abundance and are in **Supplemental Material** and **Supplemental Figure 4**)]. In addition, the ratio of *Firmicutes* to *Bacteroidetes*, which is seen as a marker of dysbiosis in some studies, was decreased in the PTS_TDW group compared with the HC group (the average F/B ratios of the HC, PTS_TDW and PTS_LAC groups were 1.186,0.331 and 0.460 respectively; the relative abundance phylum csv file and the heatmap of clustering for phylum abundance are shown in the **Supplemental Material** and **Supplemental Figure 5**), while another study found that the *Firmicutes* to *Bacteroidetes* ratio increased after stroke (Brichacek et al., 2020); therefore, we speculated that an altered *Firmicutes* to *Bacteroidetes* ratio indicated gut dysbiosis.

Our results showed that Desulfovibrionaceae, one harmful bacterium, decreased after lactulose administration, while there is no significant difference was observed for *Bifidobacterium* and *Lactobacillus*, which is similar to previous studies (Zhai et al., 2018; Zhang et al., 2019a), although Lactulose has long been viewed as a bifidus factor.

Our data showed that lactulose treatment could decrease accumulation of some harmful metabolites, such as IS, and increase the levels of some beneficial metabolites in plasma after stroke. IS, which is a toxic uremic solute derived from tryptophan metabolism, has been widely studied in renal disease, especially chronic kidney disease (Vanholder et al., 2014). In addition to the renal system, IS affects the cardiovascular system and central nervous system (Gao and Liu, 2017; Hung et al., 2017). Many studies have found that IS promotes inflammation, oxidative damage, and fibrosis and induces gut barrier (Huang et al., 2020) and endothelial cell dysfunction. Our study showed that IS was elevated after stroke, and lactulose significantly decreased its accumulation. Recently, a study found that stroke may induce kidney dysfunction (Zhao et al., 2020); therefore, IS accumulation may be a potential mechanism of stroke-induced kidney dysfunction.

The beneficial metabolites induced by lactulose supplementation, which were correlated with some taxa, included EPA, allantoin, taurine, and D-mannose. A network and a hierarchical clustering heatmap were generated to intuitively exhibit correlations among the differentially expressed fecal microbiota and plasma metabolites (**Figures 8A, B**).

EPA, an omega-3 polyunsaturated fatty acid, exerts cardiovascular protective effects *via* its anti-inflammation and antioxidative stress activities, inhibition of platelet activity, and ability to decrease plasma triglyceride levels (Innes and Calder, 2018). The presence of EPA in the network was striking because it was connected with many florae. Many studies (Nakase et al., 2015; Aung et al., 2018; Alvarez Campano et al., 2019) have shown that EPA supplementation can improve the prognosis of patients and prevent cardiovascular and cerebrovascular

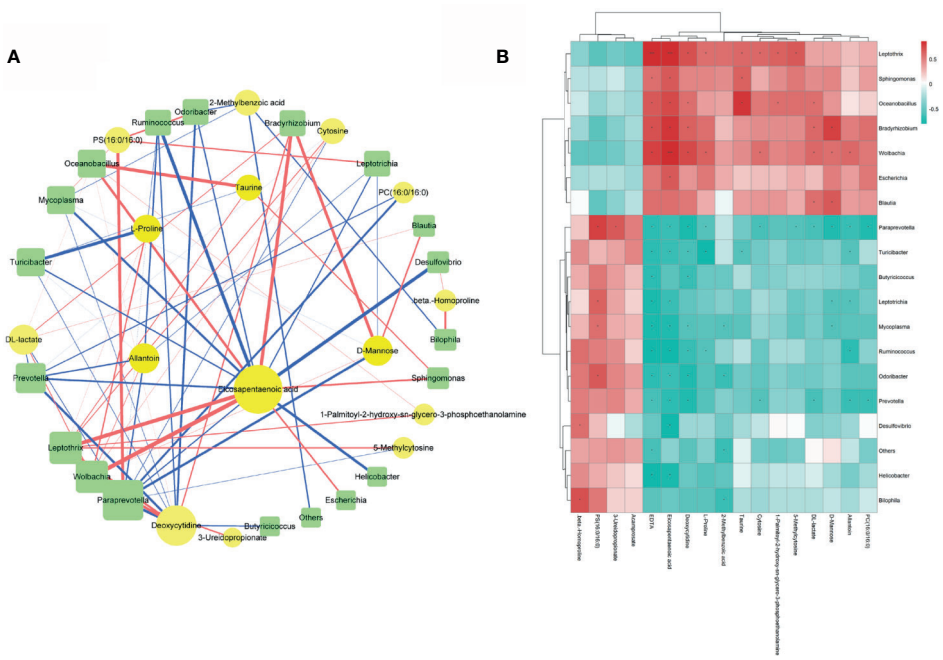

FIGURE 8 | **(A)** Spearman Correlation network among the differentially expressed fecal microbiota and plasma metabolites; circles represent metabolites and squares represent microbiota. Different line colors represent positive (red) and negative (blue) correlation coefficients, and the line width is proportional to the absolute value of the correlation coefficient. **(B)** Hierarchical clustering heatmap of differentially expressed fecal microbiota and plasma metabolites.

diseases. Therefore, increased EPA levels might lead to good outcomes after stroke. In most mammals, other than humans, uric acid is quickly degraded to allantoin by urate oxidase in purine metabolism (Maiuolo et al., 2016). Uric acid is also seen as a promising biomarker to reflect the oxidative status (Seet et al., 2011). Studies have found that uric acid therapy is effective for ischemic stroke treatment (Kand'ár et al., 2006; Llull et al., 2015). However, no stroke-related study has shown that allantoin has a similar effect to uric acid, but allantoin therapy has been used to treat other diseases, such as gastric ulcers (da Silva et al., 2018) and gastritis (Eslami-Farsani et al., 2018), which may offer a novel therapeutic opportunity for stroke. Therefore, further study is urgently needed. Taurine has cytoprotective, antioxidative stress, anti-inflammatory, and barrier integrity-maintaining effects (Schaffer and Kim, 2018; Jakaria et al., 2019). In animal experiments and clinical trials, taurine has been used to treat neurological (Hou et al., 2018; Ohsawa et al., 2019), cardiovascular (Katakawa et al., 2016), and metabolic diseases (Obrosova et al., 2001), especially stroke (Guan et al., 2011; Sun et al., 2012; Jin et al., 2018). Our study found that taurine was decreased after stroke and increased by lactulose, which perhaps could explain the good neurological performance of the PTS_LAC group. D-mannose is also a beneficial metabolite. Dunfang Zhang et al. (2017) found that D-mannose activated TGFβ expression, promoted T regulatory cell differentiation, and inhibited inflammation. The activation of TGFβ expression in both the brain and gut found in our study may be related to the increase in D-mannose.

Therefore, we speculated that an increase in beneficial metabolites and a decrease in harmful metabolites may have led to improved neurological outcomes after stroke. To our knowledge, this is the first study to examine the effects of lactulose on stroke outcomes using omics technologies (16S sequencing and metabolomics). However, inclusion of a group to explore how lactulose affects healthy mice and extension of the duration of lactulose administration would strengthen our results.

CONCLUSIONS

In summary, ischemic stroke led to inflammatory reactions in both the brain and gut, resulting in gut barrier disruption and dysbiosis, which could be partly alleviated by lactulose. The effects of lactulose may be attributable to repressing harmful bacteria and metabolic disorder, repairing gut barrier disruption, and inhibiting inflammatory reaction after stroke in mice.

DATA AVAILABILITY STATEMENT

The raw data has been deposited and made public in the NCBI-SRA database with the accession number SRP298849.

ETHICS STATEMENT

The animal study was reviewed and approved by Tianjin Medical University General Hospital Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TY: experimental design and gave final approval of manuscript. QY: experimental design, wrote the manuscript, performed experiments, analyzed data and prepared figures. LX, SH, YS, RW, XL, JW, and RL: performed experiments, analyzed data and prepared figures. 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.2021.644448/full#supplementary-material

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OPINION
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The Impact of Gut Microbiota on Post-Stroke Management

Junyi Zhao[1,2†], *Siyu Liu*[1,2†], *Jingyi Yan*[3†] *and Xinzhou Zhu*[1,2*]

[1] *The Brain Cognition and Brain Disease Institute (BCBDI), Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China,* [2] *Shenzhen-Hong Kong Institute of Brain Science-Shenzhen Fundamental Research Institutions, Shenzhen, China,* [3] *Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden*

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****Correspondence:***
Xinzhou Zhu
xz.zhu@siat.ac.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

According to the recent Global Burden of Disease (GBD) study, stroke is the leading cause of death and disability, particularly in aged population (Collaborators, 2019). In the last decades, the mortality rate of stroke has significantly decreased, and the disability-adjusted life year (DALY) and years lived with disability (YLD) have been controlled (Collaborators, 2019). Nevertheless, stroke remains as a major health concern in both developed and developing countries (Collaborators, 2019). Novel therapeutic methods and improved management in stroke prevention and post-stroke recovery are still urgently demanded.

In a long period, most studies focused on the cardiovascular and neurological aspects of stroke, while only a small group of researchers kept an eye on the pathological alterations in gastrointestinal tract of stroke patients (Schaller et al., 2006). These studies mainly discussed the consequences of impaired nutritional status in stroke events (Schaller et al., 2006). Notably, in the past ten years, the ecosystem of microbiota in gastrointestinal tract has been linked to various physiological and pathological processes (Donaldson et al., 2016). Increasing evidences have demonstrated that the compositional changes of gut microbiota complexity are involved in diverse gastrointestinal disorders (Manichanh et al., 2006) and metabolic dysfunctions such as obesity and diabetes (Wen et al., 2008; Ridaura et al., 2013), which may also contribute to the nutrition status after stroke. Moreover, gut microbiota is recently considered to communicate with central nervous system in a bidirectional pattern (Collins et al., 2012). The metabolic products of gut microbiota regulate not only normal brain development but also various brain disorders through neural, immunological, endocrinal and metabolic pathways (Collins et al., 2012). Therefore, deep insights into the relationship between gut microbiota and stroke could provide novel avenues to improve post-stroke recovery and prevent stroke recurrence.

More than 85% of stroke events are caused by the blockage of blood flow, namely ischemic stroke (Moskowitz et al., 2010), thus we focused on ischemic stroke in this review. We summarized recent advances in the interactions between commensal gut microbiota and ischemic stroke: how stroke insult changes gut microbiota composition and how these shifts reversely influence stroke outcome and prognosis. We also attempted to figure out the clues from latest literatures by which gut microbiota may affect the major aspects during post-stroke management, including the controls of body temperature, blood glucose, blood pressure, oxygen and hydration (Bhalla et al., 2001). The concerns on gut microbiota will provide researchers novel therapeutic potentials for ischemic stroke and remind clinicians for special cares in post-stroke management.

LITERATURE SEARCH STRATEGY

We used PubMed and Google Scholar to search recent advances in the relationship between gut microbiota and ischemic stroke. "Gut microbiome" and "intestinal flora" were used as synonyms of "gut microbiota". As for the progresses in the main aspects of post-stroke management, key words including "immune response", "inflammation", "body temperature", "hypothermia", "hyperthermia", "blood glucose", "hypoglycermia", "hyperglycermia", "blood pressure", "hypotension", "hypertension", "oxygen", "hypoxia", "hyperoxia", "hydration", "dehydration" and "overhydration" were combined with "gut microbiota" and its synonyms to search related references.

Studies in the field of gut microbiota-stroke relationship burst mainly in 2016, therefore we focused on the research articles in this year and afterwards. Review articles were only included when they provided novel insights in this area. Background knowledge may be referred to high-quality research articles or review articles before 2016. In a total of >200 references were included in the first round of literature search. After removing duplicating information or unsolid studies, eventually 86 references were selected and cited.

GUT MICROBIOTA AND BRAIN DISORDERS

In a healthy human, over 100 trillion microorganisms reside predominantly in gastrointestinal tract (Qin et al., 2010). Among gut microbial community, *Bacteroidetes* and *Firmicutes* are two main phylotypes which constitute more than 90% of the core microbiome shared by all individuals, while *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* dominate the remaining part (Qin et al., 2010). Interestingly, in murine gastrointestinal tract, the composition of microbiome is of high similarity as that in human, with *Bacteroidetes* and *Firmicutes* as the most dominant phyla (>90%), and *Proteobacteria*, *Cyanobacteria*, *Tenericutes*, *Actinobacteria* and *Deferribacteres* largely occupying the rest proportion (Cho et al., 2012; Gu et al., 2013). Although the core microbiome between mouse and human gut share a high qualitative similarity, the species are more variable at lower taxonomic level. In addition, the abundance of specific phyla and species is also observed to be different between mouse and human (Krych et al., 2013). Therefore, it remains challenging to establish a humanized gnotobiotic mouse model which authentically represents the compositional and metabolic alterations of human gut microbiota after intervention. Many careful considerations are required in model designing, including the isolation, storage and transplantation conditions of human feces, the genetic background of mouse, and the diet ingredients of both mouse and human (Park and Im, 2020).

The concept of gut-brain axis (GBA) has been established for a long history. GBA consists of bidirectional communications between gastrointestinal digestive functions and brain activities, and gut microbiota interacts with GBA in a complex pattern involving autonomic, endocrinal and immune crosstalk (Cryan and Dinan, 2012). On one hand, cerebral neuroendocrinal changes triggers the homeostasis of gut microbiota through hypothalamic-pituitary-adrenal (HPA) axis; on the other hand, gut microbiota conversely coordinates brain functions through their metabolic products and the modulation of immune cells (Fung et al., 2017). In the past decade, massive studies have unraveled diverse roles of gut microbiota not only in normal brain development (Diaz Heijtz et al., 2011), but also in cerebral pathological conditions, including acute brain injuries, chronic neurodegeneration and mood disorders (Houlden et al., 2016; Wu et al., 2017; Zheng et al., 2019).

THE INTERPLAY BETWEEN STROKE AND GUT MICROBIOTA

Emerging evidence have revealed that the dysbiosis of gut microbiota can be induced by ischemic stroke in both rodents and patients. Ischemic stroke can cause massive goblet cell and enteric nerve loss, breakdown of mucus layer and disruption of gut barrier, leading to subsequent dysbiosis and translocation of gut microbiota (Durgan et al., 2019). In a mouse model of ischemic stroke, the injury showed a remarkable impact on reshaping gut microbiota population, including the most abundant phylotypes *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* as mentioned above (Singh et al., 2016). The species diversity of gut microbiota was also reduced upon injury (Singh et al., 2016). In another model of pig stroke, the abundance of the *Proteobacteria* dramatically increased after stroke, while *Firmicutes* and *Lactobacillus* decreased accordingly (Jeon et al., 2020). In clinical studies, intestinal dysbiosis was consistently observed in patients with acute ischemic stroke, when comparing with healthy control group (Xia et al., 2019; Xu et al., 2021). Among the major gut microbiota populations, *Parabacteroides*, *Oscillospira* and Enterobacteriaceae were enriched in stroke patients, while *Prevotella*, *Roseburia* and *fecalibacterium* were in contrast reduced (Xia et al., 2019). Notably, in an early stage of stroke recovery, Enterobacteriaceae enrichment was observed to highly correlate with high risk and poor outcome, making it as a potential biomarker of ischemic stroke (Xu et al., 2021). Moreover, the detrimental effects of post-stroke dysbiosis are not restricted in gastrointestinal tract, but also in other organs as a consequence of translocation and dissemination. For instance, typical intestinal bacterial species such as *Enterococcus* spp., *Escherichia coli* and *Morganella morganii* can translocate into lung and cause severe infection after ischemic injury (Stanley et al., 2016).

On the other hand, dysregulated compositions of gut microbiota can in turn influence stroke outcomes. When stroke induces dysbiosis of gut microbiota, Enterobacteriaceae in gut microbiota can also accelerate systematic inflammation thus exacerbate brain damage in both mouse model and patients samples, which may serve as a promising therapeutic target (Xu et al., 2021). In another animal study, atorvastatin restored gut microbiota homeostasis, contributing to its anti-inflammatory functions after stroke (Zhang et al., 2021).

Based on the evidence in both animal models and clinical studies, the alterations of gut microbiota phyla are believed to

present a strong correlation with ischemic stroke, thus may be used as indicators of stroke incidence, progress and prognosis (**Table 1**). In addition, dysbiosis and infections in other major organs should be carefully considered and prevented in stroke patients. Meanwhile, stroke-induced dysbiosis of gut microbiota can also exacerbates brain injury and negatively influence stroke outcome, which may serve as novel biomarkers and therapeutic targets of stroke (**Table 1**).

GUT MICROBIOTA REGULATES KEY MANAGEMENT ASPECTS DURING POST-STROKE RECOVERY

In stroke patients, body temperature, blood glucose, blood pressure, oxygen and hydration status are the key parameters in post-stroke management (Bhalla et al., 2001). These parameters may change either upwards or downwards after stroke, and require careful balance to reach favorable outcome and prognosis (Bhalla et al., 2001). When stroke reshapes the population of gut microbiota, they might in turn modulate these physiological parameters *via* diverse molecular and cellular mechanisms according to the recent advances.

Immune Response

Neuroinflammation is a hallmark of ischemic stroke. In acute phase of stroke, neuroinflammation is considered to be partially beneficial by scavenging damaged tissues and promoting neuroregeneration (Yong et al., 2019). However, it predominantly exacerbates brain injury from sub-acute to chronic phase, and elevates the risk of stroke occurrence and recurrence (Esenwa and Elkind, 2016). Post-stroke population of gut microbiota can modulate neuroinflammatory responses through various pathways, with either advantageous or disadvantageous aspects. In 2016, three independent studies have demonstrated that intestinal dysbiosis can regulate cytokines and T cell functions in different patterns, by which they eventually influence stroke outcome. In the first study, the dysbiosis of gut microbiota induced by stroke has been reported to increase pro-inflammatory cytokines IL-17 and IFN-γ in recipient germ-free mice after fecal transfer, and lead to unfavorable outcome in recipient mice (Singh et al., 2016). In another report, however, antibiotic–induced dysbiosis of gut microbiota promoted anti-inflammatory cytokines IL-10 from T_{reg} cells and simultaneously suppressed pro-inflammatory cytokine IL-17 from γδT cells, thereby improved stroke outcome (Benakis et al., 2016). Interestingly, in the third study, gut microbiota depletion by antibiotics before injury can dramatically reduce survival rate after stroke, whereas either continuous subsequent antibiotic treatment or recolonization of gut microbiota can both increase animal survival (Winek et al., 2016). Regardless of stroke outcome, antibiotic-induced microbiota depletion suppressed B cells and several subtypes of T cells, resulting in a general immunodepression (Winek et al., 2016). Considering the diversity and the quantity of major populations in gut microbiota were both significantly reduced in these depletion models, the opposite effects indicate the complexity and uncertainty how gut microbiota influences stroke outcome. Moreover, a recent study has revealed that, the fecal transplants from severe stroke patients can enhance IL17 positive γδT cell number in recipient

TABLE 1 | Evidences of the correlation between gut microbiota and ischemic stroke.

Animal model or clinical study	Key findings and potential therapeutic strategies	References
Mouse MCAO	Gut micriobota dysbiosis induces pro-inflammatory T cells in gut and ischemic brain, which may serve as a target to reduce ischemic infaction.	(Singh et al., 2016)
Mouse MCAO	IL-17+ γδ T can be used as an immunomodulatory target to restore gut microbiota and promote stroke recovery.	(Benakis et al., 2016)
Mouse MCAO	Stroke can induce gut barrier permeability and dysfunction, which further promote the translocation and dissemination of gut microbiota to peripheral tissue, leading to post-stroke infections.	(Stanley et al., 2016)
Mouse MCAO	Healthy fecal transplatation with homeostatic gut microbiota before stroke can pevent ischemic damage.	(Winek et al., 2016)
Mouse MCAO	Anti-inflammatory atorvastatin can restore gut microbiota and repair gut barrier after stroke, which contributes to anti-inflammatory responses in stroke recovery.	(Zhang et al., 2021)
Mouse MCAO	Gut microbiota-derived SCFAs can improve stroke recovery and neurological outcomes	(Sadler et al., 2020)
Pig MCAO	Systmatic inflammation and dysbiosis of gut microbiota are observed in acute phase of stroke.	(Jeon et al., 2020)
Mouse photothrombotic stroke model	Lactulose repairs gut barrier injury and improves gut microbiota dysbiosis after stroke; lactulose can also improve post-stroke neurological outcomes.	(Yuan et al., 2021)
Clinical cohorts and mouse MCAO	Gut microbiota dysbiosis can be used as a index to predict stroke outcome in both animals and patients.	(Xia et al., 2019)
Clinical cohorts and mouse MCAO	In both patients and animals, rapid and dynamic gut dysbiosis were observed after stroke; Enterobacteriaceae in turn induces post-stroke inflammation and can serve as biomarker or therapeutic target.	(Xu et al., 2021)
Clinical cohorts and mouse study	Gut microbiota-derived TMAO promotes inflammation and predicts high risk of cardiovascular events in stroke patients, making it a promising biomarker of poor outcome and prognosis.	(Haghikia et al., 2018)

MCAO, middle cerebral artery occlusion; SCFA, short-chain fatty acids; TMAO, trimethylamine N-oxide.

mice (Xia et al., 2019), providing potential clinical evidences to support the conclusions from the first study (Singh et al., 2016). However, more detailed analysis on the microbial colonization and the impact of specific bacterial species are required in future studies before any medical translation.

Notably, the metabolic products of gut microbiota may play also important roles in post-stroke inflammatory responses. Short-chain fatty acids (SCFAs) are typically produced during gut bacteria fermentation, of which the major components consists of acetate, propionate and butyrate (Sun et al., 2018). Gut microbiota-derived SCFAs can dramatically stimulate IL-10 production from Th1 cells (Sun et al., 2018), which has been widely reported as a key factor in favorable stroke outcome (Garcia et al., 2017). The similar effects of gut microbiota metabolites on IL-10 are also consistent with the findings in the second report (Benakis et al., 2016). A recent report has revealed that gut microbiota-derived SCFAs can significantly alter contralesional cortex connectivity thereby improve neurological outcome in mouse stroke model (Sadler et al., 2020).

In addition, gut microbiota may also modulate the risk factors resulting in primary and secondary stroke. For instance, gram negative bacteria in gut can activate TLR-4 in brain endothelial cells, thereafter drives cerebral cavernous malformations *via* TLR4-MEKK3-KLF2/4 pathway and significantly increases the occurrence and recurrence risk of stroke (Tang et al., 2017).

To sum up, it is believed that gut microbiota can regulate post-stroke immune responses through their byproducts and pathogen associated molecular patterns (PAMPs) to modulate immune cells and inflammation-related cytokine. However, it is still controversial whether gut microbiota drives immune responses to pro-inflammatory direction or anti-inflammatory direction. The factors and contexts determining their immune-modulatory functions remain largely unknown and require to be unraveled before applying gut microbiota-based therapy to clinical use.

Body Temperature

Fever is a common symptom after ischemic stroke and usually associated with elevated mortality and morbidity (Saini et al., 2009). The mechanisms of hyperthermia-induced poor clinical outcome and prognosis involve injuries on intestinal barrier, increased pro-inflammatory cytokines and dysfunctions of blood-brain barrier (Zaremba, 2004; Oliver et al., 2012). Firstly, hyperthermia is widely known to disrupt intestinal mucosa barrier (Oliver et al., 2012), which leads to substantial changes in the community of gut microbiota (Stanley et al., 2018). The dysbiosis of gut microbiota can contribute to complex inflammatory responses as discussed above. Also, the dysfunction of intestinal barrier may lead to the translocation of gut microbiota to other organs, which increases the risk of systematic infection during hospitalization within first weeks after stroke (Fugate et al., 2014; Stanley et al., 2016). Furthermore, gut microbiota is indispensable to maintain the permeability of blood-brain barrier (BBB) by elevating the expression levels of tight junction proteins (Braniste et al., 2014). While BBB breakdown is exacerbated by hyperthermia and leads to a worsen outcome of ischemic stroke (Ginsberg and Busto, 1998), gut microbiota may provide beneficial effects in post-stroke recovery by maintaining the integrity of BBB. On the other hand, however, the thermoregulation function of gut microbiota is highly dependent on UCP1 signaling in brown adipose tissue (Li et al., 2019). The depletion of gut microbiota after antibiotic treatment could blunt toxin-induced hyperthermia by regulating UCP1 and TGR5 gene expressions in brown adipose tissue and skeletal muscle (Ridge et al., 2019), implying a positive effect of gut microbiota on hyperthermia.

In contrast to hyperthermia, slightly decreased body temperature predicts good functional outcome after stroke (Jorgensen et al., 1999). In fact, therapeutic hypothermia is one of the most encouraging methods in neuroprotection after acute brain injury including ischemic stroke (Wu and Grotta, 2013). The efficacy of hypothermia has been successfully validated in pre-clinical animal models, while its limitations remain to be solved in clinical trials (Wu and Grotta, 2013). The natural hypothermia, namely hibernation, has been demonstrated to alter the major populations in gut microbiota, including *Bacteroidetes*, *Firmicutes, Verrucomicrobia*, *Deferribacteres*, *Cyanobacteria* and *Actinobacteria* (Chevalier et al., 2015). Conversely, cold-responding microbiota orchestrates energy homeostasis by modulating insulin sensitivity (Chevalier et al., 2015), which is one of the key concerns during stroke recovery (see below, the section of 'blood glucose'). In addition, microbiota-depletion mice also showed impaired UCP1-dependent thermogenesis upon cold exposure (Li et al., 2019). However, clinical evidence remain insufficient to confirm the link between hypothermic-induced gut microbiota alteration and stroke outcome. Only one clinical study analyzed gut microbiota composition in therapeutic hypothermia-treated patients after hypoxic-ischemic encephalopathy (HIE) (Watkins et al., 2017), a similar ischemic brain injury occurring mainly in neonates. As reported by Watkin's *et al.*, the diversity in global microbial richness and the proportion of *Bacteroidetes* were both significantly reduced in hypothermia-treated HIE patients when comparing to healthy individuals (Watkins et al., 2017).

To summarize, the population of gut microbiota is significantly altered when body temperature changes. Hyperthermia-induced dysbiosis may exacerbate stroke severity, whereas hypothermia-induced dysbiosis may ameliorate post-stroke symptoms, in consistent with the clinical management strategies of body temperature in stroke patients. In addition, gut microbiota may in turn regulate body temperature through UCP1 signaling in brown adipose tissue, which provides a novel insight for body temperature management after stroke.

Blood Glucose

The dysregulation of blood glucose level is a common post-stroke symptom. Both hyperglycemia and hypoglycemia can result in detrimental brain damage and lead to a unfavorable clinical outcome (Lindsberg and Roine, 2004; Dave et al., 2011). In particular, diabetic patients often suffer a higher risk and poorer outcome and prognosis of ischemic stroke than non-diabetic population, while a high proportion of stroke patients also bear hyperglycemia even without diabetic history (Luitse et al., 2012). Large-scale sequencings have revealed the dysbiosis patterns of gut microbiota in type-II diabetic patients, including the reduced abundance of some butyrate-producing bacteria

(Qin et al., 2012; Karlsson et al., 2013), which may result in decreased insulin sensitivity (Gao et al., 2009). Moreover, the dysbiosis of gut microbiota may lead to the dysregulation of CD4 T cell homeostasis and induce glucose intolerance as well as insulin resistance (Garidou et al., 2015). In contrast, transplantation of gut microbiota from healthy donors can increase insulin sensitivity in patients with metabolic dysregulation (Vrieze et al., 2012). Additionally, microbiota-derived SCFAs have been suggested to maintain cardiovascular health by several mechanisms including the regulation of glucose homeostasis (Chambers et al., 2018). Their effects on cardiovascular functions may contribute to a reduced risk of stroke occurrence and recurrence. Based on current findings, healthy gut microbiota may assist the homeostasis of blood glucose, which favors stroke recovery and prognosis. Interestingly, the role of gut microbiota in glucose homeostasis is independent from their role in adaptive thermogenesis (Krisko et al., 2020), indicating that we may uncouple their functions in regulating body temperature and blood glucose during post-stroke management to meet individual therapeutic demands.

Blood Pressure

Similar to blood glucose, blood pressure is also commonly dysregulated and associated with adverse prognosis after ischemic stroke (Potter et al., 2009). Both hypertension and hypotension require immediate intervention to drive blood pressure to normal level (Potter et al., 2009). Excessive salt intake (> 5g/day) is one of the main risk factors of stroke by elevating blood pressure (Li et al., 2018b). Therefore, low-sodium dietetic control is recommended in post-stroke management, which favors recovery and reduces the risk of stroke recurrence. Recently, specific species of gut microbiota such as *Lactobacillus murinus* have been identified to prevent salt-sensitive hypertension by regulating T_H17 cell functions (Wilck et al., 2017). A clinical report supports the conclusion by showing certain *Lactobacillus* species are negatively associated with sodium intake and blood pressure, although the overall gut taxonomic composition showing no obvious correlation (Palmu et al., 2020). Furthermore, gut microbiota-producing metabolites like SCFAs and trimethylamine N-oxide (TMAO) are also highly involved in the regulation of blood pressure (Marques et al., 2018). While SCFAs tend to exert protective functions to avoid hypertensive cardiovascular injury (Bartolomaeus et al., 2019), TMAO on the other hand promotes hypertension and results in atherosclerosis and cardiovascular diseases (Wang et al., 2011; Wang et al., 2015). Actually, increased TMAO production has been considered as a potential biomarker to predict high risk of cardiovascular diseases (Nam, 2019). The changes of gut microbiota community are also observed in arteriosclerotic patients (Karlsson et al., 2012). To our current knowledge, only specific bacteria species in gut microbiota can regulate blood pressure by immunomodulation and metabolite-induced effects. Species like *Lactobacillus* may serve as a therapeutic target to control post-stroke blood pressure. While the effects of diverse metabolites may drive the disease progress in opposite directions, therefore accurate analysis on specific species and their metabolic products are required to deepen our understanding on gut microbiota and blood pressure control after stroke.

Oxygen

Insufficient oxygen supply (hypoxia) occurs in ischemic stroke due to the blockage of cerebral blood flow and the lack of oxygen store in brain, which results in subsequent neurological deficiency (Ferdinand and Roffe, 2016). However, the efficacy and safety of oxygen supplementation, which leads to hyperoxia, have also become controversial based on the results of recent clinical trials (Rincon et al., 2014; Roffe et al., 2017). The control of oxygen level in stroke patients remains as a dilemma. As commensal gut microbiota physiologically reside in a hypoxic to anoxic environment, the elevation of oxygen concentration in both mouse and human can break the homeostasis of gut microbiota and lead to dysbiosis (Albenberg et al., 2014), which could have a strong impact on stroke as discussed above. In hyperbaric oxygen therapy, hyperoxia may also induce dysbiosis of gut microbiota through matrix metalloproteinase-9 (MMP-9) (Cummins and Gentene, 2010; Rodrigues et al., 2012). Until now, only a few studies are involved in the mechanisms how gut microbiota influences oxygen level. In insect, gut bacteria can reduce gut oxygen level as a signal for larvae development (Coon et al., 2017). In rodent model, gut microbiota-producing butyrate activates PPAR-γ signaling pathway and facilitates nitrate production, through which oxygen is excluded outside of the colon lumen and prevents dysbiosis in gut ecosystem (Byndloss et al., 2017). Notably, depletion of butyrate-producing *Clostridia* in gut microbiota can also lead to an aerobic luminal expansion (Rivera-Chavez et al., 2016). As a typical translocating species after stroke (Stanley et al., 2018), *Clostridia* may also modulate oxygen microenvironment in peripheral tissues and organs. A recent study has revealed that lung and gut microbiota communities can be significantly altered by hyperoxia (Ashley et al., 2020). Gut microbiota can correlate with lung inflammation and in turn protect lung from oxygen-induced injury (Ashley et al., 2020), which may indicate certain functions of translocated gut microbiota after stroke (Stanley et al., 2016). However, more evidence are demanded for the precise interpretation of the crosstalk between gut ecosystem and systematic and localized oxygen modulation during post-stroke management.

Hydration

Water balance is also highly concerned in the management of stroke recovery. In elderly stroke patients, both overhydration and dehydration status exist (O'Neill et al., 1992). Dehydration is of high frequency and particularly associated with poor outcome and prognosis of stroke (Rowat et al., 2012). Secreted chloride anion (Cl^-) from gut epithelial cells facilitates water transport and hydration, in which the composition of gut microbiota is significantly shifted (Keely et al., 2012; Musch et al., 2013). Unfortunately, little data is available on whether gut microbiota reversely affects hydration status. In general, investigations on how gut microbiota regulates osmolality are still very limited, thus more studies need to be performed not only to describe phenotypes but also to elucidate underlying mechanisms before we can take advantage of gut microbiota in hydration homeostasis in post-stroke management.

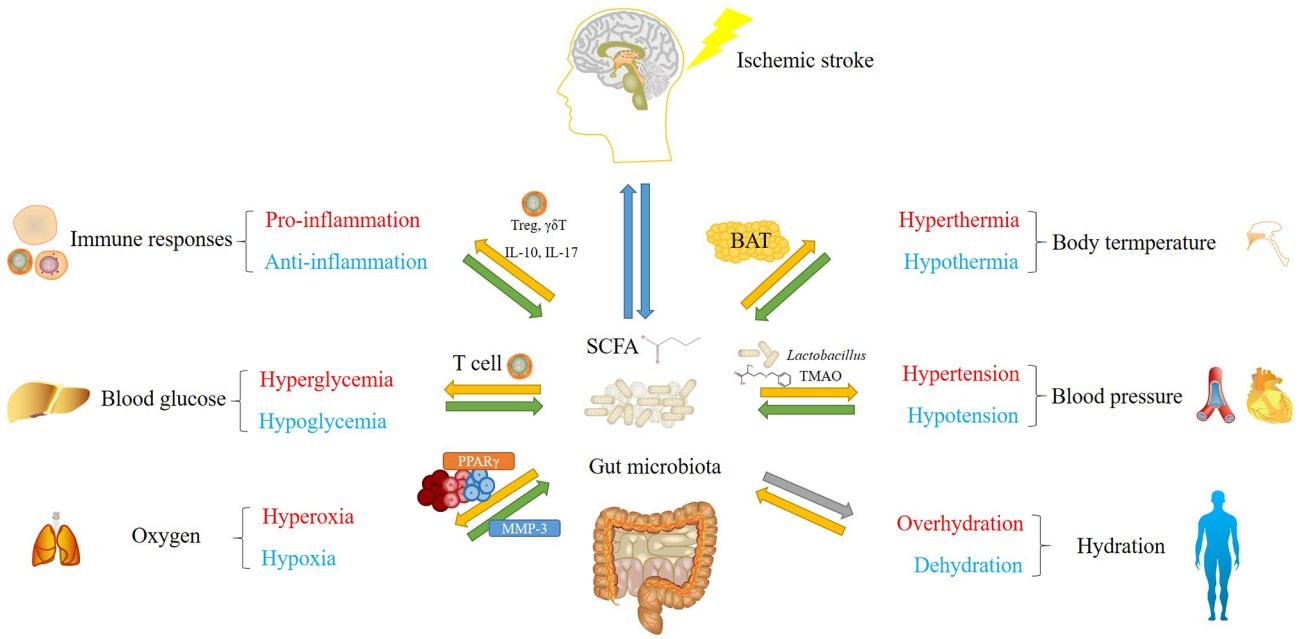

FIGURE 1 | An overview of major concerns in post-stroke management influenced by gut microbiota. Green arrows represent solid functions, yellow arrows represent opposite functions or functions with relatively weaker evidence, gray arrows show effects without solid evidence. Gut microbiota and their metabolic products, mainly short-chain fatty acid (SCFA), regulate immune responses, body temperature, blood glucose, blood pressure and oxygen level after stroke. Their populations and diversities are also altered by these factors. Gut microbiota-mediated modulation involve mechanisms including the regulation of T cell (immune responses and blood glucose), the regulation of UCP1 gene expression in brown adipose tissue (BAT) (body temperature), SCFA and trimethylamine N-oxide (TMAO)-mediated opposite effects on blood pressure, and PPARγ-mediated oxygen exclusion. Although gut microbiota populations are considered to be influenced by altered hydration status, whether they conversely regulate hydration remains unclear.

CONCLUSION

Accumulating evidence support a crucial role of commensal gut microbiota in stroke prevention and recovery management. Notably, gut microbiota and their metabolites could be involved in post-stroke regulation of immune responses, body temperature, blood glucose and blood pressure in both directions, while it remains unclear whether gut microbiota could regulate oxygen and hydration levels after stroke (**Figure 1**). Considering the complexity of the populations and metabolites in gut microbiota, their effects on ischemic stroke could be highly variable, making it difficult to apply gut microbiota directly to stroke recovery. Therefore, instead of using fecal transplantation containing entire gut microbiota populations from healthy donors, discovering specific functional bacteria species or metabolic compounds from homeostatic gut microbiota could be a wise strategy to promote stroke recovery in an efficient and safe manner. Actually, researchers have already attempted to develop drugs modulating gut microbiota in the stroke therapy using rodent models. For instance, *Panax Notoginsenoside* extract has been shown to protect rat brain from ischemic stroke by regulating GABA-β receptors *via* the modulation of gut microbiota populations, particularly through *Bifidobacterium longum* (Li et al., 2018a). Interestingly, oral administration of *Bifidobacterium longum* has achieved similar neuroprotective effects as *Panax Notoginsenoside* extract (Li et al., 2018a), which serves as an example how we could take advantage of specific species from gut microbiota for stroke recovery. In addition, atorvastatin and lactulose have already been demostrated to repair gut barrier, reduce gut inflammation and restore gut microbiota after stroke, providing novel startegies to improve stroke outcome (Yuan et al., 2021; Zhang et al., 2021). In the future, both basic research and clinical studies on gut microbiota will open novel avenues not only for stroke therapy, but also for other brain disorders.

AUTHOR CONTRIBUTIONS

XZ conceived the idea and wrote the manuscript. JZ, SL, and JY assisted with reference collection, figure and table preparation. All authors contributed to the article and approved the submitted version.

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Fecal Microbiota Transplantation Relieves Gastrointestinal and Autism Symptoms by Improving the Gut Microbiota in an Open-Label Study

Ning Li[1†], Hongyan Chen[2†], Yi Cheng[1†], Fenghua Xu[1], Guangcong Ruan[1], Senhong Ying[1], Wen Tang[1], Lu Chen[1], Minjia Chen[1], LinLing Lv[1], Yi Ping[1], Dongfeng Chen[1] and Yanling Wei[1*]*

[1] Department of Gastroenterology, Daping Hospital, Army Medical University (Third Military Medical University), Chongqing, China, [2] Department of Gastroenterology, North-Kuanren General Hospital, Chongqing, China

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Edited by:
Tingtao Chen,
Nanchang University, China

Reviewed by:
Zikai Wang,
People's Liberation Army General Hospital, China
Bota Cui,
Nanjing Medical University, China

***Correspondence:**
Dongfeng Chen
chendf1981@126.com
Yanling Wei
lingzi016@126.com

[†]These authors have contributed equally to this work

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Autism spectrum disorder (ASD) is a severe brain development disorder that is characterized by deficits in social communication and restricted, repetitive and stereotyped behaviors. Accumulating evidence has suggested that gut microbiota disorders play important roles in gastrointestinal symptoms and neurodevelopmental dysfunction in ASD patients. Manipulation of the gut microbiota by fecal microbiota transplantation (FMT) was recently shown to be a promising therapy for the treatment of various diseases. Here, we performed a clinical trial to evaluate the effect of FMT on gastrointestinal (GI) and ASD symptoms and gut microbiota alterations in children with ASD. We found that there was a large difference in baseline characteristics of behavior, GI symptoms, and gut microbiota between children with ASD and typically developing (TD) control children. FMT could improve GI symptoms and ASD symptoms without inducing any severe complications. Similarly, FMT significantly changed the serum levels of neurotransmitters. We further observed that FMT could promote the colonization of donor microbes and shift the bacterial community of children with ASD toward that of TD controls. The abundance of *Eubacterium coprostanoligenes* pre-FMT was positively correlated with high GSRS scores, whereas a decrease in *Eubacterium coprostanoligenes* abundance induced by FMT was associated with the FMT response. Our data suggest that FMT might be a promising therapeutic strategy to improve the GI and behavioral symptoms of patients with ASD, possibly due to its ability to alter gut microbiota and highlight a specific microbiota intervention that targets *Eubacterium coprostanoligenes* that can enhance the FMT response. This trial was registered at the Chinese Clinical Trial Registry (www.chictr.org.cn) (trial registration number ChiCTR1800014745).

Keywords: gut microbiota, fecal microbiota transplantation, autism spectrum disorders, clinic trial, microbiome-gut-brain axis

INTRODUCTION

Autism spectrum disorder (ASD) is a severe brain development disorder that is characterized by deficits in social communication and restricted, repetitive and stereotyped behaviors. Studies have shown that the morbidity of ASD is approximately 147/10,000 in Western countries, approximately 264/10,000 in Eastern countries, and 120/10,000 in China (Wan et al., 2013), with the male being affected more frequently than females. The pathogenesis of ASD is unclear and is currently thought to be related to genetic factors, immunomodulatory disorders, inflammation, and exposure to environmental toxins (Han et al., 2021).

Increasing reports have identified gut microbiota as an important regulator of brain development, function, and behavior, it is identified to be involved in ASD (Fung et al., 2017). Animal models have shown that transplantation of fecal microbiota from patients with neurological disorders would lead to typical disorder symptoms in germ-free mice (Davies et al., 2021), clinical evidence also indicated that interactions exist between gut microbiota and ASD behavior. A large number of studies have shown that patients with ASD have various gastrointestinal symptoms, such as distension, abdominal pain, diarrhea, and constipation, and that these symptoms are closely related to the severity of ASD (Vargason et al., 2019; Davies et al., 2021). Thus, a possible link between gut microbiota and ASD has been proposed. Moreover, changes in the composition of the gut microbiota and metabolites have been found in both ASD patients and animal models (Kushak et al., 2016; Bermudez-Martin et al., 2021). Meanwhile, toxins produced by abnormal gut microbiota can increase intestinal permeability and aggravate ASD symptoms, for example, *Fusobacterium* produces some nerve factors and exerts systemic effects. These findings suggest that gut microbiota disorders and metabolism play important roles in gastrointestinal symptoms and neurodevelopmental dysfunction in ASD patients.

The microbiota-gut-brain axis (MGBA) was recently proposed based on many studies. Through the MGBA, the gut microbiota and its metabolites can affect the body, and the body can regulate the gut microbiota composition through neural, immune, and endocrine pathways to adapt to environmental changes and maintain a microecological balance (Powell et al., 2017). An increasing number of studies have shown that deficits in the MGBA are one of the pathogenic mechanisms of ASD. Recent studies have shown that the gut microbiota is involved in the bidirectional regulation of the intestinal and central nervous systems through neural, endocrine, metabolic, and immune pathways (Osadchiy et al., 2019). It is believed that microecological therapies that improve the gut microbiota can alleviate some gastrointestinal symptoms and ASD symptoms in ASD patients, so far, some reports have already proved that gut microbiota intervention therapy is helpful for ASD improvement (Wang et al., 2020; Kong et al., 2021).

Fecal microbiota transplantation (FMT) refers to the transplantation of fecal microbiota from healthy donors to patients. It is a novel method that has been used in recent years to treat diseases such as infection, immune diseases, liver diseases, intestinal encephalopathy, and cancer (Bajaj et al., 2017; Routy et al., 2018; Wortelboer et al., 2019). Unlike probiotic therapy or others, FMT targets the entity of gut microbiota and serves as a safe and effective method for gut microecology reconstruction (Ianiro et al., 2018). Additionally, preliminary clinical studies and research on animal models have shown that FMT can significantly alleviate neurological disorders such as Parkinson's disease and act as a protective treatment against neuroinflammation (Vendrik et al., 2020). However, whether FMT has a therapeutic effect on ASD and how it works to improve neurological symptoms are not thoroughly understood. Since there is a growing need to achieve measurable and long-term improvements in children with neuropsychiatric disorders, we hypothesize that FMT in ASD children might induce improvement in stereotyped symptoms with good safety. In this study, we conducted an open-label clinical trial to investigate the safety and efficacy of FMT for gastrointestinal and behavioral symptoms in children with ASD and explored the underlying mechanism of FMT-induced ASD improvement through the microbiome-gut-brain axis.

MATERIALS AND METHODS

Study Design

This study was an open-label clinical trial involving 40 children with ASD (age 3-17 years) who were diagnosed by the Autism Diagnostic Interview-Revised (ADI-R) and accompanied with symptoms of the gastrointestinal tract (such as constipation, diarrhea, etc.), whose custodian can fully understand and accept the informed consent, all participants were able to undergo follow-up examination. Moreover, children with fever, have reliance on tube feeding, and accompanying emergent gastrointestinal disease that needs prompt medication, diagnosed with severe malnutrition or excessive weight loss or severe immunodeficiency disease, have a history of severe allergies, monogenic diseases, mental disorder or depression were exclude from the study. Besides, children who were undergoing probiotics or had antibiotics 7 days before screening were also excluded.

Apart from ASD children, 16 sex- and age-matched typically developing control (TD) children without gastrointestinal (GI) disorders were included in this trial as well. TD control children had no gastrointestinal symptoms in the last 1 month and had no antibiotics therapy for the last 3 months or any medication that would affect gut microbiota (such as proton pump inhibitors, gastrointestinal stimulants, steroids, and aspirin). Meanwhile, the Bristol stool score was 4 for each TD children.

All children participated in the study for 12 weeks, which included a 4-week FMT treatment phase and an 8-week follow-up observation phase after the treatment. The TD children were monitored for 12 weeks without any intervention. There were

two routes of administration for FMT, 27 children received oral route of FMT through freeze-dried capsules while 13 children received colonoscopic FMT. In this trial, participants who were not capable of swallowing capsules were chosen for colonoscopy, while the remaining participants could choose oral capsules, moreover, all participants were allowed to switch to another intervention if they had a strong preference regarding the route of administration. The researchers were not blinded to the group allocation or outcome assessment. **Figure 1** illustrates the study design. Neither vancomycin nor proton pump inhibitors (PPIs) was given before FMT. All participants received 2 liters of GOLYTELY (polyethylene glycol) before FMT and remained fasting until the scheduled treatment. To improve the tolerance of patients to GOLYTELY, we applied it by fractionated dose: 1 L was given one day before enema and the rest was given on the day of enema. For patients with poor tolerance (such as abdominal distention and vomiting), saline was added for bowel preparation. All the participants had clinic visits at weeks 0, 4, 8, and 12.

This clinical study was reviewed and approved by the Ethics Committee at the Army Medical Center of PLA. Written informed consent was obtained from each participant before enrollment. In addition, the study was registered at the Chinese Clinical Trial Registry (www.chictr.org.cn); the trial registration number was ChiCTR1800014745, through which the trial protocol can be accessed.

Standardized Human Gut Microbiota and FMT Preparation

One rigorously screened donor volunteered to provided stool for all participants. The screening involved review of medical history, serological examinations to screen infectious disease, stool examination, gut microbiota sequencing, and confirmation of the absence of gastrointestinal disorders and other neurodevelopmental problems, meanwhile, *Helicobacter pylori* was also detected through C13 breath tests. The serological examination was performed to exclude hepatitis A, B, and C infections, human immunodeficiency virus-1 infection, human immunodeficiency virus-2 infection, TB infection of T cells, TORCH virus infection, and syphilis infection. Fasting glucose levels, lipid levels, liver function, renal function, and C-reactive protein levels were also assessed. The stool used for the preparation was tested for the presence of bacterial pathogens (*Escherichia coli* O157, *Shigella*, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Yersinia*, *Vibrio parahaemolyticus*, and *Vibrio cholera*), infection with viruses (rotavirus A, adenovirus, and norovirus), infection with fungi (*Candida albicans*) and the presence of parasites (Giardia, Cryptosporidium, Cyclospora, and Isospora).

The donated stool samples were collected under anaerobic and sterile conditions. The samples were mixed with sterile normal saline and then homogenized immediately. The homogenates were then filtered through 20 μm nylon filters to remove large particles and fibrous matter. The filtered suspensions were then centrifuged at 6000 g for 5 min at 4°C with a centrifuge (Sorvall SS-34). The supernatant was removed, and the precipitate was dissolved in normal saline, finally, a fecal bacterial solution of 60 mg/mL was obtained and used for future manufacture of FMT capsules or colonoscopic transplantation (10 g/50 kg per child). FMT capsules were prepared as previously reported (Xu et al., 2021), lyophilized protectant was added to the fecal bacterial solution and frozen at -80°C, which was then

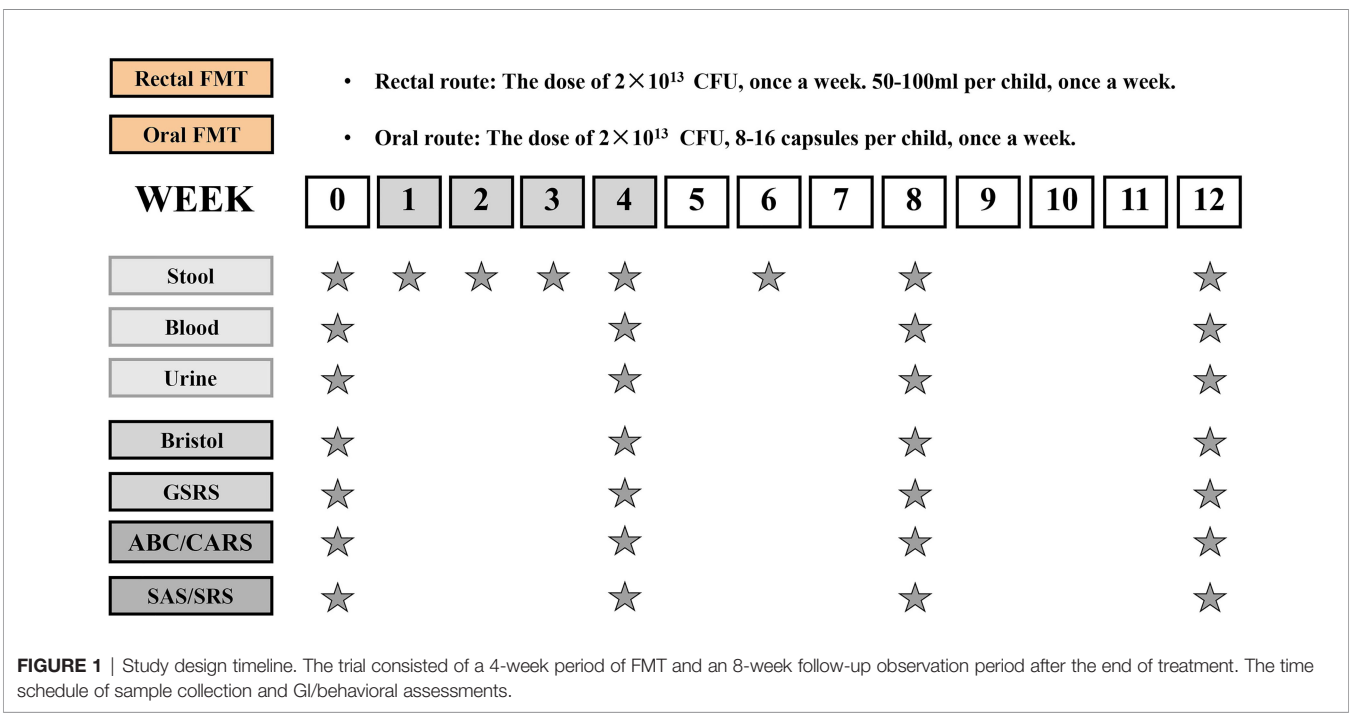

FIGURE 1 | Study design timeline. The trial consisted of a 4-week period of FMT and an 8-week follow-up observation period after the end of treatment. The time schedule of sample collection and GI/behavioral assessments.

freeze-dried into powder using a cryogenic lyophilizer and placed in a capsule for further use.

The participants received 2 liters of GOLYTELY (polyethylene glycol) the night before transplantation. Both the oral capsule administration group and the rectal administration group received the same dose (approximately 2×10^{14} CFU per patient) once a week for 4 weeks.

Evaluation and Sample Collection

During the study, the physical examination of participants was carried out at week 0. Blood samples were collected at week 0, 4, 8, and 12. Stool samples from the participants were collected at week 0, 1, 2, 3, 4, 6, 8, 10, and 12 for 16s rRNA sequencing to determine the composition and abundance of gut microbiota. For each participant, an interview by phone was conducted after FMT treatment. If the patients had any questions or adverse symptoms after FMT, more interviews or consultations would be carried out.

Assessments of Gastrointestinal Symptoms

To evaluated the gastrointestinal symptoms of each participant, their parents were asked to complete the Gastrointestinal Symptoms Rating Scale (GSRS) and the Bristol Stool Form Scale at week 0, 4, 8, and 12. The GSRS is a self-management questionnaire that is a 7-point Likert scale with the following descriptive indicators: 1 (asymptomatic), 2 (slight), 3 (mild), 4 (moderate), 5 (moderate to severe), 6 (severe), and 7 (very severe). It was originally a hierarchical scale used for face-to-face questioning, mainly to evaluate common gastrointestinal symptoms, and was modified to generate a self-management questionnaire. The 15 items can be divided into 5 dimensions: abdominal pain (3 items), reflux (2 items), dyspepsia (4 items), diarrhea (3 items), and constipation (3 items). The questions were all regarding the severity of symptoms in the last 2 weeks. The score for each dimension was expressed as the average score of all items in the dimension, with the lowest score being 1 and the highest score being 7. The Bristol Stool Form Scale was used to assess the condition of the participant's recent stool.

Assessments of Autism and Related Symptoms

Autism and ASD related symptoms were assessed by the Childhood Autism Rating Scale (CARS), Social Responsiveness Scale (SRS) and Autism Behavior Checklist (ABC). The CARS is a 15-item scale that can be used to both diagnose ASD and assess the overall severity of the symptoms. The ABC assesses problem behaviors in five areas that are common in children with ASD, including irritability, lethargy, stereotypy, hyperactivity, and inappropriate speech. Additionally, we assessed parents' levels of anxiety with the Self-Rating Anxiety Scale (SAS). The SAS is a 20-item scale that measures the severity of anxiety in parents of children with ASD. The CARS, ABC, SRS, and SAS were administered at baseline (week 0), at the end of treatment (week 4), and at the end of the observation period (week 12).

Microbial DNA Extraction and 16S rRNA Sequencing

We collected fecal samples, stored them in a refrigerator at -80°C, and then delivered them to the G-Bio Sequencing Company (www.igeneseq.com) in dry ice, for further 16S rRNA sequencing. Microbial DNA was extracted from the fecal samples from ASD children, TD controls, and donors by using the PowerSoil DNA Isolation Kit (Mobio Carlsbad, CA). The purified PCR products were used to prepare a sequencing library using the TruSeq DNA Kit from Illumina following the manufacturer's manual and were subjected to gene sequencing using Reagent Kit v3 on the MiSeq sequencer (Illumina, San Diego, CA, USA). The Quantitative Insights into Microbial Ecology 2 (QIIME2, version 2019.7) platform within a condo environment was used to process the sequencing data on our Linux server. Bioinformatics analysis was performed according to the official "Moving Pictures" tutorial provided by the QIIME2 website (https://docs.qiime2.org/2019.7/tutorials). Linear discriminant analysis effect size (LEfSe) was used to select significantly enriched candidates at the genus level. We compared the relative abundance of taxa between the two groups and within each group at different periods using the nonparametric Mann-Whitney U test followed by linear discriminant analysis (LDA) to estimate the effect size of each microbial feature with differential abundance. A taxon was considered significantly enriched if it had an LDA score greater than 2.0 and p value < 0.05.

Serum 5-HT, GABA, and DA Measurements

Blood samples were taken from ASD children on week 0, 4, 8, and 12 in vacutainer tubes without anticoagulant, and were centrifuged at 3000 r/min for 10 min at room temperature. After centrifugation, the serum (supernatant) was transferred to clean tubes and was immediately stored at -80°C for future experiments. Each serum sample was kept at 4°C for 12 hours for thawing before use. In this study, ELISA kits (Thermo Fisher Scientific, USA) were used to quantify the serum concentration of 5-hydroxytryptamine (5-HT), dopamine (DA), and γ-aminobutyric acid (GABA) of each ASD child, based on the manufacturer's instruction.

Statistical Methods

For statistical analysis, SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA) was used in this study. Measurement data are validated by a normality test and presented as mean ± SD. Comparisons of the measurement data were performed by t-test, Mann-Whitney U test, and one-way ANOVA (analysis of variance), and comparisons of categorical data were performed by chi-square test. Unless otherwise indicated, $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of ASD and TD Subjects

Forty children who were diagnosed with ASD based on critical inclusion criteria, and 16 typically developing (TD) children from different families were enrolled in this study. All children with ASD received FMT treatment for consecutive 4 weeks and then underwent 8 weeks of follow-up study (the TD children were not treated during the whole trial) (**Figure 1**). The TD children had no relatives with any mental disorders. All participants enrolled in this trial were comparable in most demographic characteristics at baseline (**Table 1**), including ages, gender distributions, body mass indexes (BMIs). We also evaluated the consumption of the nutrients among participants, the consumption of carbohydrates and fat was comparable between children with ASD and TD cohort, while protein consumption was higher in ASD children (51.95 ± 9.84 g) than TD controls (48.44 ± 11.36 g) ($p < 0.05$). Moreover, children in the ASD cohort took more rounds of antibiotics during the first 3 years of life (5.75 ± 1.86 *vs* 10.35 ± 3.36, $p<0.001$), and suffer from a higher rate of food allergy incidence (6.25% *vs* 60.00%, $p < 0.001$).

Fecal Microbiota Transplantation Improves GI Symptoms and ASD Symptoms

During the follow-up phase, we found that children after FMT showed obvious improvement on symptoms like abdominal pain, constipation, or diarrhea, some children relived a lot from reflux as well. The overall GI symptoms of participants after treatment were measured using the GSRS. The average GSRS scores of ASD children decreased 35% after 4 weeks of FMT treatment and last for the next 8 weeks (**Figure 2A**), indicating that symptoms including abdominal pain, reflux, indigestion, diarrhea, and constipation were significantly improved after FMT. The Bristol Stool Form Scale and Daily Stool Record (DSR) were used to evaluate changes in stool properties. The occurrence of no stool, hard stool (type 1 or 2), and soft/liquid stool (type 6 or 7) was significantly decreased at the end of treatment compared to baseline, and this improvement persisted 8 weeks after FMT (**Figure 2B** and **Table 2**).

In addition to GI symptoms, ASD symptoms were also improved after FMT treatment. Scores on the ABC, which includes 57 items that assess mood, behavior, emotion, and language, were significantly alleviated by the treatment, and no obvious reversion was observed during 8 weeks after FMT (**Figure 2C**). Scores on the CARS, which evaluates core ASD symptoms, were decreased by 10% at the end of the treatment and remained decreased by 6% after 8 weeks (**Figure 2D**). The SAS was used to assess the level of anxiety in the parents of children with ASD. We found that parents' anxiety levels decreased with the improvement of gastrointestinal symptoms and autism-like symptoms in the children with autism but returned to baseline levels by 8 weeks after the end of treatment (**Figure 2E**). Furthermore, children with ASD exhibited improvements in scores on the SRS, which assesses social skill deficits, at the end of treatment, but these improvements were reversed after 8 to 12 weeks without further treatment (**Figure 2F**).

Moreover, FMT was generally safe and only induced minimal adverse effects, including hyperactivity and aggression. Nausea/vomiting, major changes in blood chemistry, or long-term adverse effects were observed during follow-up (**Table 3**),

TABLE 1 | Demographic characteristics of study participants and their medical history.

Category	TD controls (n = 16)	ASD children (n = 40)	P value
Gender			
Female (n)	1	3	
Male (n)	15	37	
Age range	3-15	3-17	
2-3 years old (n)	1	2	
4-6 years old (n)	6	17	
7-11 years old (n)	7	14	
12-17 years old (n)	2	7	
Age (years), mean (SD)	7.13 ± 3.20	8.03 ± 3.73	0.564
BMI, (mean ± SD)	16.90 ± 2.52	17.96 ± 4.05	0.446
Autism severity, n (%)			
Mild		14 (35.00)	
Moderate		13 (32.50)	
Severe		13 (32.50)	
Food allergy (%)	6.25	60.00	P < 0.001
Oral antibiotic use during first 3 years of life (total rounds)	5.75 ± 1.86	10.35 ± 3.36	P < 0.001
Carbohydrate consumption (g)	106.56 ± 17.20	115.95 ± 16.20	0.059
Fat consumption (g)	54.06 ± 14.05	56.80 ± 15.50	0.543
Protein consumption (g)	48.44 ± 11.36	51.95 ± 9.84	P < 0.05

All values are mean ± standard deviation (SD). P value is calculated by one-way ANOVA analysis.

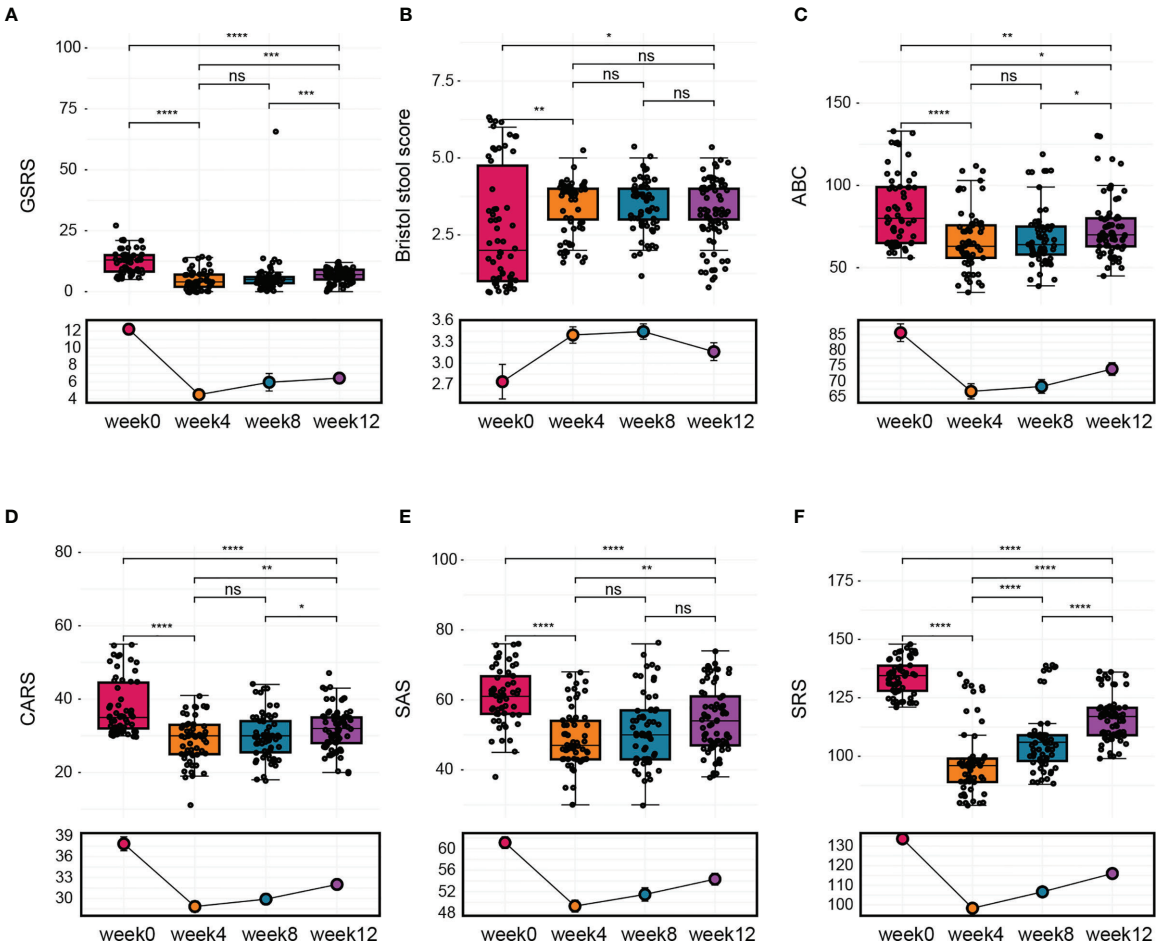

FIGURE 2 | The change in GI symptoms and ASD symptoms after FMT. Children were treated with FMT for 4 weeks and underwent follow-up evaluation for 8 weeks after treatment ended. **(A)** Changes in average GSRS scores after FMT. **(B)** Bristol stool scores. **(C)** Results of ABC assessment at different time points. **(D)** CARS scores before treatment, after treatment, and 8 weeks after treatment. **(E)** Total SAS scores before treatment, after treatment, and 8 weeks after treatment. **(F)** Total SRS scores before treatment, after treatment, and 8 weeks after treatment. The two-tailed Wilcoxon signed-rank test was used to determine the significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, no significance.

TABLE 2 | The percent of stool style of ASD is based on the daily stool record and the Bristol Stool Form Scale (p valve by two-tailed χ^2 test).

Category	Week 0 (%)	Week 4 (%)	Week 12 (%)	P value (Week 0 *vs.*)	
				Week 4	Week 12
No stool	0	0	0	NA	NA
Hard stool (Type 1 or 2)	60	17.5	10	P < 0.001	P < 0.001
Soft/liquid stool (Type 6 or 7)	10	0	0	0.040	0.040
Abnormal stool (Hard/soft/liquid stool or no stool)	70	17.5	10	P < 0.001	P < 0.001

NA, not applicable.

suggesting that FMT was well tolerated. Thus, our data implied that FMT could improve GI symptoms and ASD symptoms without eliciting any severe complications and that the beneficial effects were gradually lost within a few weeks of the end of therapy, suggesting that extended treatment with FMT is needed.

Oral and Rectal Route of FMT Induced Similar Effect on ASD Children

In this trial, FMT treatment was performed through oral or rectal (colonoscopic) route based on the condition of participants. To understand whether the administration route would lead to a

TABLE 3 | Adverse effects of oral and rectal administration.

Adverse effect	Incidence (%)	
	Oral	Rectal
Rash	0	0
Fever	3.7	0
Hyperactivity	3.7	7.7
Tantrums/aggression	3.7	0
Nausea/vomiting	0	0

different outcome on FMT recipients, we also analyzed the clinical indicators of oral and rectal FMT subgroups (**Supplementary Figure 1**).

Participants in the rectal FMT subgroup had a higher Bristol stool score at week 0, showing that children in the rectal subgroup tended to be suffered from diarrhea while the oral subgroup suffered more from constipation. At week 4, 8 and 12, the Bristol score of the two subgroups was also statistically different, interestingly, the discrepancy between the oral and the rectal subgroup decreased after 4 weeks of FMT treatment, and the score of the two subgroups get closer to 3 to 4 after FMT simultaneously, indicating that both oral and rectal FMT improved stool characteristics in ASD children. As for GSRS, the score of the rectal subgroup was significantly higher than the oral subgroup at week 0, this is consistent with the fact that children in the rectal subgroup had more severe symptoms and were not capable of swallowing capsules, however, after 4 weeks of FMT treatment, GSRS score of oral and rectal subgroup both decreased remarkably and showed no significance in comparison, this effect continued during the whole follow-up phase after FMT. This result implies that both oral and rectal routes of FMT could induce significant improvement in GI symptoms, and no significant difference was observed between oral or rectal FMT administration.

Consistent with GI symptoms, autism in participants in both oral and rectal subgroups showed similar change after FMT. At week 0, children in the rectal subgroup had a higher score of ABC and CARS, showing the severity of autism in rectal children, while children in the oral subgroup had a higher SRS score. After 4 consecutive weeks of FMT treatment, ABC, CARS, and SRS score of children in both subgroups decreased significantly and had no statistical difference at week 4, which sustained to week 8 and 12.

FMT Alters the Serum Levels of 5-HT, DA, and GABA in Children With ASD

Neurotransmitters are important in neurological regulation and can affect mood, cognition, and behavior in human beings, abnormality of neurotransmitters would lead to autistic behavior and other neurodevelopmental disorders, alteration of several neurotransmitters are also found in serum or plasma samples of ASD children (Marotta et al., 2020). To further evaluated the effect of FMT on ASD children, we next measured the serum levels of neurotransmitters after FMT. We found that 5-HT and GABA concentrations in the serum decreased after treatment, while the level of DA increased after 4 weeks of FMT (**Figure 3**). Similar to the GI symptom scores, the change of these neurotransmitters in serum was maintained weeks after FMT. During the whole follow-up, the level of 5-HT, DA and GABA changed most significantly at week 4 when FMT treatment was just completed and did not go further since then. Moreover, correlation analysis between the serum level of neurotransmitters and scales for clinical outcomes reveal that 5-HT had a negative correlation with Bristol stool score ($r = -0.434$, $p = 0.001$) while GABA had a positive correlation with Bristol score ($r = 0.527$, $p < 0.001$) (**Supplementary Table 1**), these results suggested that alteration of serum neurotransmitters could have a therapeutic outcome on clinical symptoms on ASD children. Furthermore, at week 8 and 12, these neurotransmitters were still at an altered level significantly but tend to get closer to the original level (**Figure 3**).

Gut Microbiota Changes After FMT

We first evaluated alpha diversity and found that there were no significant differences in alpha diversity between TD and ASD

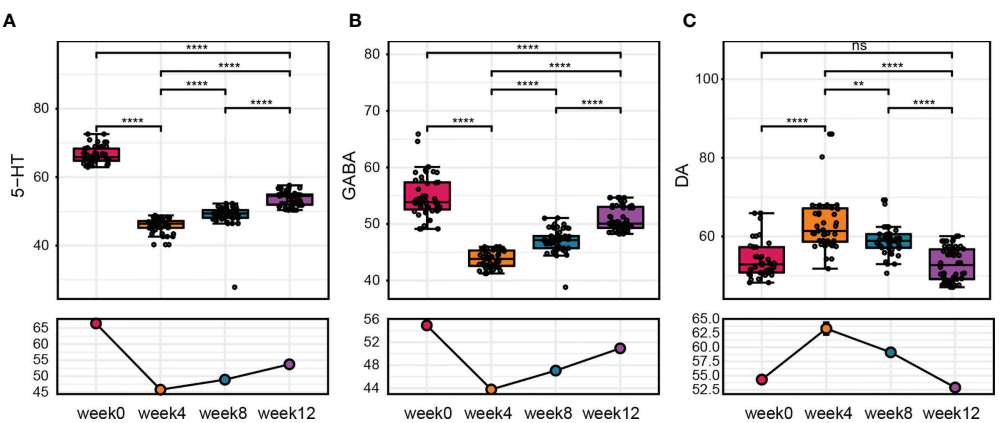

FIGURE 3 | The change in neurotransmitter levels after FMT. **(A–C)** Changes in 5-HT, GABA, and DA levels at before treatment, after treatment, and 8 weeks after treatment. The Wilcoxon signed-rank test was used to determine the significance. **P < 0.01, ****P < 0.0001, ns, no significance.

groups at baseline (**Supplementary Figure 2**). To examine the differences in the microbiota between the two groups, we calculated beta diversity and found significant differences in beta diversity between the two groups (**Supplementary Figure 3**). At both the phylum and genus levels, the microbiota composition of patients with ASD differed from that of the TD controls (**Supplementary Figure 4**). To further identify the bacterial taxa with differential abundance between the ASD group and TD controls at baseline, we performed LEfSe analysis and identified eight genera with significant difference, we found that the abundance of gut microbiota in ASD children differs from TD controls, children in ASD group had a relative higher abundance of *Christensenellaceae*, *Akkermansiaceae* in family level, and *Christensenellaceae R 7 group*, *Akkermansia*, *Coprococcus 2*, *Eisenbergiella*, and *Tyzzerella 3* in genus level, while TD cohorts had a higher abundance of *Peptostreptococcaceae* in family level, and *Romboutsia*, *Fusicatenibacter*, *Eubacterium eligens group* in genus level (**Supplementary Figure 5**).

After FMT treatment for 4 weeks, although the gut microbiota diversity in ASD children remained unaltered, suggesting that FMT did not affect the overall structure of gut microbial communities (**Figure 4A**), the unweighted UniFrac distances of the recipient patient samples were significantly decreased compared with those of the TD controls and donor after 4 weeks of FMT (**Figures 4B, C**). Eight weeks after the FMT treatment (week 12), the distances increased to the level observed before FMT (**Figures 4B, C**), suggesting that FMT could promote the colonization of donor microbes and shift the bacterial community of patients with ASD toward that of TD controls or donor.

Association of the Specific Gut Microbiota With the FMT Response

Because the pre-existing intestinal landscape can dramatically affect the response to FMT (Ericsson et al., 2017; Zuo et al., 2018), we next assessed the composition of the gut microbiota pre-FMT and analyzed its correlations with clinical outcomes post-FMT therapy. Children with ASD who achieved a less than 50% reduction in average GSRS score were defined as nonresponders. We found that FMT responders and nonresponders clustered separately based on orthogonal partial least squares discriminant analysis (OPLS-DA) (**Figure 5A**). The variable importance in projection (VIP) score for the gut microbiota showed that several genera contributed significantly to group separation (**Figure 5B**). Further comparisons of the relative abundance of all the significant bacteria between responders and nonresponders were performed. A significantly lower relative abundance of *Eubacterium coprostanoligenes* was reported in the responder group than in the nonresponder group (**Supplementary Figure 6**), whereas no significant differences in the abundance of the other bacterial genera were found (**Supplementary Figure 6**).

Furthermore, we observed a significant negative correlation between the pre-FMT relative abundance of *Eubacterium coprostanoligenes* and GSRS scores post-FMT in the FMT recipients (**Figure 5C**). We further assessed the changes in *Eubacterium coprostanoligenes* abundance after FMT treatment and found that the reduction in *Eubacterium coprostanoligenes* abundance was positively correlated with a reduction in GI symptoms, as indicated by the change in GSRS score (**Figure 5D**). Besides, the correlation analysis of neurotransmitters between gut microbiota showed that *Eubacterium coprostanoligenes*

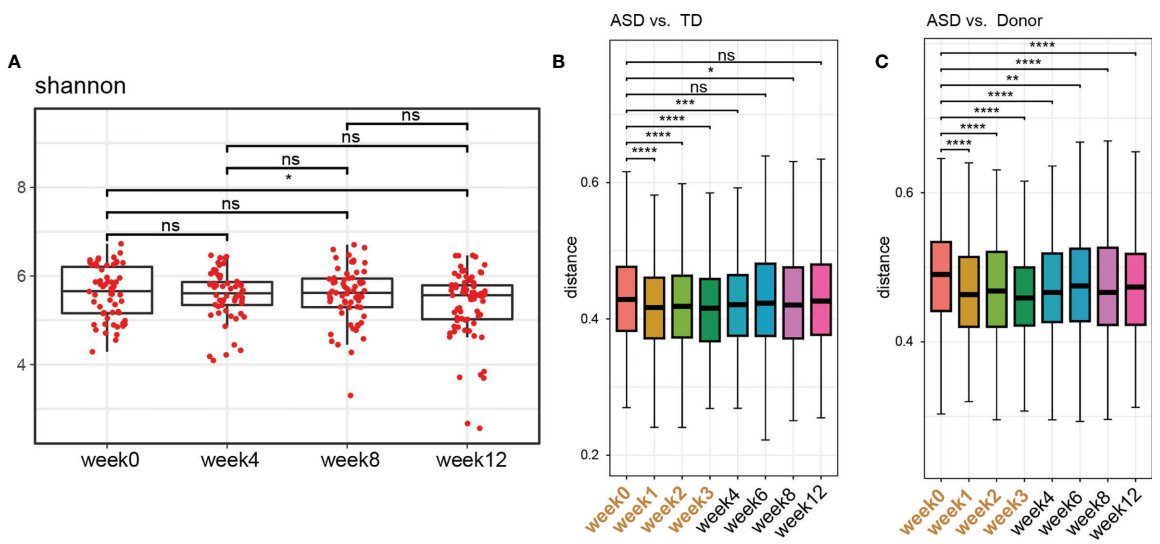

FIGURE 4 | Gut microbiota changes after FMT. **(A)** Changes in alpha diversity were determined using Shannon indices in stool samples from children with ASD collected at different time points. The two-tailed Wilcoxon signed-rank test was used to analyze the difference. *P < 0.05. ns, not significant. **(B)** Unweighted UniFrac distance between ASD children treated with FMT at each timepoint and TD controls. **(C)** Unweighted UniFrac distance between ASD children treated with FMT at each timepoint and donors. The two-tailed Wilcoxon signed-rank test was used to analyze the difference. *P < 0.05, **P < 0.01, ***P < 0.0001. ****P < 0.00001. NS, not significant.

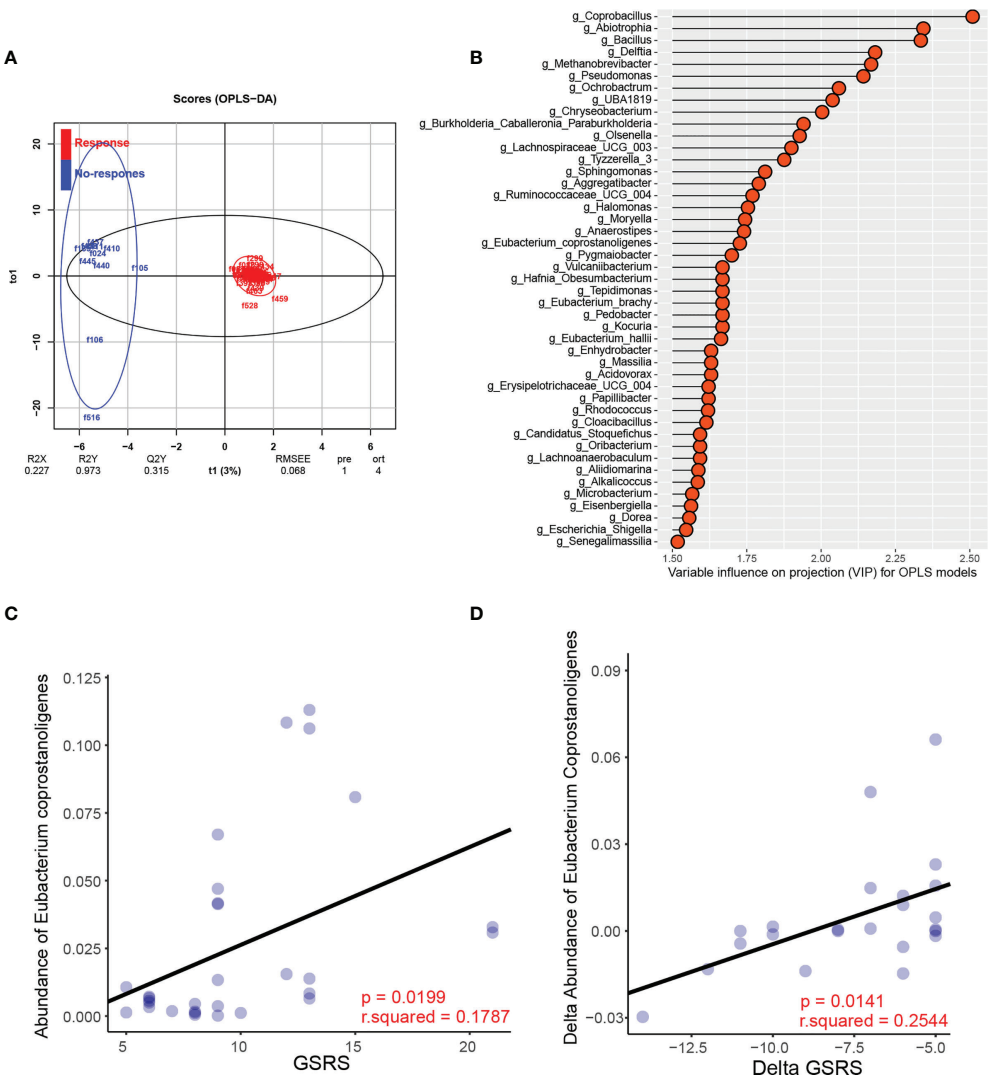

FIGURE 5 | Specific microbial changes are associated with the response to FMT. **(A)** OPLS-DA analysis of the responder group and nonresponder group pre-FMT. **(B)** Variable influence on projection (VIP) for OPLS models. **(C)** Correlation between the abundance of *Eubacterium* and GSRS scores post-FMT in the FMT recipients. **(D)** Correlation between the change in the relative abundance of *Eubacterium* after FMT and the change in GSRS scores after FMT.

had a negative correlation at week 12 (r = 0.341, p = 0.048) with serum concentration of GABA. This result indicated that FMT might act partially to treat ASD by reducing the abundance of *Eubacterium coprostanoligenes* and that the genus *Eubacterium coprostanoligenes* is a potential regulator of FMT treatment response in children with ASD.

DISCUSSION

In this trial, to better understand how the gut microbiota works in ASD through the microbiome-gut-brain axis, we performed FMT by oral and rectal routes in ASD children who have associated gastrointestinal disorders and analyzed the gut microbiota and serum levels of several neurotransmitters. As our data suggested, ASD children with gastrointestinal symptoms suffered from gut dysbiosis, and FMT could serve as a protective treatment for reconstructing gut microbiota at both the phylum and genus levels and had a therapeutic outcome on autism symptoms and on gastrointestinal disorders. In addition, FMT also induced a desirable effect on recovering the serum levels of 5-HT, GABA, and DA in the ASD cohort, indicating that FMT might play an important role in modulating neurotransmitters through the MGB axis.

ASDs are complex neurobiological disorders that impair social interactions and communication and represent one of the pervasive developmental disorders. According to a recent report, the prevalence of ASD is 0.6% to 1.7% in children and adolescents, posing a heavy burden on public health (Iglesias-Vazquez et al., 2020). In ASD patients, symptoms are heterogeneous from one

individual to another, and their disorders could be identified by two main features, regardless of sex, race, ethnicity, or other factors: social communication and restricted, repetitive sensory-motor behaviors (Lord et al., 2018). It is generally believed that various hazardous factors are associated with the onset of ASD, including both genetic and environmental factors, such as nutritional deficiencies or overloads, exposure to viruses, errors during embryonic neural tube closure, dysfunctional immune systems, and allergies (Bhandari et al., 2020). The genetic factors related to ASD are complex, and more than 100 genes and genomic regions have been reported and identified to be involved in the etiology of ASD (Li et al., 2017).

Apart from the main disorders, ASD patients often suffer from comorbidities, such as intellectual disability and gastrointestinal symptoms, and gastrointestinal disorders are reported to be highly prevalent. Many subjects with ASDs have significant gastrointestinal dysfunctions, including altered bowel habits and chronic abdominal pain, that accompany their neurological alterations (Madra et al., 2020). The incidence of constipation, diarrhea, abdominal pain, vomiting, and flatulence ranges from 9% to 90% in patients with ASD (Vuong and Hsiao, 2017). Gastroenterology disorders can be of great threat to children's quality of life, and some typical presentations, such as diarrhea or constipation, easily affect well-being. Additionally, some less attractive and nontypical presentations can be of great harm to children's growth; for example, maladaptive behaviors, sleep disorders, aggressive behavior, irritability, and self-injury may result from symptoms in ASD children who are undergoing gastrointestinal disorders (Patusco and Ziegler, 2018).

Although the cause and effect of gastrointestinal dysfunction in ASD children have not been fully elucidated, increasing evidence has shown that gut microbiota plays an important role in this process. The gut microbiota, which is composed of bacteria, fungi, and viruses, is essential in maintaining a healthy gut microenvironment for gastrointestinal function and immune and endocrine homeostasis (Osadchiy et al., 2019). Abnormal alterations in gut microbiota can lead to intestinal malfunction, thus resulting in increased intestinal permeability (also known as leaky gut), inflammation, and metabolism dysfunction (Powell et al., 2017), thus affecting distant organs or systems of the host. Increasing reports have indicated that the gut microbiota participates in a bidirectional signaling pathway between the gastrointestinal system and the brain, which is known as the microbiota-gut-brain (MGB) axis (Naveed et al., 2021). Recent studies reported that alterations in the gut microbiota composition or gut microbiota dysbiosis in children with ASD may contribute to both gastrointestinal and nervous system symptoms (Hughes et al., 2018), while several studies have suggested a potential link between the microbiota and ASD: great differences exist in the gut microbiota between children with ASD and TD controls (Fattorusso et al., 2019).

Consistent with numerous studies of diverse cohorts that have reported differences in the microbiome profiles of individuals with ASD and TD controls, we found significant differences in the gut microbiota between individuals with ASD and TD controls in this trial. We analyzed the diversity and abundance of gut microbiota in ASD populations and TD controls and observed dysbiosis in children with ASD. As our data presented, alpha and beta diversity did not show any significant differences between the TD cohort and ASD; however, gut microbiota at the phylum and genus levels were found to be variable between the two groups. We speculated that although the diversity was not statistically different, the microecology in the intestinal tract was disturbed due to alterations in several microorganisms of low abundance. In particular, in ASD populations, *Verrucomicrobia* at the phylum level was remarkably increased compared to that in TD children, which has been identified as an upregulated intestinal microorganism in many neurological disorders, such as Parkinson's disease. It has also been reported by many other publications that the abundance of *Verrucomicrobia* is altered significantly in autism populations. In addition, at the genus level, *Ruminococcus*, *Corprococcus*, *Akkermansia*, and *Christensenellaceae* were found to be increased in ASD children, while *Romboutsia*, *Fusicatenibacter*, and *Eubacterium coprostanoligenes* were found to be significantly decreased.

To date, there is no confirmed evidence showing that autism can be cured by any medication. Currently approved and recommended treatments for ASD essentially include rehabilitation, educational therapy and psychopharmacological approaches (Famitafreshi and Karimian, 2018). Recently, microbiota intervention treatment by nutritional management and probiotic therapy has attracted increasing attention, since some trials have already reported that by reconstruction of the gut microbiota, gastrointestinal disorders, along with autism symptoms, can be alleviated (Patusco and Ziegler, 2018). Among microbiota therapies, fecal microbiota transplantation (FMT) is thought to be a preferred method due to its capacity to target the entire gut microbiota with good safety. FMT is mainly applied as an effective treatment for recurrent *Clostridium difficile* infection (Kelly et al., 2016) and has also been developed as an alternative therapy for many diseases complicated by gastro intestinal disorders. Recent studies have shown that FMT also has a therapeutic effect on neurological disorders, both in animal models and clinical trials. In 2017, Kang et al. reported that microbiota transplantation from healthy donors improves gastrointestinal and autism disorders in an open-label study (Kang et al., 2017).

To better understand the efficacy and mechanism of FMT in improving ASD symptoms, we carried out this trial, and our data showed that FMT by both the rectal route and oral administration had a significant beneficial clinical effect in children with ASD, especially in young children, which lasted for 8 weeks without inducing severe complications. ASD children who participated in this trial were allocated to oral or rectal FMT subgroups based on whether they could comply with capsule swallowing. During enrollment, we found that children with more severe symptoms (both gastrointestinal disorders and autism) tend to be not capable of cooperating and it is difficult for them to swallow FMT capsules, this leads to the variation in baseline characteristics of clinic symptoms between the oral and rectal group. Children in the rectal group suffered more from diarrhea and have more severe GI symptoms (such as abdominal pain, reflux, flatulence), also, the scale on autistic symptoms

showed that they have a higher score on ABC, CARS, and lower score on SRS. Fortunately, oral FMT and rectal FMT both lead to improvement of GI symptoms and autism in participants, furthermore, after 4 consecutive weeks of FMT therapy, no significant difference in GI symptoms or autism was observed between the two subgroups, this result verified the equality of FMT through oral and rectal routes. When applying FMT for ASD children, the oral or rectal route would not cause a prominent difference in the therapeutical outcome, whether the recipient is capable of taking FMT capsules by oral is the key factor that should be taken into consideration.

Gut microbiota diversity after FMT treatment in ASD children also showed significant differences, which implied that improvement of symptoms is associated with the change in gut microecology. It is believed that the bidirectional interaction between the brain and gut through the MGB axis is associated with neuroendocrine, neuroimmune, and autonomic nervous system mechanisms. An increasing number of publications have reported that gut microbiota can affect the metabolism of neurotransmitters. For example, some metabolites derived from gut microbiota can be absorbed and enter the blood circulation, and these metabolites can cross the blood-brain barrier and modulate cerebral function (Fung et al., 2017). *Lactobacillus rhamnosus* YS9 can produce gamma-aminobutyric acid (GABA), an important inhibitory neurotransmitter in the system, while monoamines, such as noradrenaline, dopamine, and serotonin, are also produced by several strains of bacteria colonizing the intestinal tract (Strandwitz, 2018). Take GABA as an example, which is derived from glutamate thanks to the action of glutamate decarboxylase, it is important in neuronal excitability, alterations in gabaminergic and glutaminergic systems would cause a disrupted excitatory/inhibitory balance, and it is found that plasma level of GABA in ASD children is altered compared to healthy cohorts (Marotta et al., 2020). As for 5-HT, it intervenes in multiple brain functions, studies have revealed that the 5-HT transporter (SERT or 5-HTT) or 5-HT levels were higher in autistic individuals both in clinic studies and in animal models (Muller et al., 2016). Based on current evidence, we evaluated the serum level of neurotransmitters in participants to better understand the possible mechanism by which FMT showed a therapeutic effect on ASD. We found that FMT in the ASD cohort induced a significant change in neurotransmitters in serum; 5-HT and GABA decreased after FMT, while DA levels were elevated. As our results show, we speculate that FMT might be helpful in modulating the central nerve through the MGB axis by regulating neurotransmitters.

In this trial, we also found that the gut microbiota α diversity did not change significantly, suggesting that FMT did not affect the overall structure of gut microbial communities, or at least, was not sufficient to induce a response in each participant. In addition, FMT treatment could promote the colonization of donor microbes and shift the bacterial community of individuals with ASD toward that of TD controls or donors. Because the pre-existing intestinal landscape can dramatically affect the outcome of FMT, we assessed the composition of the gut microbiota pre-FMT and divided the participants from the ASD group into two subgroups, responders and nonresponders to FMT, according to the microbiota analysis. We found that responders and nonresponders clustered separately and that a high abundance of *Eubacterium coprostanoligenes* in ASD patients prior to FMT was associated with high GSRS scores. We thus hypothesized that in FMT clinical responders, FMT might reduce the abundance of *Eubacterium coprostanoligenes*, thus improving GI symptoms in individuals with ASD. We further found that the reduction in *Eubacterium coprostanoligenes* abundance was positively correlated with a reduction in GI symptoms, indicating an association between clinical improvements and the changes in bacterial profiles following FMT in ASD patients. Moreover, a negative correlation exists between *Eubacterium coprostanoligenes* abundance and serum GABA concentration, indicating that FMT might have an influence on neurotransmitters that would regulate mood, behavior, and neurodevelopment, and this might be a possible explanation that why FMT induced improvement not only GI symptoms but also autistic symptoms on ASD children.

In conclusion, our work proved that FMT was well tolerated and effective in improving gastrointestinal symptoms and autism-like behaviors in children with ASD. FMT seemed to induce the production of a microbiota that was significantly different from the pre-FMT microbiota and much more similar to those of the healthy donor and typically developing children. However, this study did not include children under other therapies as the control group, and only include participants and donor in southwest China, whether geological factors would have impact on the therapeutical outcomes in ASD children is still lack of evidence. Our findings identified specific bacteria *Eubacterium coprostanoligenes* that might be associated with therapeutic outcomes, which should be further explored in future FMT trials in ASD patients.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI Sequence Read Archive, accession number PRJNA758217.

ETHICS STATEMENT

The present clinical trial study was reviewed and approved by the Ethics Committee at the Army Medical Center of PLA. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NL, DC, and YW conceived the idea. NL, HC, YC, FX, SY, LC, MC, LL, YP, and WT performed the experiments and analyzed the data. YC, NL, and WT wrote the manuscript. DC and YW supervised the study. NL, HC, and YC contributed equally to this

work. 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.2021.759435/full#supplementary-material

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CORRECTION
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Corrigendum: Fecal Microbiota Transplantation Relieves Gastrointestinal and Autism Symptoms by Improving the Gut Microbiota in an Open-Label Study

Ning Li[1†], Hongyan Chen[2†], Yi Cheng[1†], Fenghua Xu[1], Guangcong Ruan[1], Senhong Ying[1], Wen Tang[1], Lu Chen[1], Minjia Chen[1], LinLing Lv[1], Yi Ping[1], Dongfeng Chen[1] and Yanling Wei[1*]*

[1] Department of gastroenterology, Daping Hospital, Army Medical University (Third Military Medical University), Chongqing, China, [2] Department of Gastroenterology, North-Kuanren General Hospital, Chongqing, China

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Edited and reviewed by:
Tingtao Chen,
Nanchang University, China

***Correspondence:**
Dongfeng Chen
chendf1981@126.com
Yanling Wei
lingzi016@126.com

[†]These authors have contributed equally to this work

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Keywords: gut microbiota, fecal microbiota transplantation, autism spectrum disorders, microbiome-gut-brain axis, clinic trial

A Corrigendum on:

Fecal Microbiota Transplantation Relieves Gastrointestinal and Autism Symptoms by Improving the Gut Microbiota in an Open-Label Study

By Li N, Chen H, Cheng Y, Xu F, Ruan G, Ying S, Tang W, Chen L, Chen M, Lv L, Ping Y, Chen D and Wei Y (2021). Front. Cell. Infect. Microbiol. 11:759435. doi: 10.3389/fcimb.2021.759435

In the original article, there was a mistake in ***Figure 1*** as published. **The dose of oral route FMT was mislabeled.** The corrected ***Figure 1*** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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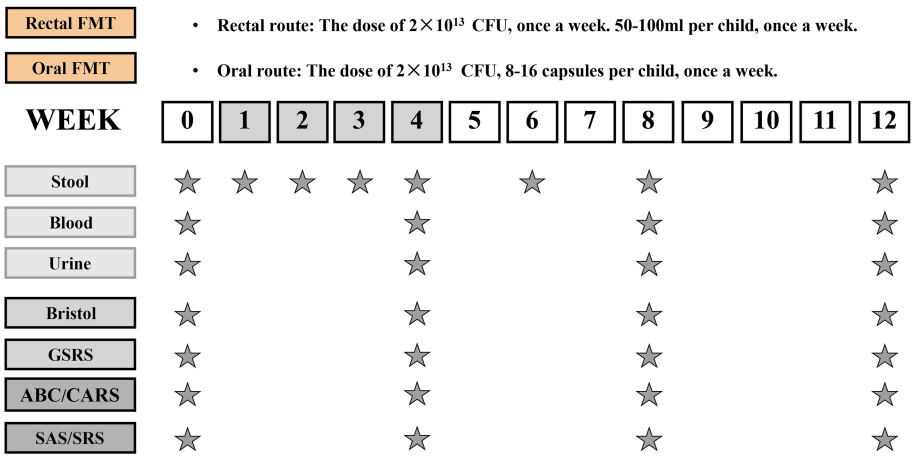

FIGURE 1 | Study design timeline. The trial consisted of a 4-week period of FMT and an 8-week follow-up observation period after the end of treatment. The time schedule of sample collection and GI/behavioral assessments.

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Porphyromonas gingivalis-Induced Cognitive Impairment Is Associated With Gut Dysbiosis, Neuroinflammation, and Glymphatic Dysfunction

Li Chi[1†], Xiao Cheng[1†], Lishan Lin[2,3], Tao Yang[1], Jianbo Sun[1], Yiwei Feng[4], Fengyin Liang[2,3], Zhong Pei[2,3] and Wei Teng[1*]*

[1] Hospital of Stomatology, Guangdong Provincial Key Laboratory of Stomatology, Institute of Stomatological Research, Guanghua School of Stomatology, Sun Yat-sen University, Guangzhou, China, [2] Department of Neurology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China, [3] Guangdong Provincial Key Laboratory of Diagnosis and Treatment of Major Neurological Diseases, National Key Clinical Department and Key Discipline of Neurology, Guangzhou, China, [4] Department of Neurology, Huashan Hospital, Fudan University, Shanghai, China

Background: Periodontal pathogen and gut microbiota are closely associated with the pathogenesis of Alzheimer's disease (AD). *Porphyromonas gingivalis* (Pg), the keystone periodontal pathogen, can induce cognitive impairment. The gut has a connection and communication with the brain, which is an important aspect of the gut–brain axis (GBA). In the present study, we investigate whether Pg induces cognitive impairment through disturbing the GBA.

Methods: In this study, Pg was orally administered to mice, three times a week for 1 month. The effects of Pg administration on the gut and brain were evaluated through behaviors, gut microbiota, immune cells, glymphatic pathway clearance, and neuroinflammation.

Results: Pg induced cognitive impairment and dysbiosis of gut microbiota. The α-diversity parameters did not show significant change after Pg administration. The β-diversity demonstrated that the gut microbiota compositions were different between the Pg-administered and control groups. At the species level, the Pg group displayed a lower abundance of *Parabacteroides gordonii* and *Ruminococcus callidus* than the control group, but a higher abundance of *Mucispirillum schaedleri*. The proportions of lymphocytes in the periphery and myeloid cells infiltrating the brain were increased in Pg-treated animals. In addition, the solute clearance efficiency of the glymphatic system decreased. Neurons in the hippocampus and cortex regions were reduced in mice treated with Pg. Microglia, astrocytes, and apoptotic cells were increased. Furthermore, amyloid plaque appeared in the hippocampus and cortex regions in Pg-treated mice.

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Tingtao Chen,
Nanchang University, China

Reviewed by:
Ming Li,
Dalian Medical University, China
Yaping Pan,
China Medical University, China

***Correspondence:**
Zhong Pei
peizhong@mail.sysu.edu.cn
Wei Teng
tengwei@mail.sysu.edu.cn

[†]These authors have contributed equally to this work

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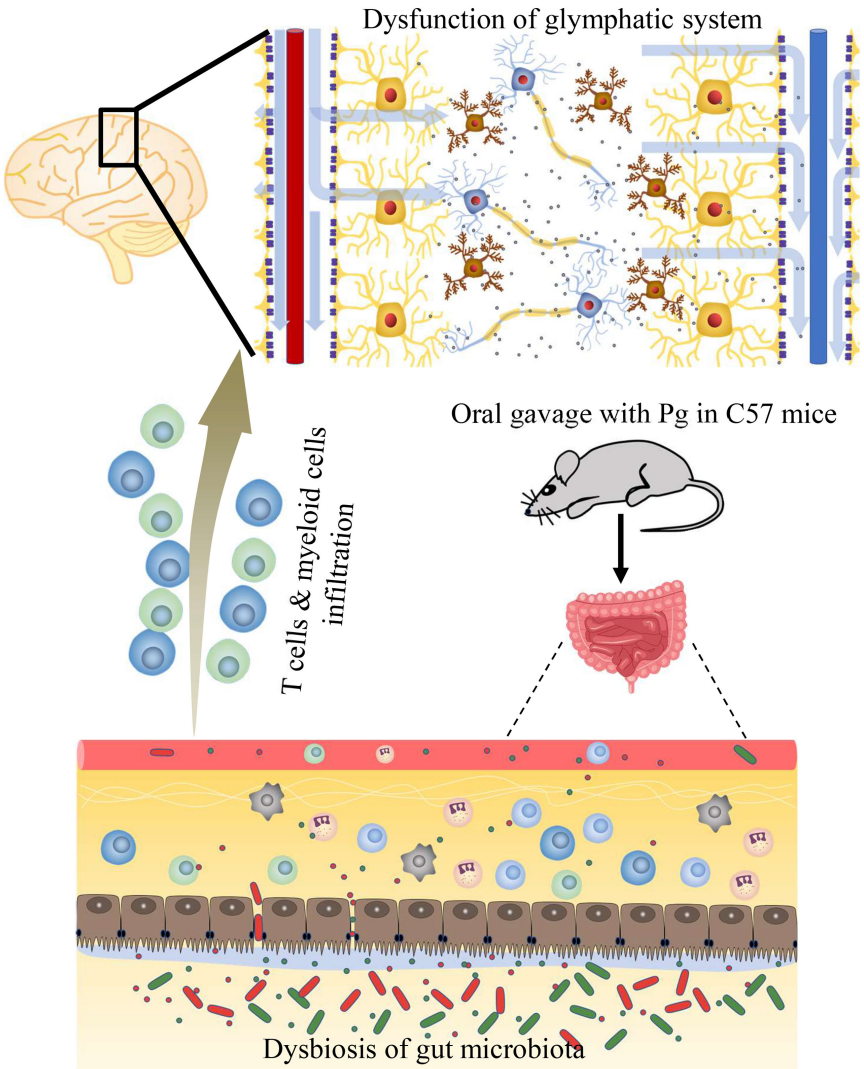
Conclusions: These findings indicate that Pg may play an important role in gut dysbiosis, neuroinflammation, and glymphatic system impairment, which may in turn lead to cognitive impairment.

Keywords: ***Porphyromonas gingivalis*, cognitive impairment, neuroinflammation, glymphatic system, gut–brain axis**



GRAPHICAL ABSTRACT | The periodontal pathogen *P. gingivalis* induces cognitive impairment by disturbing gut-brain axis.

INTRODUCTION

Porphyromonas gingivalis (Pg) is a keystone pathogen in periodontitis (Yang et al., 2019). In addition to its oral effect, Pg is closely related with the occurrence and development of numerous systemic diseases, such as atherosclerosis (Xie et al., 2020), diabetes (Tian et al., 2020), and Alzheimer's disease (AD) (Diaz-Zuniga et al., 2020). Cognitive impairment is an early symptom of AD and Pg was found to be closely related to cognitive impairment (Noble et al., 2009; Zhang et al., 2018). Amyloid plaques are aggregation of beta-amyloid peptides (Aβ) that accumulate in the brain, damaging and destroying neurons and resulting in progressive cognitive impairment. It is reported that Pg can not only promote the Aβ deposit in the central system (Dominy et al., 2019), but also induce macrophages to produce Aβ, which may contribute to the central deposition

(Nie et al., 2019). Virulence factors of Pg such as LPS and gingipain can induce inflammatory responses. Pg-LPS can induce neuronal inflammation through the TLR4/NF-κB pathway (Zhang et al., 2018). Gingipain induces the migration of microglia to the site of infection and leads to neuroinflammation (Nonaka and Nakanishi, 2020). Inhibitors of Pg virulent factors could ameliorate infection and reduce amyloid plaque production and neuroinflammation (Dominy et al., 2019).

Pg of oral origin can induce the dysbiosis of gut microbiota (Kato et al., 2018; Ohtsu et al., 2019). The oral–gut connection of Pg occurs during common activities such as chewing and swallowing. Gut microbiota composition plays a part in the regulation of brain functions, including social behavior, motor dysfunction, and cognitive functions *via* the gut–brain axis (GBA) (Erny et al., 2015; Rogers et al., 2016; Sampson et al., 2016; Cryan et al., 2020). The GBA is regarded as a bidirectional connection between the central nervous system (CNS) and the gastrointestinal tract of the body. It contains various direct and indirect pathways between the cognitive center in the brain and peripheral intestinal function. Regulation of the GBA is critical for maintaining homeostasis, including that of the CNS. The regulatory effects of gut microbiota on the brain can be mediated by the immune aspect of the GBA (van Sadelhoff et al., 2019). Peripheral immune cells in brain parenchyma are maintained at a low level under normal condition. In the state of disease, infiltrated lymphocytes and myeloid cells often turn to damage CNS tissue (Gate et al., 2020; Dressman and Elyaman, 2021; Savinetti et al., 2021).

Neuroinflammation is a general characteristic of the CNS in neurological disorders and is considered as a potential factor of cognitive impairment (Gilhus and Deuschl, 2019). The neuroinflammatory responses, such as activation of gliocytes and expression of proinflammatory cytokines, could exacerbate the CNS microenvironment in diseases and may make a contribution to acceleration of cognitive impairment. Deposition of Aβ is considered as one of the pathological features of AD. In normal physiological conditions, Aβ production and clearance are maintained at a balanced level. In the past, the CNS is believed to be immune privileged, lacking a classic drainage of the lymphatic system. But now, as is known to all, the CNS goes through continuous immune surveillance (Louveau et al., 2015). The glymphatic system has a significant effect on the clearance of brain metabolic wastes (Abbott et al., 2018). The clearing efficiency of the glymphatic pathway can be influenced by sleep deprivation (Nedergaard and Goldman, 2020), some drugs, and neuroinflammation (Sundaram et al., 2019). The glymphatic pathway includes the perivascular space (PVS) influx of cerebrospinal fluid (CSF) into the brain interstitial fluid (ISF), followed by the clearance of ISF along draining veins (Iliff et al., 2012). The continuous movement of fluid through the interchange between the CSF and ISF is critical to clear interstitial solutes. Dysfunction of the glymphatic pathway leads to metabolic waste accumulation, such as Aβ, which is considered to contribute to AD (Arbel-Ornath et al., 2013; Plog and Nedergaard, 2018).

Therefore, we hypothesized that Pg might induce cognitive impairment through regulating the GBA in middle-aged mice.

METHOD

Animals

All experiments were approved by the Institutional Animal Care and Use Committee, Sun Yat-Sen University (Guangzhou, China; approval no. 000439). In this study, 9- to 10-month-old male C57BL/6J mice were acquired from Vital River (Beijing, China). All animals were raised in a specific pathogen-free facility of Sun Yat-Sen University, with ad libitum food and water. All animals were randomly assigned to two groups: control and Pg group (n = 15).

Oral Administration of Pg

Mice were given by oral gavage 10^9 colony-forming units (CFU) of Pg in total, and the Pg was resuspended in 0.1 ml phosphate buffered saline (PBS) with a concentration of 2% carboxymethyl cellulose (CMC) (Sigma Aldrich, St. Louis, MO, USA). This suspension was given three times a week for 4 weeks. The control group was given a suspension without Pg.

Morris Water Maze Test

The Morris water maze (MWM) test was conducted, based on the protocol previously described (Akers et al., 2014). The maze consisted of a round pool with a platform, and the platform was placed 1 cm under the water surface. The test contained two parts: the first one was place navigation trainings (5 days) and the other was spatial probe tests. Briefly, mice were put in the water from four quadrants of the maze every day, lasting for 5 days. The aim was to train the mice to locate the platform. When the mice failed to locate the platform in 60 s, they were guided to swim to the platform and remained there for 10 s of each trial. On the last day, the platform was taken out. Mice were put into the maze at the place opposite to the original location of the platform and were taken out after 60 s. The test parameters were recorded with an automated equipment (San Diego Instruments, San Diego, CA, USA).

Rotarod Test

Briefly, mice ran on the accelerated rod three times a day, lasting for 3 days, with 2 days of training. The rod accelerated from 4 to 40 rpm in 300 s (Xin Ruan, Shanghai, China). Each mouse was allowed to rest for 30 min between experiments. The time of mice falling from the rod was recorded and the average was taken of the three tests.

Open Field Test

In this experiment, the open field consisted of a white plastic box (45 × 45 × 45 cm). Locomotor activity was captured by a fixed camera and processed by a software (Xin Ruan, Shanghai, China). The animals were subjected to the open field test (OFT) for 5 min. The box was cleaned after each trial.

Fecal Microbiota Analysis *via* 16S rRNA Sequencing

DNA of the fecal samples was extracted. Amplification of the V3 and V4 regions of the 16S rRNA gene was performed. Paired-end reads were generated on an Illumina MiSeq platform by following standard instructions. The tags consisted of high-quality paired-end reads and were clustered to operational taxonomic unit (OTU) at the level of 97% sequence similarity using the software USEARCH v7.0.1090. OTU taxonomy was divided on the basis of comparison with the Greengenes database (Fadrosh et al., 2014). According to the OTU abundance, Venn diagram was acquired by VennDiagram of software R (v3.1.1). The ACE, Chao1, Simpson, and Shannon parameters of α-diversity were analyzed. β-Diversity analysis was performed by partial least squares discriminant analysis (PLS-DA).

Flow Cytometry

Periphery blood, spleens, and brains of mice treated with or without Pg were collected. Tissues of spleens and brains were ground and filtered through sterile cell filters. For blood and spleens, erythrocytes were lysed using RBC lysis buffer (CWBIO, Beijing, China) according to the instructions of the manufacturer and were then washed twice with PBS. A single-cell suspension of tissue was prepared. Anti-mouse CD16/32 monoclonal antibody (BioLegend) was used for blockage of Fc receptors. Dead cells were labeled with Zombie NIR Fixable Viability Kit (BioLegend). Cells were stimulated with Cell Activation Cocktail (BioLegend) and fixation/permeabilization was applied before intracellular staining. The antibodies were utilized for flow cytometry as follows: anti-mouse CD45 (clone 30-F11), anti-mouse CD11b (clone M1/70), anti-mouse CD3 (clone 145-2C11), anti-mouse CD4 (clone 145-2C11), anti-mouse CD8 (clone 53-6.7), and anti-mouse IFNγ (clone XMG1.2). All data were collected on a CytoFLEX (Beckman Coulter, USA) and analyzed with FlowJo software (version X, USA).

Function Assessment of the Glymphatic Pathway

An *in vivo* two-photon microscope was used to assess the clearance function of the glymphatic pathway. The mice were anesthetized with pentobarbital (1%, 50 mg/kg). A slender cranial window was created about 3 mm in diameter using a stereotaxic device (RWD, Shenzhen, China). The view of the glymphatic pathway was observed by the two-photon microscope (Leica, Germany). Ten microliters of cerebrospinal fluid (CSF) tracer (FITC, Sigma-Aldrich, Germany) was injected into the cisterna magna with a duration of 10 min at a concentration of 1%. In order to make the blood vessels visible, rhodamine B dextran (Sigma, USA) was given by intravenous injection at a dosage of 0.2 ml per mouse. The operation was repeated at 5, 10, 15, 20, 25, 30, 45, and 60 min after the injection of the tracer. We analyzed the three-dimensional (3D) vectorized reconstruction of the distribution of the FITC tracer to observe its movement. For interstitial clearance, mean pixel intensities were also measured. All data acquisition was obtained by the Leica Lite software. The mean pixel intensities were measured in regions of interest throughout the time course and were normalized at the time of 5 min.

TUNEL Staining

The TUNEL staining kit (Roche, USA) was utilized to assess the apoptotic neurocytes in the hippocampus and cortex. The procedures were conducted based on the instructions of the manufacturer. The number of TUNEL-positive nuclei was measured with ImageJ software. As a control, sections of brain tissue were operated by the same procedures in the absence of TdT enzyme.

Immunofluorescence Staining

Sections of the brain were incubated with the following primary antibodies, including anti-IBA1 antibody (catalog number 019-19741, Wako, Japan), anti-Aβ1–42 (catalog number SIG-39142, BioLegend), and anti-GFAP (catalog number nG3893, Sigma-Aldrich), overnight at 4°C. The next day, the sections were incubated with secondary antibodies (catalog number 4408, 4413, Cell Signaling Technology) at room temperature for 1 h. The number of cells was calculated by two individuals using ImageJ software (version 1.46r, MD, USA).

Data and Statistical Analyses

Two-way repeated measures ANOVA was used for the MWM measurements and the glymphatic system results, with Sidak's test for multiple comparisons conducted. The difference between the two groups was evaluated by performing a *t*-test for normally distributed data and a non-parametric Mann–Whitney test for non-normal distribution. Data were expressed as means ± SEM, and p-value <0.05 was judged as significant difference (SPSS 19.0 software, USA; Prism 6, GraphPad, USA).

RESULTS

Porphyromonas gingivalis Caused Behavioral Changes in Mice

Pg administration had no negative effect on body weight (**Figure S1**). The MWM test was used to examine the learning and spatial memory of mice. The results of the 5-day training are shown in **Figure 1**. Pg-administered mice presented a longer escape latency on day 2 to day 5 (**Figure 1A**). Although the difference of latency was not significant, the longer latency during the training day somewhat reflected a slowed rate of spatial learning after Pg administration. Moreover, the distance that the Pg group traveled to locate the platform was significantly increased compared with the control group on day 5 (**Figure 1B**). The probe trial confirmed the presence of a spatial memory impairment in Pg-administered mice. The number of times crossing the target area was significantly decreased in the Pg group (3.22 ± 0.32) than that in the control group (5.67 ± 0.78, $p < 0.05$; **Figure 1C**). The time mice spent in the target quadrant

was also significantly decreased in the Pg group (17.85 ± 2.18 s) than that in the control group (25.99 ± 2.63 s, $p < 0.05$; **Figure 1D**). Pg-treated mice did not recall the location of the platform and explored other quadrants (**Figure 1F**). There was no significant difference of the swimming speeds between the two groups (**Figure 1E**). Taken together, our results demonstrated that Pg worsens the function of spatial cognition of mice.

To evaluate the general locomotor activity of the mice and their willingness to explore, the OFT was carried out. Indeed, Pg-administered mice spent less time in the central region of the box and showed little interest in exploring when compared with the control mice (**Figure 1J**). Moreover, Pg-treated mice showed a significant reduction in the central distance traveled (1.42 ± 0.21 m), when compared with the control group (2.63 ± 0.49 m, $p < 0.05$; **Figure 1H**). There was no significant difference in total moving distance between the control and Pg group (**Figure 1G**). The representative trajectories of both groups are shown in **Figure 1I**.

To assess motor function and fatigue level, performance on the accelerating rotarod was recorded. The latency of falling from the rotarod of both groups was 272.30 ± 9.57 s (control) and 191.00 ± 7.26 s (Pg). Administration with Pg significantly decreased the riding time by 29.9% compared with that of the control group ($p < 0.001$; **Figure 1K**).

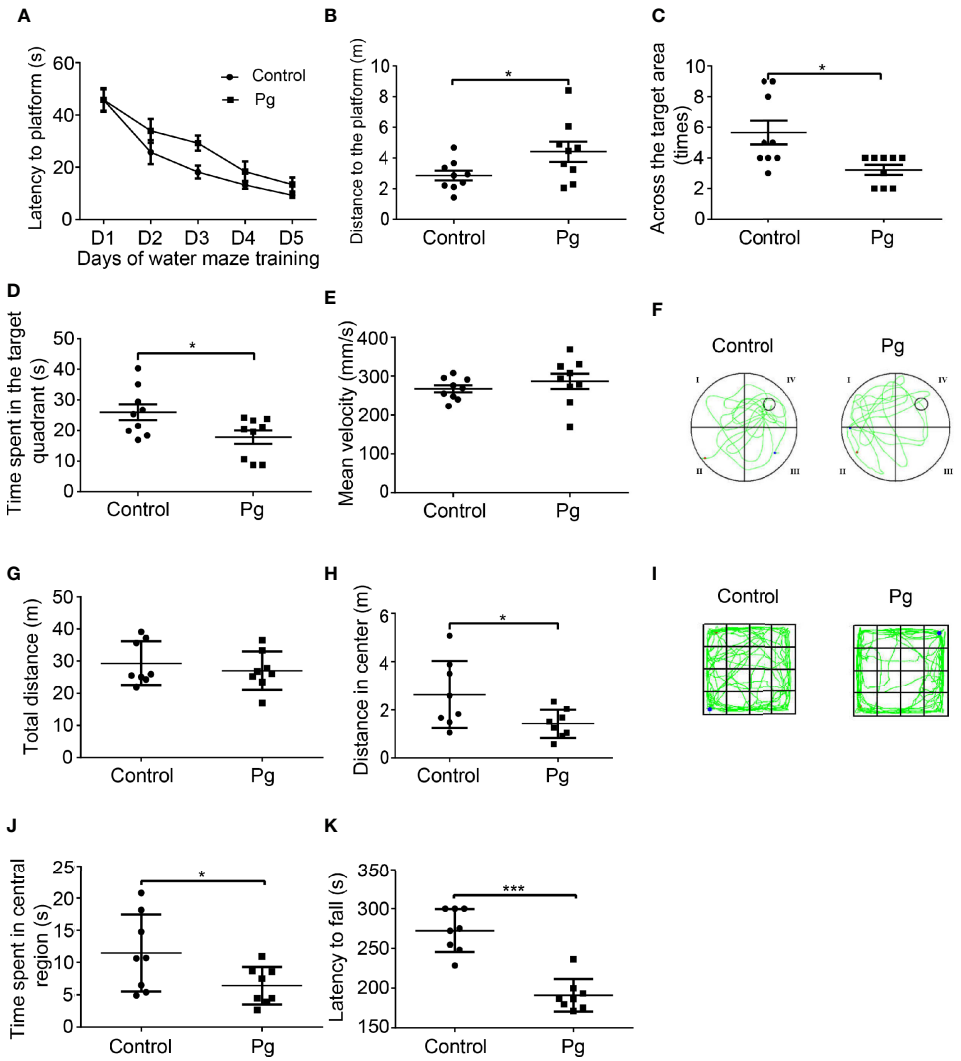


FIGURE 1 | The effects of *Porphyromonas gingivalis* (Pg) on behavioral changes of mice. **(A)** Escape latencies in spatial acquisition trial of the Morris water maze (MWM). **(B)** The distance of mice to locate the platform on day 5. **(C)** The number of times the platform was crossed in the probe trial of the MWM. **(D)** Target quadrant movement time in the probe trial of the MWM. **(E)** Mean velocity of mice in the probe trial of the MWM. **(F)** Representative trajectories of each group in the MWM. **(G)** Total moving distance in the open field test (OFT); **(H)** distance in the central region of the OFT. **(I)** Representative trajectories of each group in the OFT. **(J)** Time spent in the central region of the OFT. **(K)** Latency to fall in the rotarod test. Each dot represents data from a mouse. Data were shown as means ± SEM. $^{*}p \leq 0.05$; $^{***}p \leq 0.001$.

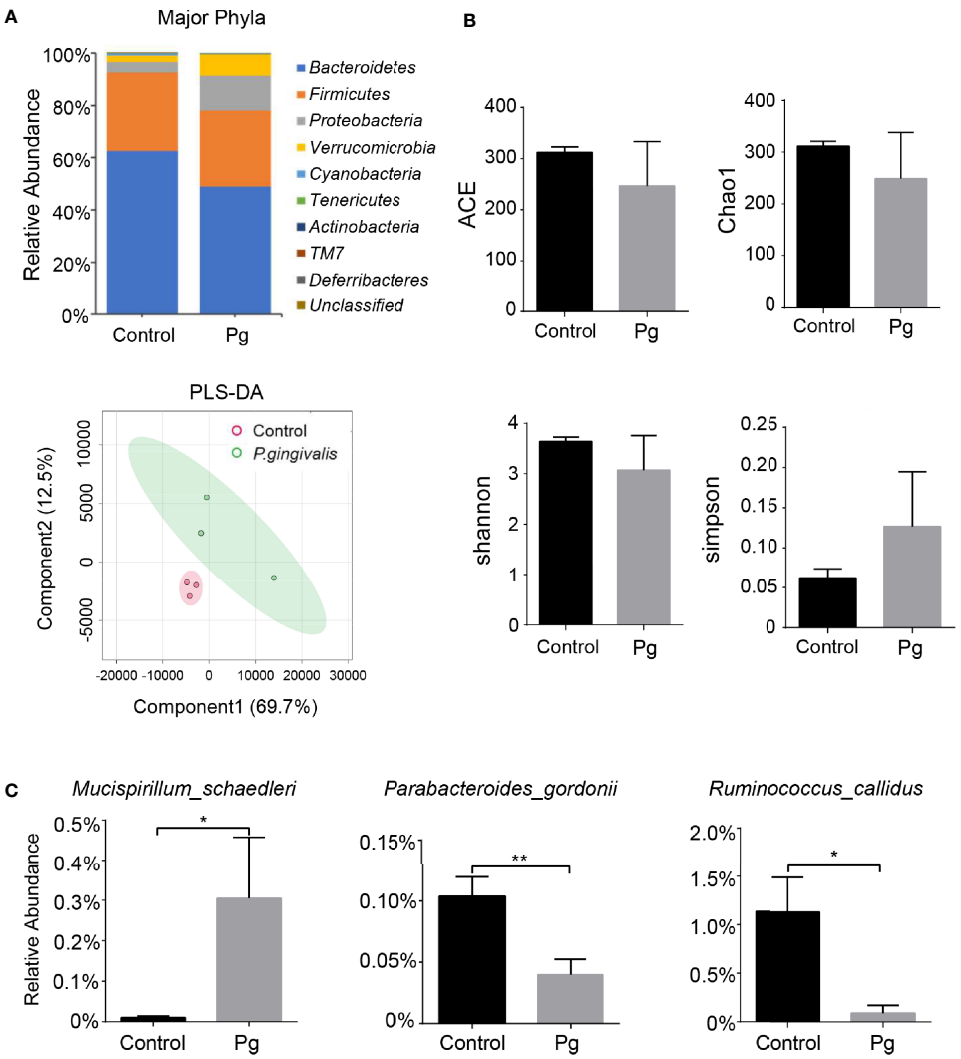


FIGURE 2 | Influence of oral gavage with Pg on the composition of gut microbiota. Mice were subjected to oral gavage with either 10^9 CFU of Pg or CMC three times a week for 4 weeks. Stool samples were used for 16S rRNA sequencing. **(A)** At the phyla level, the relative abundance of bacteria in the Pg-administered and control groups. **(B)** Alpha- and beta-diversity of the gut microbiota in the Pg-administered and control groups. **(C)** The significant differences in relative abundance of species between the two groups. $n = 3$, data were shown as means ± SEM. $^*p \leq 0.05$; $^{**}p \leq 0.01$.

Oral Administration of Pg Altered Gut Microbiota Composition

To evaluate the influence of Pg gavage on gut microbiota, feces were analyzed for their microbiota composition. The composition ratio of the gut microbiota changed (**Figure 2A** and **Figures S2A, B**). The number of shared OTUs in both groups was 334 as shown in the Venn diagrams, and the unique OTUs of the two groups were 60 in the control group and 52 in the Pg group, respectively (**Figure S2C**). The α-diversity parameters, including ACE, Chao1, Shannon, and Simpson, were analyzed. None of the parameters showed a significant change by repeated administration of Pg (**Figure 2B**). The result of PLS-DA analysis displayed that the samples could be divided into two parts. This demonstrated that the gut microbiota compositions were different between the Pg-administered and control groups (**Figure 2B**). At the phylum level, the proportion of *Tenericutes* was significantly increased in Pg-treated mice than in control ones, and the proportion of *Actinobacteria* was slightly decreased (**Figure S2D**). At the class level, the proportion of *Coriobacteriia* was significantly decreased in the Pg group than in the control group, while the proportion of *Mollicutes* was significantly increased (**Figure S2D**). At the order level, the proportion of *Coriobacteriales* was significantly decreased in Pg-treated mice than in control ones (**Figure S2D**). At the family level, the proportions of *Clostridiaceae*, *Coriobacteriaceae*, and *Prevotellaceae* were significantly decreased in the Pg group, and that of *S24-7* was slightly decreased. At the genus level, the proportion of *Prevotella* was significantly decreased in the Pg group than in the control group (**Figure S2D**). At the species level, the proportions of *Parabacteroides gordonii* and *Ruminococcus callidus* were

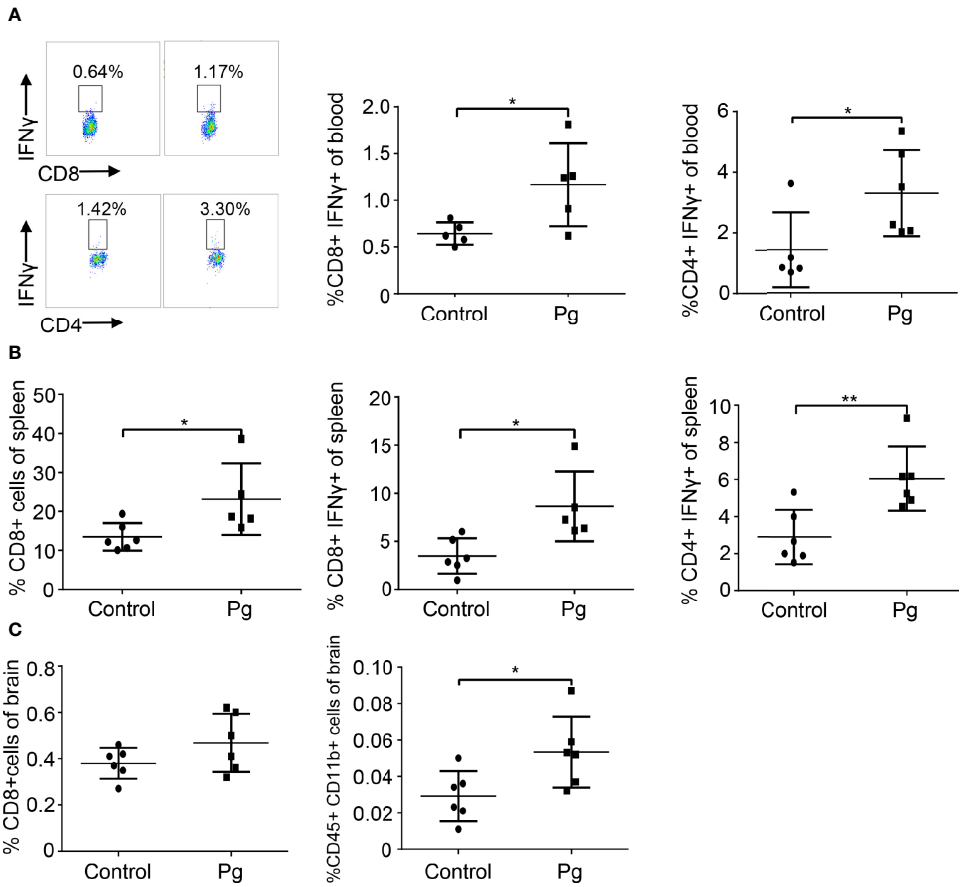


FIGURE 3 | Pg-administered mice show changes in proportions of periphery lymphocytes and brain-infiltrating immune cell subsets. (A) Flow cytometry is used to analyze the composition of blood cells in the control and Pg-administered group. Numbers represent the percentage of the target cell group in blood cells. (B) Flow cytometry is used to analyze the composition of spleen cells. (C) Flow cytometry is used to analyze the composition of brain-infiltrating immune cells. Each dot represents data from a mouse. Data were shown as means ± SEM. *p ≤ 0.05; **p ≤ 0.01.

significantly decreased in Pg-treated mice than in control ones, whereas the proportion of *Mucispirillum schaedleri* was significantly increased (**Figure 2C**). Besides, the ileum of the Pg group showed partial intestinal gland destruction and inflammatory cell infiltration. These results demonstrated that gut microbiota dysbiosis caused by Pg administration can induce intestinal inflammatory response. However, there was no histopathologic change in the colon (**Figure S3**).

Pg Changed the Immune Environment After Gut Microbiota Dysbiosis

To address whether Pg contributed to brain disorders by affecting the immune pathway of the GBA, we detected immune cells from the blood, spleen, and brain of mice. The proportions of $CD4^+IFN\gamma^+$ T cells and $CD8^+IFN\gamma^+$ T cells were increased in the blood and spleen of mice with Pg gavage compared with those of the control mice (**Figures 3A, B**). The proportion of $CD8^+$ T cells of the spleen was significantly increased in the Pg group, while that of the brain was slightly increased. However, the proportion of $CD45^+CD11b^+$ myeloid cells was significantly increased in the brain of the Pg group (**Figure 3C**).

Dysfunction of the Glymphatic System

The clearance function of the glymphatic system was measured in mice. The CSF tracer was given to the cisterna magna by infusion and the blood was visualized by intravenous injection of rhodamine B dextran (**Figure 4A**). The CSF tracer ran to the cortex along the permeating arterioles and went into ISF of the parenchyma through PVS. The CSF tracer in the PVS of the permeating arteries was analyzed 100 μm under the surface of the cortex (**Figure 4B**). In control mice, the measurement of the CSF tracer in pixel intensity at 5 min was set as a baseline. The relative pixel intensity along the PVS in control mice was gradually decreased over time. In contrast, the CSF tracer was accumulated along the PVS in Pg-treated mice, and the relative pixel intensity was significantly increased at 25, 30, 45, and 60 min (**Figure 4C**). These results indicated that oral gavage with Pg decreased the CSF–ISF exchange of the brain. We also analyzed the pixel intensity of the CSF tracer in brain parenchyma. In

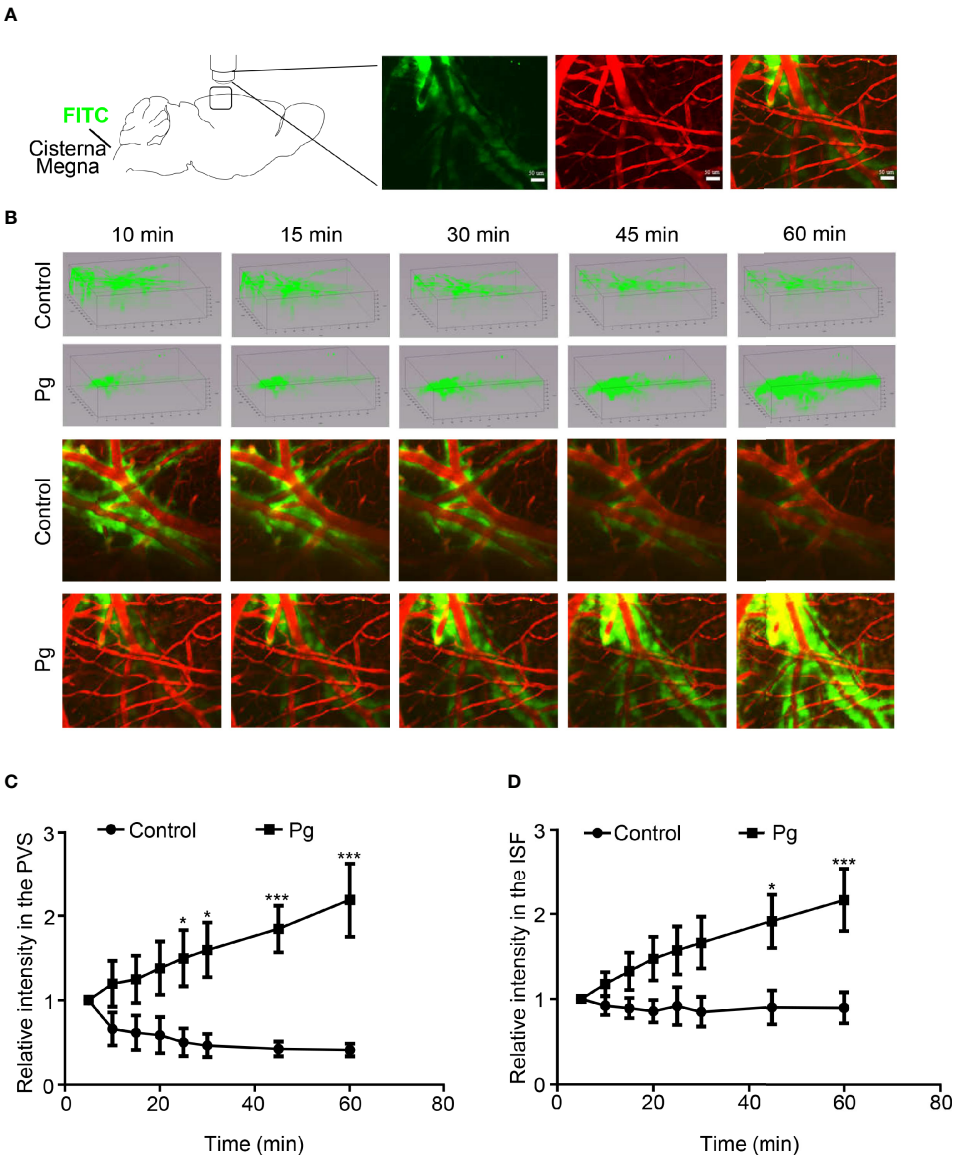


FIGURE 4 | Clearance function of the glymphatic system, including inflow of the cerebrospinal fluid (CSF) through PVS–ISF exchange and the outflow of ISF drainage. **(A)** Diagram representing the two-photon microscopic image of the CSF tracer into the cisterna magna. **(B)** Three-dimensional images of the distribution of the CSF tracer in the Pg and control groups. Representative picture of the CSF tracer entering the brain parenchyma along the PVS. **(C)** Comparison of the relative fluorescence intensity in the PVS. **(D)** Comparison of the relative fluorescence intensity in the ISF between the control and Pg group. $n = 4$, data were shown as means ± SEM, $^{*}p \leq 0.05$; $^{***}p \leq 0.001$. Scale bar, 50 μm.

control mice, the relative pixel intensity stayed nearly at the same level during the testing time. However, in the Pg group, the relative pixel intensity was significantly increased at 45 and 60 min (**Figure 4D**). It indicated that Pg of oral origin impaired the ISF drainage of the brain.

Pg Aggravated Neuroinflammation in the Brain

Overactivation of neuroinflammation is reported to be associated with neurodegeneration in AD (Heneka et al., 2015). We conducted immunofluorescence staining to explore the histopathologic changes induced by Pg. We gauged and compared the positive cells of TUNEL, neurons (NeuN), microglia (Iba-1), and astrocytes (GFAP) in the cortex and hippocampus regions of mice in different groups. The dysbiosis of gut microbiota and infiltration of immune cells can promote inflammatory activation of glial cells. Pg increased over 16.35% of the number of microglia and 39.12% of the number of astrocytes in the hippocampus region than those in the control group (**Figures 5B, F**). Neuroinflammation may induce apoptosis of neurocytes. Pg increased the number of TUNEL-positive cells in the hippocampus and cortex regions than those

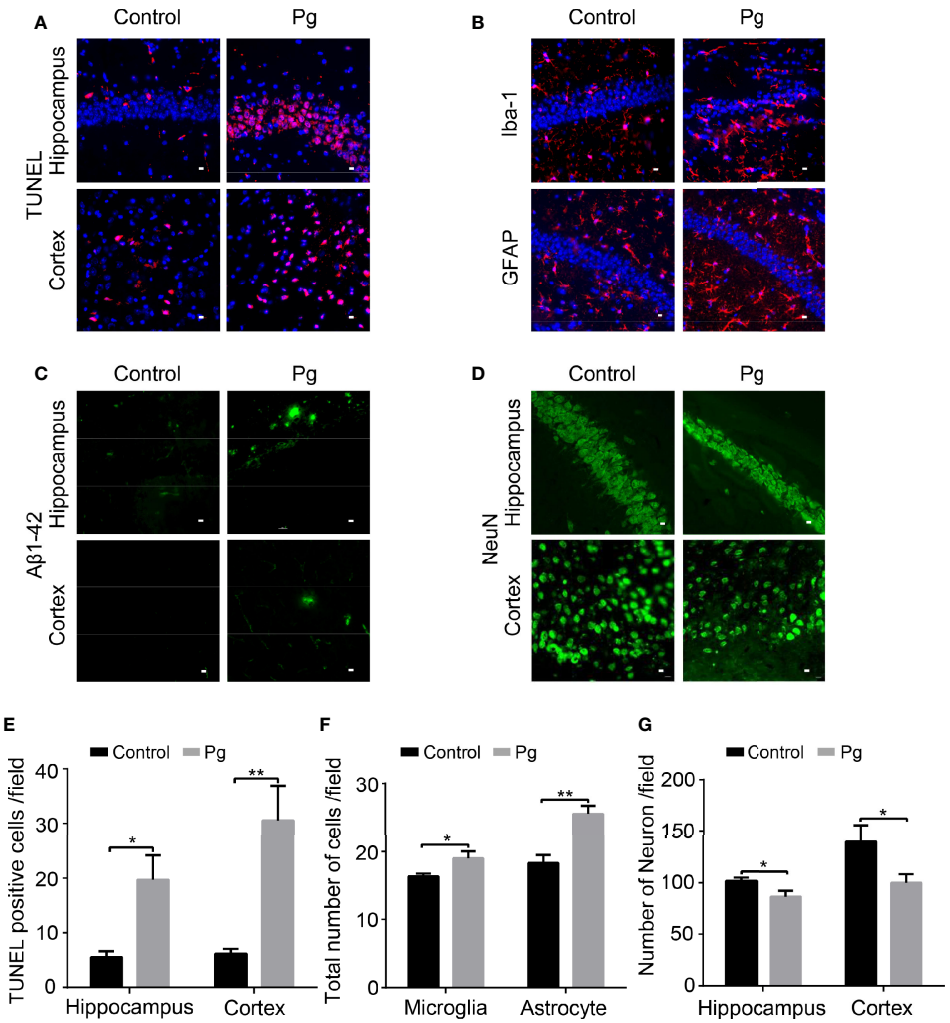


FIGURE 5 | Immunohistochemical staining of the hippocampus and cortex. **(A)** Representative image presenting TUNEL-positive cells in the hippocampus and cortex. **(B)** Representative image presenting Iba-1 and GFAP-immunopositive cells in the hippocampus. **(C)** Representative section showing amyloid plaque in the hippocampus and cortex. **(D)** Representative image presenting NeuN-positive cells in the hippocampus and cortex. **(E)** Comparison of the difference in the number of TUNEL-positive cells. **(F)** Comparison of the difference in the number of GFAP and Iba-1-positive cells in the hippocampus. **(G)** Comparison of the difference in the number of NeuN-positive cells in the hippocampus and cortex. $n = 6$, data were shown as means ± SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$. Scale bar, 10 μm.

in control mice (**Figures 5A, E**). Moreover, the number of neurons in the hippocampus and cortex regions was significantly decreased in Pg-administered mice (**Figures 5D, G**). In addition, amyloid plaque appeared in those two brain regions of the Pg group (**Figure 5C**).

DISCUSSION

In this study, Pg of oral origin induced dysbiosis of gut microbiota. Microbiota was a potent regulator of host immune responses, and the T lymphocytes and myeloid cells were increased in the peripheral and CNS, respectively. Changes of the CNS immune microenvironment exacerbated the neuroinflammation. Furthermore, the solute clearance function of the glymphatic system was reduced. Aβ plaques were shown in the brain and cognitive function was prominently impaired in mice that were subjected to oral gavage with Pg. Hence, this study provides further evidence for Pg exacerbating neuroinflammation, impairing glymphatic function, and ultimately leading to a decline in cognitive function by disturbing the GBA.

In order to mimic clinical conditions, the dose of Pg given to mice was calculated according to the quantity of microbial load and saliva swallowed by a patient with periodontitis. The time of Pg administration was based on how long periodontitis can be induced by bacteria in mice (Boyer et al., 2020). Pg can act either directly or indirectly on the brain. On the one hand, Pg is detected in the brain of AD patients. Pg in the brain may induce neuroinflammation by secreting gingipain and results in the deposition of amyloid protein (Dominy et al., 2019). In addition

to the direct action, Pg can act indirectly on neuroinflammation through gut dysbiosis. Gut dysbiosis causes an increase in inflammatory T and B lymphocytes and a subsequent systemic inflammation, thereby inducing neuroinflammation (Suganya and Koo, 2020; Carlessi et al., 2021). Second, gut dysbiosis decreases the production of short-chain fatty acids (SCFAs), which is related to inflammatory responses (Lach et al., 2018; Gudi et al., 2020). Third, gut dysbiosis exacerbates neuroinflammation by transmitting gut-derived Aβ to the brain through the vagus nerve (Chen et al., 2021).

The oral cavity–gut–multiorgan axis has been recently proposed to link periodontitis to systemic diseases (Kato et al., 2018). Indeed, Pg can be detected in the fecal collections from patients with colorectal cancers (Wang et al., 2021). It is reported that during periodontal diseases, extension of the oral microbiota can promote inflammatory bowel disease by ectopic gut colonization (Kitamoto et al., 2020). An outgrowth of potentially pathogenic bacteria and a decrease of beneficial bacteria may result in pathological changes of gut tissue. In the present study, we found inflammatory cells infiltrated in the ileum tissue and some destruction of the intestinal glands. Besides, at the species level, the proportions of *M. schaedleri*, *P. gordonii*, and *R. callidus* were changed in the Pg group. *Mucispirillum schaedleri* is detected in a variety of mammals, and it is known to have low relative abundance of the intestinal microbiota in murine feces (Herp et al., 2021). An increasing number of certain bacterial species have been associated with inflammatory conditions in the gut (Selmin et al., 2021). *Parabacteroides gordonii* is one of the several gut bacteria with anti-inflammatory attributes (Abais-Battad et al., 2021). *Ruminococcus callidus* which produces SCFAs is considered as a biomarker for improving health (Sanchez-Tapia et al., 2020). Moreover, the relative abundances of both bacteria were decreased in the Pg group. The changes of the above three bacterial species were associated with inflammatory pathology of the ileum. In addition, Pg administration has been documented to modulate gut microbiota and gut immune system. Pg could shift the proportion of T lymphocytes to inflammatory T cells in mesenteric lymph node by disturbing the gut microbiota, and the level of proinflammatory cytokine in sera is also increased. Besides, oral gavage with Pg can impair the barrier function of intestinal epithelium and decrease the expression of tight junction protein ZO-1 (Kato et al., 2018; Feng Y. K. et al., 2020; Tsuzuno et al., 2021). Further studies are urgently needed to clarify the pathway how Pg causes pathologic and microbiological changes of the gut.

In accordance with a previous study, gut microbiota played an important role in behavior (Oh et al., 2015), and animals in the Pg group were more prone to fatigue in the rotarod test. In addition, Pg-treated mice showed little interest in exploration, indicating anxiety-like behavior. These results indicated that dysbiosis of gut microbiota caused by Pg administration could disturb the brain function. The gut microbiota is critical for the maturation and proper function of microglia, while dysbiosis of gut microbiota induces neuroinflammation (Erny et al., 2015). AD mice exhibit altered gut microbiota compositions, which is positively correlated with enhanced astrogliosis and microgliosis in the brain (Shukla et al., 2021). Conversely, rescuing the dysbiosis of gut microbiota can suppress the microglia activation and downregulate the production of proinflammatory cytokines (Shen et al., 2020). Consistently, we found that Pg of oral origin could induce astrogliosis and microgliosis in the brain. Dysbiosis of gut microbiota has been strongly involved in the development of neurodegenerative disorders through modulating the GBA (Stefano et al., 2018; Cryan et al., 2020; Zhu et al., 2020). The gut microbiota can modify immune cells and promote the production of proinflammatory cytokines (Hegazy et al., 2017; McCoy et al., 2017; Zhao and Elson, 2018). Thereafter, immune cells along with inflammatory mediators may infiltrate the brain (Campos-Acuna et al., 2019). In this study, the proportions of $CD4^+IFN\gamma^+$ T lymphocytes and $CD8^+IFN\gamma^+$ T cells were increased in the blood and spleen of Pg-treated mice, and the peripheral Th1 ($CD4^+IFN\gamma^+$) cells are associated with M1 microglia activation and contribute to neuroinflammation (Wang et al., 2019). A study limitation is that we did not examine the levels of IFNγ in the sera, gut, and brain. Furthermore, we observed that Pg promoted the infiltration of myeloid cells ($CD45^+CD11b^+$) in the brain. The mechanisms by which myeloid cells activate neuroinflammation are still under examination. Further studies are needed to clarify whether removing Pg can rescue the damage of the brain.

In addition, the gut microbiome imbalance and associated neuroinflammation may disrupt CNS fluid flow, which leads to the breakdown of the glymphatic system (Rustenhoven et al., 2021). The recently discovered glymphatic system in brain parenchyma and the meningeal lymphatics are recognized as vital pathways for clearance of toxic solutes from the brain (Da Mesquita et al., 2021). The dysfunction of the glymphatic CSF–ISF exchange has been implicated in the initiation and progression of AD and Parkinson's disease (Wood, 2021). In our study, the clearance rate of both the PVS and ISF bulk flow in the Pg group was much slower than that in the control group. This accelerated the accumulation of interstitial waste from the brain parenchyma. In correspondence, the deposition of Aβ plaque in the brain was observed in the Pg group. The bidirectional interaction between Aβ deposition and neuroinflammation resulted in the apoptosis of neurocytes and then impaired cognitive function in the Pg group. It is reported that inhibition of Pg-induced neuroinflammation can decrease the deposition of Aβ (Dominy et al., 2019; Liu et al., 2020).

The glymphatic system mainly consists of astrocytes where aquaporin-4 (AQP4) water channels locate (Mestre et al., 2018). Astrocytes are a group of glial cells in abundance in the CNS that have significant homeostatic maintenance and disease-promoting function. Immune cells that are licensed by the gut microbiota can modulate the function of astrocytes (Sanmarco et al., 2021). Moreover, in this study, reactive proliferation of astrocytes was observed in the Pg group, which is related with the dysfunction of the glymphatic system. Furthermore, the localization of AQP4 is highly polarized to perivascular endfeet of astrocytes that facilitate the periarterial CSF influx and the perivenous ISF clearance pathways (Feng W. et al., 2020; Hablitz et al., 2020). Dysfunction of astrocytes, including reactive astrogliosis, causes

abnormal production and position of AQP4, which disturbs the clearance function of adverse solutes in the brain in turn. Future work will address whether Pg can modify the polarization of AQP4 water channel by interacting with the GBA.

CONCLUSIONS

Our results indicate that periodontal pathogen Pg induces cognitive decline, accompanied by gut microbiota dysbiosis, neuroinflammation, and glymphatic system impairment. In conclusion, the present study suggests a potential role of Pg-induced dysfunction of the GBA in the pathophysiology of cognitive impairment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available in Figshare: DOI: 10.6084/m9.figshare.17081648.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee, Sun Yat-Sen University.

AUTHOR CONTRIBUTIONS

LC, ZP, and WT designed the studies. LC and XC performed the experiments, analyzed the data, and wrote the manuscript. LL, TY, JS, YF, and FL performed some of the experiments. ZP and WT supervised the project and revised and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.755925/full#supplementary-material

Supplementary Figure 1 | Physiological measures of body weight after the first Pg administration.

Supplementary Figure 2 | (A) The composition of bacteria at the Class level between the Pg-administered and control group. **(B)** The composition of bacteria at the Genus level between the Pg-administered and control group. **(C)** Venn diagrams are used to show the number of common or unique OTUs between Pg and control group. Each circle represents the number of OTUs for each group. The overlapping area represents the shared OTUs in both groups. **(D)** Bar plots for phylum, class, order, family and genus that showed differences in the Pg-administered and control group. n=3, data were shown as means ± SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

Supplementary Figure 3 | Histological aspects of ileum and colon. **(A)** Representative histological section of ileum. Rectangular box represents the gland destruction in the gut and inflammatory cells infiltration. **(B)** Representative histological section of colon. Scale bar, 200 μm.

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