GUT MICROBIOTA AS A THERAPEUTIC TARGET IN NEUROPSYCHIATRIC DISORDERS: CURRENT STATUS AND FUTURE DIRECTIONS

EDITED BY: HuaLin Cai, Xia Chen, Aurelijus Burokas and Rafael Maldonado

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GUT MICROBIOTA AS A THERAPEUTIC TARGET IN NEUROPSYCHIATRIC DISORDERS: CURRENT STATUS AND FUTURE DIRECTIONS

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Editorial: Gut microbiota as a therapeutic target in neuropsychiatric disorders: current status and future directions

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Editorial on the Research Topic
Gut microbiota as a therapeutic target in neuropsychiatric disorders: current status and future directions

This Research Topic, started with the aim to explore the roles and provide further insights for gut microbiota involved in the pathophysiology and treatment of neuropsychiatric disorders, has now included 12 research and review articles. There is an undoubtedly complex communication between the microorganisms in the gastrointestinal tract and the central nervous system (CNS), since the gut microbiota constitutes the major component of the "gut-brain axis" that includes various pathways that enable communication between the gut and the CNS. Among those pathways, although emerging evidence implicates the dysregulated kynurenine pathway in the pathophysiology of mood disorders, whether the metabolites of the kynurenine pathway can serve as modulators linking the gut microbiota with the CNS is largely unclear. Bibliometric analysis is a statistical method can be applied to quantitatively analyze and visualize scientific output, research hotspots, and developing trends, by utilizing public literature databases (Zhang et al., 2020). With the help of bibliometric analytic method, here Zhu X. et al. comprehensively evaluate the general aspects and future trends connecting kynurenine pathway and the gut microbiota in research of mood disorders. The mounting evidence suggests that the balance between kynurenic acid and quinolinic acid plays a key role in the pathophysiology of mood disorders under the control of the gut microbiota. Researches focusing on the gut microbiota-brain axis were also identified as frontiers and hotspots in this field. Under the circumstances, many recent studies have reported that the gut microbiota influences cognitive function through the gut-brain axis, which is involved in the pathophysiology of neurodegenerative and mental disorders, including Alzheimer's disease (Kesika et al., 2021) and schizophrenia (Zeng et al., 2021). Similarly, in this topic Dai et al. summarized the emerging evidence of alterations in the gut microbial composition of patients with bipolar disorder, suggesting that gut microbial dysbiosis contributes to disease progression and cognitive impairment.

They indicated that gut microbiota modulates neurological function in the brain through various pathways such as productions of microbial-derived metabolites, neurotransmitters, and gastrointestinal hormones, affecting patients' cognitive function. Meanwhile, gut microbiota-related end-products may influence Parkinson's disease pathology through creating peripheral and systemic inflammatory environments (Perez-Pardo et al., 2017). Here, Hill et al. collected the associative evidence from case-control studies and functional evidence from animal models which support for microbiota causing Parkinson's disease. They concluded that two main routes linking gut microbiota can influence Parkinson's disease pathophysiology, the neural and humoral routes. The neural route involves alpha-synuclein misfolding peripherally in the enteric nerves which can then be transported to the brain via the vagus nerve. The humoral route involves transportation of bacterial products and proinflammatory cytokines from the gut via the circulation which can cause central alpha-synuclein misfolding by inducing neuroinflammation.

Except for the interactions between gut microbiota and the pathophysiological mechanisms of these neuropsychiatric disorders, several lines of data demonstrate that complex interactions with the gut microbiota may also explain some of the cognitive and/or metabolic side effects of certain psychiatric medications. Herein, Zhu Z. et al. explored the mechanism by which olanzapine-induced lipid disturbances through the gut microbiota-brain axis. They found that olanzapine increased the *Firmicutes/Bacteroides* (F/B) ratio in the gut, which can be even aggravated by subphrenic vagotomy, reduced the abundance of short-chain fatty acids and 5-hydroxytryptamine levels in the rat cecum, and increased the gene and protein expression of the appetite-related neuropeptide Y/agouti-related peptide in the hypothalamus. These results suggest that abnormal lipid metabolism caused by olanzapine may be closely related to the vagus nerve-mediated gut microbiota-brain axis. On the one hand, the data reinforce the idea that antipsychotic drugs induced changes in these gut microbiota-derived molecules can directly activate the vagus nerve or be transported into the brain to influence appetite (Oliphant and Allen-Vercoe, 2019). On the other hand, accumulated evidence implied that these molecules can also regulate related lipid metabolism via peripheral signaling pathways. Keep this in mind, here Chen et al. reviewed the mechanisms of second-generation antipsychotic drug-induced disorders of lipid metabolism mediated by the gut microbiota, not just from the CNS but also from the periphery. They reported that antipsychotic related peripheral regulations of lipid metabolism can be exerted by shorts-chain fatty acids, bile acids, leptin and glucagon-like peptide 1. The major signaling pathways including AMPK, MAPK, PI3K/Akt/mTOR, and cAMP/PKA/CREB-P work systematically in concert to regulate fatty acid oxidation, fatty acid synthesis, protein synthesis, mitochondrial oxidative phosphorylation and energy metabolism. Apart from antipsychotic-induced metabolic side effects, surgery and anesthesia medication related perioperative neurocognitive dysfunction (PND) is receiving increasing attention nowadays, and there is a strong link between gut microbiota and PND that depends on the gut-brain axis. Here, Lu et al. reviewed the potential mechanisms of the dysbiosis of gut microbiota underlying PND. They concluded that surgery and anesthesia

may disrupt gut microbiota homeostasis in a direct or indirect manner. Then gut microbiota dysbiosis leads to altered levels of neurotransmitters, causes abnormal gut microbiota-host co-metabolites, and affects intestinal mucosal permeability ("leaky gut"), resulting in an increased entering of Aβ proteins into bloodstream and subsequent neuroinflammation. Related to the above, more preclinical and clinical researches are needed in the future to focus on the role of specific gut microbiome and specific targets of gut microbiota, in order to provide new approaches for coping with these neuropsychiatric disorders and side effects of certain medications.

As a matter of fact, probiotics play an increasingly important role in acting as alternative medicines/drugs, filling the gap in the therapeutics of different psychopathologies, and contributing to the pharmacological response with fewer adverse effects (Morkl et al., 2020). Herein, Rehman et al. investigated the effects of probiotic formulations (*Lactobacillus fermentum* NMCC-14 and *Bacillus clausii*, 10^{10} colony forming unit/day/animal, per oral) in acute (up to day 7) and subacute (days 8–14) restraint-stressed and normal mice through behavioral paradigms. The results showed improvements in behavioral tests, cortisol and adrenocorticotropic hormone levels, hippocampal neurodegenerative status, monoamine levels, and mRNA expression of dopamine receptor subtypes in probiotic-treated restraint-stressed mice. They concluded that in comparison, *Bacillus clausii* showed greater stress suppressant activity than *Lactobacillus fermentum* NMCC-14. However, both probiotics can be better and safer therapeutic alternatives for stress-related ailments than currently available drugs. Accordingly, another study conducted by Zhang et al. has indicated that the chronic stress-induced behavioral disorders can be ameliorated by treatment with probiotic *Clostridium butyricum* (*C. butyricum*). The data showed that chronic foot shock stress resulted in downregulation of tissue-type plasminogen activator (tPA) but upregulation of plasminogen activator inhibitor 1 (PAI-1), which could contribute to the decrease in BDNF by reduced conversion from proBDNF to BDNF in the hippocampus, and *C. butyricum* RH2 attenuated stress-induced behavior via inhibiting the expression of PAI-1 without changing tPA. These findings suggest that targeting PAI-1 could be an innovative strategy for the development of new drugs to counter the effects of stress. From biological perspective, stressful conditions can result in increased levels of oxidative stress markers (Casado et al., 2011), whereas persistent state of oxidative stress may in turn lead to vulnerability to neuropsychiatric disorder produced by stressful life events (Bouvier et al., 2017). Herein, Zeng et al. investigated the effects of probiotic supplementation on plasma oxidative stress-related biomarkers and different domains of clinical symptom in patients suffering from bipolar disorder. The results showed that after 3 months of intervention, decreased levels of plasma lysophosphatidylcholines (LPCs) were found in both placebo and probiotic groups. However, six other oxidative stress biomarkers including creatine, inosine, hypoxanthine, choline, uric acid, and allantoic acid increased in patients after the two types of therapies. In addition, a positive correlation between changes of LPC (18:0) and Young Mania Rating Scale was found in patients and this association only existed in the probiotic group. Additionally, the mania symptom greatly alleviated in patients

who received probiotic supplements as compared with the placebo group. They concluded that the changes in plasma biomarkers of oxidative stress in bipolar disorder patients are trait-like, and can serve as prognostic indexes. The treatment with probiotics may be a promising adjunctive therapeutic strategy for bipolar disorder patients especially in manic episode.

In recent years, the interaction between the bioactive ingredients of traditional Chinese medicine (TCM) and gut microbiota has been a focus of many fields, including oncology and neuropsychiatry, etc. When TCM enters the digestive tract, some ingredients of TCM are metabolized, or bio-transformed by gut microbiota, thereby producing new bioactive molecules, and promote medicine absorption into the circulation. At the same time, the ingredients of TCM impact the composition and abundance of gut microbiota, thereby influencing the remote function of diseased organs/tissues through the systemic action of the gut microbiota (Gong et al., 2020). Herein, Fasina et al. found that gastrodin from *Gastrodia elata* (commonly known as Tian ma in Chinese) enhances cognitive function and neuroprotection of mouse model of Alzheimer's disease via the regulation of gut microbiota. The results showed that gastrodin treatment had a positive correlation with *Firmicutes* and had a negative correlation with *Cyanobacteria*, *Proteobacteria*, and *Deferribaceters*. Importantly, the lipopolysaccharides and proinflammatory cytokines in the brain increased in Alzheimer's disease mice, but these parameters recovered to normal levels after oral administration of gastrodin. Although detailed mechanisms remain to be clarified, this study added evidence showing gastrodin improves the memory of the Alzheimer's disease mouse model via partly targeting the microbiota-gut-brain axis and mitigating neuron inflammation. Likewise, the combination of TCM may be more effective in treating cognitive impairment as compared with single ingredient from the herb. An optimized combination of TCM named Zi Shen Wan Fang (ZSWF) is composed of *Anemarrhenae Rhizoma*, *Phellodendri Chinensis Cortex*, and *Cistanches Herba*. Herein, Shi et al. used this prescription for treating diabetes-induced cognitive impairment (DCI) and found that ZSWF restored cognitive function in DCI mice and reduced levels of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. Moreover, ZSWF protected the integrity of the intestinal barrier by increasing intestinal ZO-1 and occludin protein expression and decreasing urinary lactulose to mannitol ratio, and through reversing the abundance changes of a wide range of intestinal bacteria. In contrast, removing gut microbiota with antibiotics partially eliminated the effects of ZSWF on improving cognitive function and reducing inflammation, confirming the essential role of gut microbiota in the improvement of DCI by ZSWF. These results suggest that ZSWF can be a potential Chinese medicine prescription for DCI treatment.

Despite neuropsychiatric diseases, the importance of the gut-brain axis has been implicated in overall mental health of general population. Although the pandemic of coronavirus disease 2019 (COVID-19) is a fading away recently, the uncertain future, fear of job loss, lockdown and negative news all around have taken a heavy toll on the mental health of individuals from across the world. Stress and anxiety can affect not only the COVID-19 patients but also the ordinary people even more. Here, Dhar briefly summarized the current evidence supporting that gut dysbiosis may be implicated in anxiety and depression both in COVID-19 patients and healthy individuals exposed to various stressors. The author proposed a new perspective indicating that personalized gut microbiome based nutritional strategies, if adopted by people affected by stress and anxiety due to the prevailing environment of COVID-19 and COVID-19 patients themselves, can promisingly improve the mental wellbeing and might act as an alternate mode to assist the mental healthcare infrastructure.

In summary, this collection highlights new findings from original research articles regarding data of both animal experiments and clinical studies, and review articles that help understanding the mechanisms underlying the pathophysiology of gut microbiota played in cognitive deficits, in order to give further insights in the microbiota-oriented treatment for patients with neuropsychiatric disorders and general population affected by stress.

Author contributions

HC wrote the draft. XC, AB, and RM provided comments for revisions. All authors approved the publication of this editorial.

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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.

Publisher's note

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Comprehensive Bibliometric Analysis of the Kynurenine Pathway in Mood Disorders: Focus on Gut Microbiota Research

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Background: Emerging evidence implicates the dysregulated kynurenine pathway (KP), an immune-inflammatory pathway, in the pathophysiology of mood disorders (MD), including depression and bipolar disorder characterized by a low-grade chronic pro-inflammatory state. The metabolites of the KP, an important part of the microbiota-gut-brain axis, serve as immune system modulators linking the gut microbiota (GM) with the host central nervous system.

Aim: This bibliometric analysis aimed to provide a first glimpse into the KP in MD, with a focus on GM research in this field, to guide future research and promote the development of this field.

Methods: Publications relating to the KP in MD between the years 2000 and 2020 were retrieved from the Scopus and Web of Science Core Collection (WoSCC), and analyzed in CiteSpace (5.7 R5W), biblioshiny (using R-Studio), and VOSviewer (1.6.16).

Results: In total, 1,064 and 948 documents were extracted from the Scopus and WoSCC databases, respectively. The publications have shown rapid growth since 2006, partly owing to the largest research hotspot appearing since then, "quinolinic acid." All the top five most relevant journals were in the neuropsychiatry field, such as Brain Behavior and Immunity. The United States and Innsbruck Medical University were the most influential country and institute, respectively. Journal co-citation analysis showed a strong tendency toward co-citation of research in the psychiatry field. Reference co-citation analysis revealed that the top four most important research focuses were "kynurenine pathway," "psychoneuroimmunology," "indoleamine 2,3-dioxygenase," and "proinflammatory cytokines," and the most recent focus was "gut-brain axis," thus indicating the role of the KP in bridging the GM and the host immune system, and together reflecting the field's research foundations. Overlap analysis between the thematic map of keywords and the keyword burst analysis revealed that the topics "Alzheimer's disease," "prefrontal cortex," and "acid," were research frontiers.

Conclusion: This comprehensive bibliometric study provides an updated perspective on research associated with the KP in MD, with a focus on the current status of GM research in this field. This perspective may benefit researchers in choosing suitable journals and collaborators, and aid in the further understanding of the field's hotspots and frontiers, thus facilitating future research.

Keywords: kynurenine pathway, mood disorders, depression, bipolar disorder, bibliometric analysis, gut microbiota, microbiota-gut-brain axis, immune system

INTRODUCTION

Major depressive disorder (MDD) and depressive episodes of bipolar disorder (BD), which have similar clinical presentations, are both types of mood disorders (McIntyre et al., 2019; Vöhringer and Perlis, 2016). They are both associated with high suicide rates and can pose large burdens to societies and economies, according to the Global Burden of Disease study (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). Thus, understanding the molecular mechanisms of mood disorders is highly important for the development of effective treatment. Although the relationship between inflammation and mood disorders has been well documented, the complex pathophysiology of mood disorders has not been fully elucidated. Increasing preclinical and clinical studies provide evidence of alterations in the levels of pro-inflammatory and anti-inflammatory cytokines in MDD and other mood disorders (Chistyakov et al., 2018). Thus, mood disorders can be characterized by a low-grade chronic pro-inflammatory state, possibly owing to pathophysiological dysfunctions in immune-inflammatory pathways; this state might induce brain functional and structural alterations via multiple mechanisms (Squassina et al., 2019).

The kynurenine pathway (KP) is a potential inflammation-related mechanism implicated in the pathophysiology of mood disorders. Tryptophan (TRY) is an essential amino acid that serves as a precursor for serotonin (5-HT). However, kynurenine (KYN) is a major metabolite of TRY via the KP, which is responsible for 99% of dietary TRY metabolism (Russo et al., 2003; Won and Kim, 2016). The key primary enzymes in TRY oxidation metabolism via the KP are indoleamine 2,3-dioxygenase (IDO), which is expressed in all tissues, and tryptophan dioxygenase (TDO), which is mostly localized to the liver (Clarke et al., 2012; Kennedy et al., 2017). IDO and TDO expression are highly induced by the actions of inflammatory cytokines (particularly interferon-γ) and elevated levels of glucocorticoids in response to stress, respectively (Kennedy et al., 2017; Yoshida et al., 1981). Therefore, decreased levels of TRY may potentially influence serotonergic signaling in the brain, and IDO activity may also affect 5-HT biosynthesis (Zhang et al., 2016a). Downstream of IDO, KYN has two catabolic branches involving the formation of either kynurenic acid (KYNA) or quinolinic acid (QUIN) and intermediate metabolites, such as 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HAA), which are collectively termed "kynurenines." KYNA is a well-known N-methyl-D-aspartate (NMDA) receptor antagonist with

antioxidant and neuroprotective effects, although its levels are diminished in inflammatory conditions (Lugo-Huitrón et al., 2011; Öztürk et al., 2020). In contrast, QUIN is an NMDA receptor agonist showing several neurotoxic effects, such as blocking uptake by astrocytes, whereas 3-HK is a neurotoxic compound that enhances oxidative stress and contributes to neurodegeneration, particularly in depression in late life (Leonard, 2018). The balance between KYNA and QUIN is shown in **Figure 1**. Therefore, if chronically imbalanced levels of neuroprotective and neurotoxic KP metabolites are not corrected, changes in the neuronal-glial network may result. This damage might be progressive and may make the brain more vulnerable to pathological conditions, thereby causing neuropsychiatric disorders, including depression and BD (Myint et al., 2007; Myint and Kim, 2014).

Figure 2 presents the timeline of key, seminal original discoveries that gave rise to the field of research on the KP in mood disorders and the gut-brain axis. For example, Coppen et al. (1972) reported diminished free-plasma-TRY concentrations in patients with depression, which may be a cause of low lumbar cerebrospinal fluid-TRY levels. Lapin. (1978) first discovered that intraventricularly injected QUIN produces seizures in mice, thus suggesting the central effects of kynurenines. Subsequently, Stone and Perkins. (1981) revealed that QUIN plays roles in neuronal excitation, possibly owing to its potent endogenous excitatory effects on NMDA receptors in the central nervous system (CNS). In the same year, Yoshida et al. (1981) first demonstrated that interferon induces IDO; thereafter, a study by Christen et al. (1990) suggested that the induction of IDO may stimulate a local antioxidant defense against inflammatory diseases, a response related to the antioxidant activities of some TRY metabolites in the KP. Subsequently, Perkins and Stone. (1982) discovered the opposing roles of KYNA and QUIN in antagonizing and agonizing glutamate receptors, respectively; the authors proposed that an imbalance in the KP might be associated with CNS disorders. Later, a study by Birch et al. (1988) first demonstrated that KYNA antagonizes NMDA responses via acting at the glycine site of the NMDA receptor. Notably, a recent review by Stone. (2020) concluded that there was overwhelming evidence for the action of QUIN on ionotropic glutamate receptors; conversely, no reliable or reproducible evidence for that on nicotinic receptors. A landmark study by Heyes et al. (1992) linked cerebral inflammation to a dysfunctional KP in the CNS. In the same year, van Dam et al. (1992) first demonstrated the induction of the expression of interleukin-1β in the brain in response to peripheral administration of lipopolysaccharide (LPS).
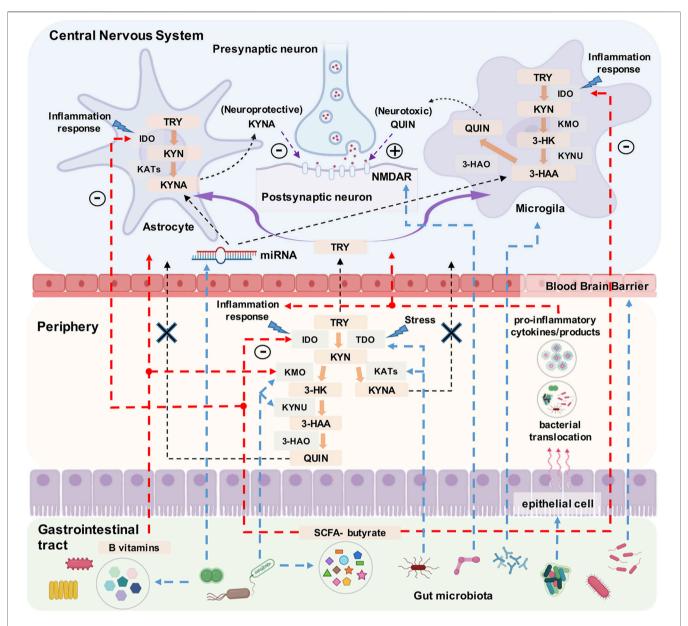
FIGURE 1 | The balance between kynurenic acid (KYNA) and quinolinic acid (QUIN) in the central nervous system and the critical impact points (presented as blue dotted lines) on the kynurenine pathway (KP) under the control of the gut microbiota. Note. TRY: tryptophan, KYN: kynurenine, 3-HK: 3-hydroxykynurenine, KYNA: kynurenic acid, 3-HAA: 3-hydroxyanthranilic acid, QUIN: quinolinic acid, IDO: indoleamine 2,3-dioxygenase, TDO: tryptophan dioxygenase, KATs: kynurenine aminotransferases, KMO: kynurenine-3-monooxygenase, KYNU: kynureninase, 3-HAO: 3-hydroxyanthranlic acid oxygenase, NMDAR: N-methyl-D-aspartate receptor.

Furthermore, Maes et al. (1993) proposed that the lower plasma-TRY availability to the brain may be related to the immune response in major depression. Direct clinical evidence from a study by Myint et al. (2007) provided support for the neurodegeneration hypothesis linking the imbalance between neuroprotective and neurodegenerative KP metabolites to the pathophysiology of major depression. In the gut microbiota field, an important study by Sudo et al. (2004) showed that germ-free mice have an exaggerated hypothalamic-pituitary-adrenal reaction to stress, as compared with the response in specific

pathogen free mice, and this response could be reversed by the colonization of specific strains of bacteria at an early stage of development. O'Connor et al. (2009a) demonstrated an essential role of the pro-inflammatory cytokines interferon-γ and tumor necrosis factor-α on the induction of IDO and subsequent depressive-like behaviors in mice in the context of chronic inflammation induced by bacille Calmette-Guérin (an attenuated form of *Mycobacterium bovis*). Notably, the findings from Bravo et al. (2011) demonstrated the potential ability of probiotics to regulate emotional behavior and central
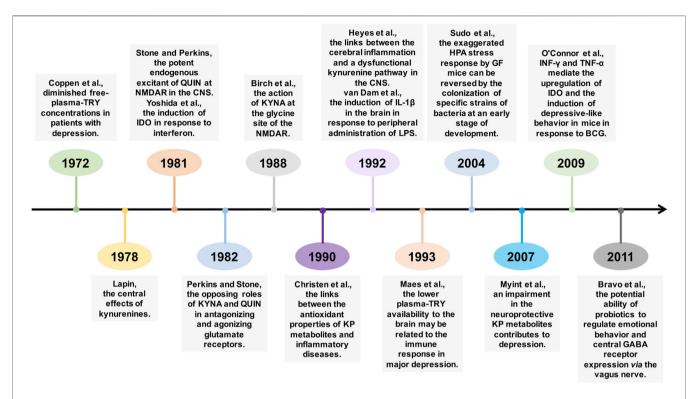
FIGURE 2 | The timeline of key, seminal original discoveries that gave rise to the fields of research on the kynurenine pathway (KP) in mood disorders and the gut-brain axis. Note. TRY: tryptophan, QUIN: quinolinic acid, NMDAR: N-methyl-ᴅ-aspartate receptor, CNS: central nervous system, IDO: indoleamine 2,3-dioxygenase, KYNA: kynurenic acid, IL-1β: interleukin-1β, LPS: lipopolysaccharide, HPA: hypothalamic-pituitary-adrenal, GF: germ-free, GABA: gamma-aminobutyric acid, TNF-α: tumor necrosis factor-α, BCG: bacille Calmette-Guérin.

gamma-aminobutyric acid receptor expression via the vagus nerve, thus highlighting the important role of bacteria in bidirectional gut-brain interactions. The further development of culture-independent molecular methods, such as 16S ribosomal RNA and metagenomic sequencing tools, has enabled researchers to better understand the structure and functions of the gut microbiome, thereby contributing to a key research hotspot, "gut microbiota," occurring in 2011 (Zhu et al., 2021).

The microbiota-gut-brain axis is a bidirectional communication pathway linking the host central nervous system and gastrointestinal tract (Rhee et al., 2009; Hao et al., 2020), and one of the key communication pathways of the microbiota-gut-brain axis is the KP (Kennedy et al., 2017). The gut microbiota has multifocal effects on the KP, including synthesizing enzymes analogous to TDO, kynureninase, and kynurenine 3-monooxygenase; regulating both QUIN production, by influencing microglia cells, and NMDA receptor expression in the CNS; reducing the activities of multiple enzymes (including colon TRY hydrolase, IDO, and kynurenine aminotransferases); producing the short-chain fatty acid butyrate, which inhibits IDO transcription; and influencing the levels of cofactors (e.g., vitamins B_{12} and B_6) of KP enzymes (Kennedy et al., 2017; Więdłocha et al., 2021). The gut microbiota can also affect the KP through epigenetic regulation of non-coding RNAs in the CNS. For example, Moloney et al. (2017) have demonstrated that the gut microbiota regulates the hippocampal

microRNA-294-5p expression associated with KP metabolism. Normally peripheral KYNA and QUIN are not considered to easily cross the blood-brain barrier (BBB) (Kennedy et al., 2017); however, the gut microbiota can decrease the BBB permeability by up-regulating the expression of tight junction proteins (Braniste et al., 2014); thus, a permeable brain in germ-free animals can markedly increase the flow of peripheral KYNA and QUIN across the BBB (Więdłocha et al., 2021). The gut microbiota is also involved in the activation of epithelial cell gene expression through Toll-like receptors, thereby inducing pro-inflammatory cytokine production and release (Elson and Alexander, 2015), whereas only Toll-like receptor-3 stimulation has been reported to be associated with elevated KP metabolites (e.g., KYNA and QUIN) in human peripheral monocytes (Orhan et al., 2016). Greater gut dysbiosis can also lead to increased intestinal permeability, bacterial translocation, and the release of pro-inflammatory bacterial products into the circulatory system (Ferrucci and Fabbri, 2018). Subsequently, these pro-inflammatory cytokines and products may influence KP metabolism in both the peripheral and central nervous systems. The critical points in the KP under the control of the gut microbiota are shown in **Figure 1**.

In the past 2 decades, human and animal studies have increasingly been conducted to reveal the links between KP and mood disorders. Several meta-analyses have been reported (Arnone et al., 2018; Ogyu et al., 2018; Bartoli et al., 2020; Marx et al., 2020); however, these reviews have focused only on the
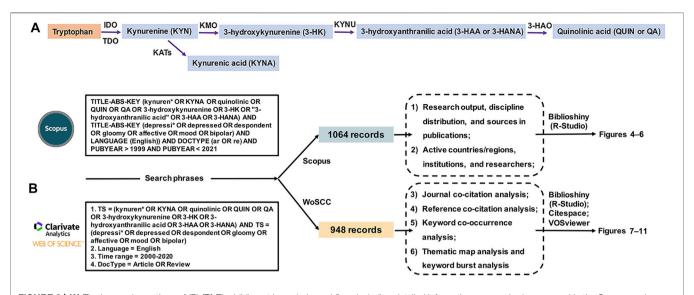
FIGURE 3 | (A) The kynurenine pathway (KP) **(B)** The bibliometric analysis workflow, including detailed information on search phrases used in the Scopus and WoSCC databases, along with the corresponding numbers of extracted records, bibliometric analysis items, analysis tools, and generated figures. Note. IDO: indoleamine 2,3-dioxygenase, TDO: tryptophan dioxygenase, KATs: kynurenine aminotransferases, KYN: kynurenine, KYNA: kynurenic acid, KMO: kynurenine-3-monooxygenase, 3-HK: 3-hydroxykynurenine, KYNU: kynureninase, 3-HAA or 3-HANA: 3-hydroxyanthranilic acid, 3-HAO: 3-hydroxyanthranlic acid oxygenase, QUIN or QA: quinolinic acid.

relationship between the KP metabolites and mood disorders and included a relatively small number of articles. Additionally, although these meta-analyses have confirmed the abnormal levels of KP metabolites in mood disorders, further research on their exact etiological roles in mood disorders, particularly involving the microbiota-gut-brain axis, is required. To the best of our knowledge, the general aspects of the links between the KP and mood disorders and the current status of gut microbiota research in this field have not been systematically studied via bibliometric and visual analysis. Thus, researchers may have difficulty in attaining a comprehensive and macroscopic view of this field, because of the diverse research domains (Chen et al., 2020). Furthermore, additional innovations and breakthroughs might be hindered, owing to potential untimely analysis of research frontiers (Zhu et al., 2021). The present bibliometric analysis fills the gap in the literature in this field.

Bibliometric analysis is a statistical method used to rapidly quantitatively analyze and visualize scientific output, research hotspots, and developing trends, by using public literature databases (Zhang et al., 2020). This method has been applied to various disciplines including the medical sciences (Thompson and Walker, 2015). In the present study, we investigate the research output; discipline distribution; publication sources; and active countries/regions, institutions, and researchers, thus helping researchers choose suitable journals and collaborators. In addition, we combine journal co-citation analysis, reference co-citation analysis, keyword co-occurrence analysis, thematic map analysis, and keyword burst analysis to map the intellectual structure and to evaluate the research foundations, hotspots, and frontiers in the KP in mood disorders, with the aim of revealing the research status of gut microbiota in this field, guiding future research, and promoting the development of this field.

MATERIALS AND METHODS

Data Sources and Search Strategy

A comprehensive search was conducted on two large, multidisciplinary citation databases, Scopus and Web of Science Core Collection (WoSCC) (Falagas et al., 2008), on a single day (January 1, 2021), to avoid the discrepancies due to daily database updates. These databases were chosen because Scopus is the world's largest abstract and citation database (Zyoud et al., 2019), and WoSCC is a curated collection of high-quality scholarly peer-reviewed literature published worldwide (Zhu et al., 2021). The search phrases associated with the KP included KYN and its key metabolites (e.g., KYNA, QUIN, 3-HK, and 3-HAA) (**Figure 3A**), and the phrases associated with mood disorders included depression and related disorders such as BD. The search timespan covered the years 2000–2020, a period considered sufficiently long enough to reflect the development trends in this field that were our research focus. The present analysis was concerned with only two types of documents, articles and reviews published in the English language, and no species restrictions were imposed. All extracted records from both databases were identified after removal of duplicates. The same search strategy was applied to both databases, and detailed information is shown in **Figure 3B**.

Data Analysis

The data from different databases were required to follow the fixed formats required by different bibliometric analysis tools. Given that converted data may be incompatible and affect subsequent analyses (Shi and Liu, 2019), the data from both databases were analyzed separately.

In the present study, the analyses of research output, discipline distribution, and sources in publications, and the active countries/regions, institutions, and researchers were conducted on the Scopus database by using the R-bibliometrix package (version 3.0.3, http://www.bibliometrix.org) in R-Studio (version 1.2.1335). Biblioshiny, a shiny app providing a web-interface for bibliometrix, was used to support the importation of metadata from databases and subsequent data management (Aria and Cuccurullo, 2017). The journal co-citation analysis, reference co-citation analysis, and keyword co-occurrence analysis were performed on the WoSCC database by using VOSviewer software (version 1.6.16, https://www.vosviewer.com/download), Citespace software (version 5.7 R5W, https://citespace.podia.com/courses/download), and the biblioshiny app, respectively. VOSviewer is a computer program developed by Nees Jan van Eck and Ludo Waltman from Leiden University (Leiden, the Netherlands) for bibliometric mapping. It can be used to conduct co-authorship analysis, keyword co-occurrence analysis, citation and co-citation analysis, and bibliographic coupling (van Eck and Waltman, 2010; Merigó et al., 2018). Citespace is a tool invented by Professor Chaomei Chen from Drexel University (Philadelphia, the United States of America) for conducting visualization analysis of scientific references. It is typically used to construct social network maps, co-citation network maps, and co-occurrence network maps (Chen, 2004; Chen, 2020). To obtain more convincing and reliable results, we performed an integrated analysis of thematic maps via the biblioshiny app and burst analysis of keywords via Citespace, on the basis of the WoSCC database, to examine the research frontiers in the field of the KP in mood disorders. A thematic map resulting from a clustering analysis of a co-occurrence network provides a Cartesian representation of the identified term clusters (Aria et al., 2020). It allows for easy visual interpretation of the research themes developed in a framework and provides information regarding emerging or declining themes (Aria and Cuccurullo, 2017). Similarly, burst analysis of keywords, which involves two attributes (i.e., the intensity and the duration of the burst), can reveal abrupt changes in keywords over a particular period, thus serving as an indicator of emerging research directions (Chen et al., 2014). The parameters used in the journal co-citation analysis via VOSviewer were as follows: counting method (fractional counting), minimum number of citations of a source (100), visualization weights (citations), normalization (association strength), clustering resolution (1.00), minimum cluster size (1), minimum line strength (200), and maximum lines (500). The parameters used in Citespace were as follows: time slicing (2000–2020), years per slice (1), term source (all selection), node type (one option chosen at a time from the reference in reference co-citation analysis, and the keyword in keyword co-occurrence analysis), selection criteria (top 40), pruning (none), and visualization ("cluster view-static" and "show merged network"). The bibliometric analysis items, as well as the corresponding databases and tools, and the figures in which the maps were visualized, are displayed in **Figure 3B**.

All statistical analyses were performed in IBM SPSS Statistics, version 25.0 (SPSS Inc., Chicago, IL, United States). Polynomial model fitting was used to predict the research output in 2021. The Spearman correlation coefficient was used to analyze the correlations between selected continuous variables. A p-value < 0.05 was considered statistically significant.

RESULTS

Research Output, Discipline Distribution, and Sources in Publications

The total numbers of collected documents in the Scopus and WoSCC databases were 1,064 and 948, respectively, without duplications. Here, Scopus was selected as the data source of analysis, because of its better coverage of literature. **Figure 4** shows that the total research output was very low before 2006, but the annual output of articles and reviews subsequently showed rapid upward trends. The annual growth rate calculated as (value in the year 2020/value in the year 2000) $^{(1/20)}$ −1, was 11.1%. A total of 781 articles and 283 reviews were retrieved from Scopus. Polynomial model fitting revealed significant correlations between the publication year and the publication output (the coefficients of determination (r^2) were 0.951, 0.948, and 0.883 for total documents, articles, and reviews, respectively). On the basis of polynomial curve fitting, the publication output is expected to reach approximately 130 in 2021, comprising 100 articles and 30 reviews.

Given that each document may involve several disciplines, the extracted studies mainly belonged to five disciplines: medicine (n = 516, 26.8%), neuroscience (n = 510, 26.5%), biochemistry, genetics and molecular biology (n = 293, 15.2%), pharmacology, toxicology and pharmaceutics (n = 230, 11.9%), and immunology and microbiology (n = 100, 5.2%). The literature included was published in 343 journals. There was a significant but weak positive relationship between the total publications (TP) of journals and their total citations (TC) (r = 0.593, p < 0.001). All the top five most relevant sources were in the field of neuropsychiatry and had impact factors (IFs) above 3: Brain Behavior and Immunity (IF $_{2019}$ = 6.663, TP = 47, TC = 1,962), Psychoneuroendocrinology (IF $_{2019}$ = 4.732, TP = 24, TC = 975), Progress in Neuro-Psychopharmacology and Biological Psychiatry (IF $_{2019}$ = 4.361, TP = 19, TC = 1,064), Journal of Affective Disorders (IF $_{2019}$ = 3.892, TP = 18, TC = 668), and Molecular Psychiatry (IF $_{2019}$ = 12.384, TP = 18, TC = 2,368). **Table 1** shows the top 20 journals that published the largest number of papers related to this field.

Active Countries/Regions, Institutions, and Researchers

The data extracted from Scopus indicated that the United States (TP = 871) was the most productive country, followed by China (TP = 319), Austria (TP = 220), Germany (TP = 213), and the United Kingdom (TP = 191). **Figure 5** shows the country/region collaboration map worldwide generated by using the biblioshiny app. There were 280 pairs of collaborating countries/regions worldwide, of which the top three were Australia and Thailand with 24 collaborations, followed by Australia and
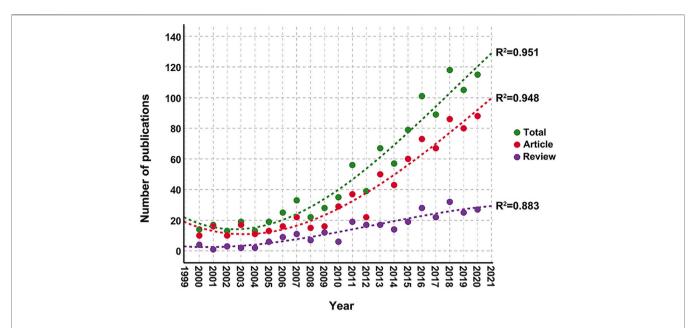
FIGURE 4 | Annual trends in publication from Scopus (2000–2020) in the field of the kynurenine pathway (KP) in mood disorders and the corresponding polynomial fitted curves.

TABLE 1 | The top 20 most relevant journals in the field of the kynurenine pathway (KP) in mood disorders between 2000 and 2020.

Rank	Source	Country	2019 JCR® category (partition)	IF_{2019}	TP	TC
1	Brain, Behavior, and Immunity	United States	Neurosciences (Q1), immunology (Q1), psychiatry (Q1)	6.633	47	1,962
2	Psychoneuroendocrinology	England	Neurosciences (Q1), psychiatry (Q1), endocrinology and metabolism (Q1)	4.732	24	975
3	Progress in Neuro-Psychopharmacology and Biological Psychiatry	England	Neurosciences (Q2), psychiatry (Q1), pharmacology and pharmacy (Q1), clinical neurology (Q1)	4.361	19	1,064
4	Journal of Affective Disorders	Netherlands	Psychiatry (Q1), clinical neurology (Q1)	3.892	18	668
5	Molecular Psychiatry	England	Neurosciences (Q1), psychiatry (Q1), biochemistry and molecular biology (Q1)	12.384	18	2,368
6	Journal of Neuroinflammation	England	Neurosciences (Q1), immunology (Q1)	5.793	17	1,095
7	PLoS One	United States	Multidisciplinary sciences (Q2)	2.740	16	400
8	Current Pharmaceutical Design	United Arab Emirates	Pharmacology and pharmacy (Q3)	2.208	15	621
9	Psychiatry Research	Netherlands	Psychiatry (Q3)	2.118	13	317
10	Frontiers in Psychiatry	Switzerland	Psychiatry (Q2)	2.849	12	163
11	Journal of Neural Transmission	Austria	Neurosciences (Q2), clinical neurology (Q2)	3.505	12	606
12	Journal of Neuroscience	United States	Neurosciences (Q1)	5.674	12	1,066
13	Neuroscience	England	Neurosciences (Q2)	3.056	12	752
14	Neuroscience and Biobehavioral Reviews	England	Neurosciences (Q1), behavioral sciences (Q1)	8.329	12	1,172
15	CNS and Neurological Disorders-Drug Targets	Netherlands	Neurosciences (N/A), pharmacology and pharmacy (N/A)	N/A	11	242
16	Journal of Psychiatric Research	England	Psychiatry (Q2)	3.745	11	1,237
17	Neuropsychopharmacology	England	Neurosciences (Q1), psychiatry (Q1), pharmacology and pharmacy (Q1)	6.751	11	1,541
18	Behavioral Brain Research	Netherlands	Neurosciences (Q3), behavioral sciences (Q2)	2.977	10	265
19	International Journal of Neuropsychopharmacology	England	Neurosciences (Q2), psychiatry (Q1), pharmacology and pharmacy (Q1), clinical neurology (Q1)	4.333	10	298
20	Neuropsychiatric disease and Treatment	New Zealand	Psychiatry (Q3), clinical neurology (Q3)	2.157	10	188

Note. JCR®: journal of citation reports in Web of Science, IF_{2019}: impact factor in 2019, TP: total publications, TC: total citations, N/A: not available.

United Kingdom with 24 collaborations, and Australia and Brazil with 20 collaborations.

The most relevant affiliations were Innsbruck Medical University (TP = 90) in Austria, followed by the Karolinska Institutet (TP = 66) in Sweden, and the University of Illinois at Urbana-Champaign (TP = 54) in the United States. The most prolific researchers, whose names have been replaced with codes according to the principles of data protection, were Author "A"

Rank	From	To	Freq	Rank	From	To	Freq
1	Australia	Thailand	24	6	USA	UK	18
2	Australia	UK	24	7	USA	Austria	16
3	Australia	Brazil	20	8	Germany	USA	16
4	USA	Sweden	19	9	Bulgaria	Thailand	15
5	UK	Thailand	19	10	Australia	Bulgaria	15
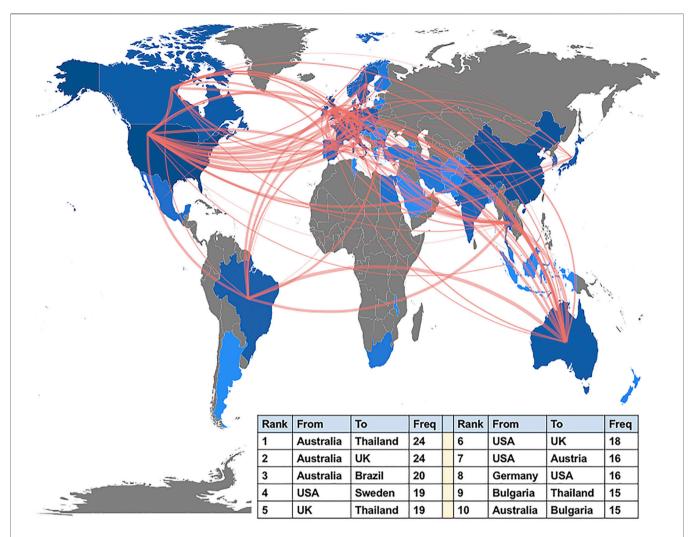
FIGURE 5 | Country/region collaboration map of studies associated with the kynurenine pathway (KP) in mood disorders, generated through the biblioshiny app. Note. USA, United States; UK, United Kingdom.

from Innsbruck Medical University (61 articles), followed by Author "B" from Chulalongkorn University in Thailand (44 publications), and Author "C" from the University of Texas, M. D. Anderson Cancer Center in the United States (34 publications). **Table 2** and **Figure 6** show the details of the top ten active researchers in this field and their productions over time, respectively.

Journal Co-citation Analysis

Journal co-citation analysis, first introduced by McCain (1991), focuses primarily on the journal-to-journal relationship and is widely used to study the interdisciplinary structure of a given academic field (Chi and Young, 2013; Hsiao and Yang, 2011; Yang et al., 2019). The network map yielded co-citation patterns of 132 journals, which were divided into three clusters, "Psychiatry (red cluster)," "Immunology, Pharmacology and Pharmacy, and Multidisciplinary Sciences (blue cluster)," and "Neuroscience (green cluster)," via manual assignment of their

descriptive labels based on the subject categories (**Figure 7**) (Najas-Garcia et al., 2018). The easily interpreted visualization of item density indicates a strong tendency toward the co-citation of journals in these dense areas, including the cluster "Psychiatry" (**Figure 8**) (van Eck and Waltman, 2010). Highly co-cited journals are those that are frequently cited together by other journals; in these journals, the published articles reflect the important research fundaments of the field (Guo et al., 2019). The top two journals on the basis of total co-citations (TCC) were Biological Psychiatry (IF $_{2019}$ = 12.095, TCC = 2009, total link strength = 1876.75), and Brain Behavior and Immunity (IF $_{2019}$ = 6.663, TCC = 1923, total link strength = 1787.65). **Figure 7** shows a strong tendency toward co-citation relationships between the journal Biological Psychiatry and other journals from these three clusters.
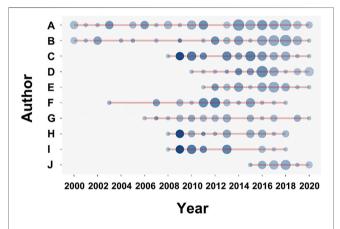
Reference Co-citation Analysis

Highly co-cited references are those that are frequently cited together by other articles, and thus, can be regarded as knowledge

TABLE 2 | The top ten most prolific authors in the field of the kynurenine pathway (KP) in mood disorders between 2000 and 2020.

Rank	Author	Organization (Country/Region)	Major fields in publications	TP	TC	H-index
1	A	Medical university of innsbruck (Austria)	Psychiatry, neurosciences, endocrinology and metabolism	61	3,119	29
2	B	Chulalongkorn university (Thailand)/Medical university plovdiv (Bulgaria)/ Deakin university (Australia)	Neurosciences, pharmacology and pharmacy, psychiatry	44	2,999	23
3	C	University of Texas dallas (United States)/UTMD anderson cancer center (United States)	Neurosciences, psychiatry, immunology	34	3,974	27
4	D	Karolinska institutet (Sweden)	Psychiatry, neurosciences, biochemistry and molecular biology	26	1,332	15
5	E	CRC scotland and london (England)/Clin res commun CRC scotland and london (England)	Neurosciences, pharmacology and pharmacy, clinical neurology	25	969	16
6	F	University of munich (Germany)	Psychiatry, neurosciences, clinical neurology	25	2,360	21
7	G	University of munich (Germany)	Psychiatry, neurosciences, clinical neurology	22	1,501	18
8	H	University of Texas health san antonio (United States)/Audie L. Murphy VA hospital (United States)	Neurosciences, psychiatry, immunology	20	2,611	18
9	I	University of Illinois system (United States)/Coll med (United States)	Neurosciences, immunology, psychiatry	18	3,045	16
10	J	University of toronto (Canada)/Center for addiction and mental health (Canada)/Deakin university (Australia)	Neurosciences, psychiatry, pharmacology and pharmacy	16	331	10

Note. TP: total publications, TC: total citations.

FIGURE 6 | The top ten active researchers in the field of the kynurenine pathway (KP) in mood disorders and their publications over time. The larger the circle, the more articles published. The deeper the color, the more citations.

bases in a particular field. In this section, the WoSCC database was used as the data source for analysis because of its coverage of high-quality literature. **Figure 9A** presents the largest seven clusters of the co-citation network of references, together with their details and the top five representative references, on the basis of a log-likelihood ratio algorithm in Citespace software: "kynurenine pathway (cluster #0)," "psychoneuroimmunology (cluster #1)," "indoleamine 2,3-dioxygenase (cluster #2)," "proinflammatory cytokines (cluster #3)," "psychosis (cluster #4)," "insulin resistance (cluster #5)," and "gut-brain axis (cluster #9)." All clusters were constructed on the basis of keywords extracted from the references. The total modularity Q-value of 0.7164 and the mean silhouette of each cluster above 0.7 indicated that the clustering structure was significant, and the results were highly credible (Guo et al., 2019). The burst detection
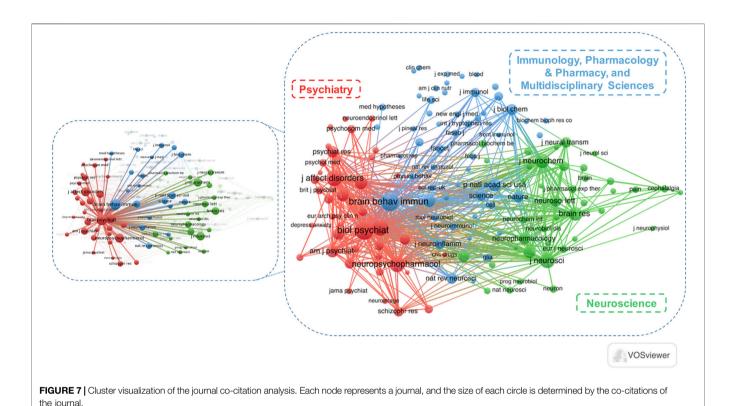
of references can reveal the abrupt changes in citations over time, thereby indicating the evolution of a knowledge domain (Synnestvedt et al., 2005). **Figure 9B** shows the references with the strongest citation bursts that are currently ongoing, most of which belonged to cluster #0, labeled "kynurenine pathway."

Table 3 shows the top ten co-cited references (four reviews, five articles, and a meta-analysis) in this field, which were found mostly in clusters #0 and #2. The review published in Nature Reviews Neurosciences by Schwarcz et al. (2012), from the University of Maryland, United States, was the most co-cited reference (TCC = 163). **Table 4** presents the representative references in cluster #9, labeled "gut-brain axis," in which the review published in Behavioral Brain Research by O'Mahony et al. (2015) had the strongest currently ongoing citation bursts (**Figure 9B**). Highly co-cited references are commonly important foundational studies that contribute to future researchers' understanding of the research foundations in the field.

Keyword Co-Ocurrence Analysis
Keyword co-occurrence analysis, aiming to investigate the co-occurring relationships between keywords in a set of publications, can reflect hot topics and help researchers deepen their understanding of scientific findings in research hotspots. "Keywords Plus" comes from a glossary defined by the Thomson Reuters editorial expertize team. This state-of-the-art keyword searching tool has been confirmed to be more broadly descriptive than the "Author Keywords," thus enhancing the power of cited-reference searching (Zhang et al., 2016b).

Figure 10A presents the top ten highest frequency keywords, on the basis of the WoSCC database, by using the "Keywords Plus" parameter via the biblioshiny app. The term "quinolinic acid" occurred in 2006 and ranked first with 189 occurrences, followed by "depression" with 174 occurrences, and "indoleamine 2,3-dioxygenase" with 160 occurrences. **Figure 10B** shows the keyword co-occurrence network among the 50 main keywords,

FIGURE 7 | Cluster visualization of the journal co-citation analysis. Each node represents a journal, and the size of each circle is determined by the co-citations of the journal.

by using the "Keywords Plus" parameter and the "Louvain" clustering algorithm via the biblioshiny app, in which three clusters with different colors (red, green, and blue) were established. The most relevant nodes belonged to the same clusters with the same colors, which represented close co-occurring relationships. The node size and link line width were proportional to the extent of co-occurrence and the strength of co-occurring relationships between nodes, respectively (Rodríguez-Sabiote et al., 2020). Additionally, the betweenness centrality was used to measure the importance of nodes in the network. The more important the node, the higher the betweenness centrality, thus indicating that more information passed through the node (Rodríguez-Sabiote et al., 2020). The hot topic "quinolinic acid," which belonged to the red cluster, notably was the largest and had the highest calculated betweenness centrality value of 57.01.

Thematic Map Analysis and Burst Analysis of Keywords

In the present study, the terms from "Keywords Plus," according to the WoSCC database, could be considered emerging topics within this field. Given that differences might exist in analysis results across bibliometric tools, an overlap analysis between the thematic map of keywords from the biblioshiny app and the keyword burst analysis from Citespace software was conducted to identify research frontiers in this field.

The thematic map was created by using a two-dimensional matrix involving two types of measurement: centrality and density. **Figure 11A** shows the thematic map of "Keywords Plus," in which the X-axis indicates the centrality, i.e., the importance of a theme, and the Y-axis represents the density, a measure of the development of a theme (Aria et al., 2020). Accordingly, the upper right quadrant (i.e., quadrant 1) pertains to motor themes that are both important and well-developed, the upper left quadrant (i.e., quadrant 2) is associated with highly developed and isolated themes, the lower left quadrant (i.e., quadrant 3) refers to emerging or declining themes, and the lower right quadrant (i.e., quadrant 4) contains transversal and basic themes (Cobo et al., 2011). The clusters are represented by bubbles within the map, which are labeled by the keywords with the highest occurrences, and their sizes are proportional to the keyword occurrences (Rodríguez-Sabiote et al., 2020). Notably, two clusters corresponding to "quinolinic acid" and "cerebrospinal-fluid," composed of 43 and 44 keywords, respectively, were positioned in quadrant 3, which was characterized by both low centrality and low density.

According to keyword burst analysis, the top 47 keywords were found to have the strongest citation bursts during the period from 2006 to 2020. **Figure 11B** shows the meaningful keywords with the strongest citation bursts that are currently ongoing, including "Alzheimer's disease," "immune," "prefrontal cortex," "inhibition," "acid," "plasma," and "association." An overlap analysis indicated that only three terms, "Alzheimer's disease," "prefrontal cortex," and "acid," were found within the clusters in quadrant three of **Figure 11A**. Among them, the term "prefrontal cortex," appearing in 2018, had the highest strength, thus
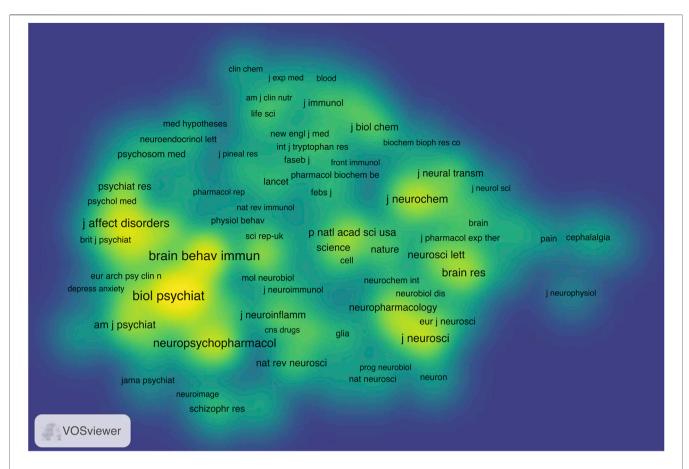
FIGURE 8 | Item density visualization of the journal co-citation analysis. The larger the number of the neighboring items and the higher the weights of the items in a point, the closer the color of the point is to yellow in the visualization.

reflecting its highest frequency of citation. Hence, these three terms were identified as research frontiers in this field.

DISCUSSION

To date, kynurenines have been implicated in a variety of diseases, particularly psychiatric disorders, owing to their neuromodulatory properties (Chen and Guillemin, 2009; Savitz, 2020). The roles of variations in the levels of kynurenines in the pathogenesis of mood disorders have gained increasing attention, thus making the KP a hotspot in the field of mood disorders in the past 2 decades. Currently, bibliometric analysis is increasingly being used to review the status and trends in a particular field (Zhang et al., 2020). However, to date, no bibliometric analyses focusing on the KP in mood disorders, particularly the gut microbiota research in this field, have been reported.

The current analysis showed that the number of publications on the KP in mood disorders increased rapidly since 2006, a result possibly associated with the many active researchers in different disciplines (e.g., Authors "A–F") with interests within this field, and several hotspots (e.g., QUIN) having occurred since then.

Most of the top relevant journals had high IFs. Moreover, a weak positive relationship between the TP and TC of journals in this field indicated that the research activity regarding the KP in mood disorders in general was favored by high-IF journals in the field of neuropsychiatry. In addition, our journal co-citation analysis showed a strong tendency toward co-citation of research in the field of psychiatry, and the journal Biological Psychiatry received many co-citations despite its few publications related to this research area, thus indicating that the studies in this journal served as important research foundations in this field.

Among countries, the United States had an absolute advantage in the research output in this field, followed by China. Remarkably, active scientific collaborations between the United States and countries such as Sweden, United Kingdom, and Austria, were found. Although China, a developing country, has shown extensive development in this field, its influence was relatively low, owing to insufficient cooperative relationships with the most prolific countries; thus, more international scientific cooperation is needed. Austria was the third most productive country, possibly because Author "A," the most active scholar in this field, is from Innsbruck Medical University, which is also a highly relevant affiliation in this field. Interestingly, active collaborative relationships between Thailand and countries
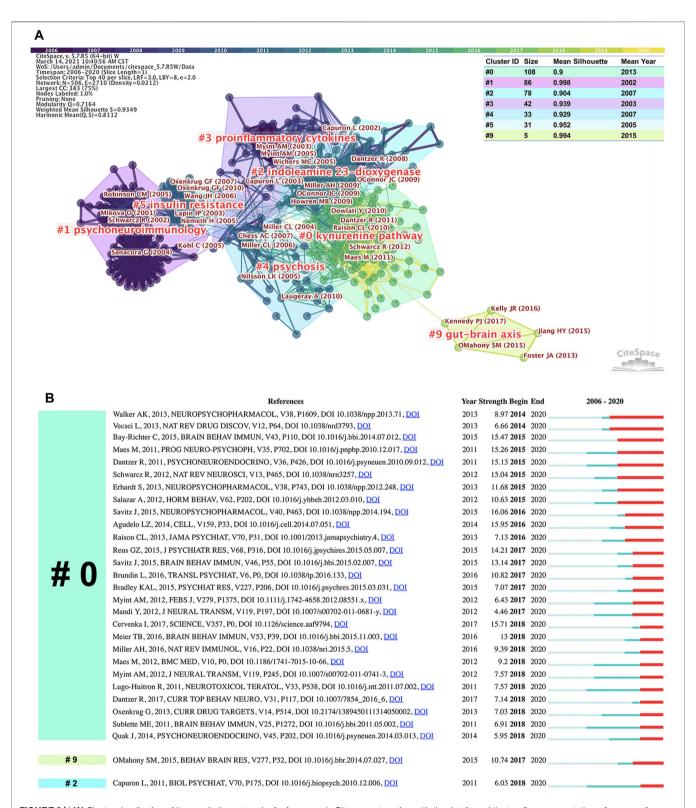
FIGURE 9 | **(A)** Cluster visualization of the co-citation network of references via Citespace, together with the details and the top five representative references of the generated clusters **(B)** The references with the strongest currently ongoing citation bursts in this field. The red bars indicate the duration of the burst and reflect references cited frequently; the green bars indicate references cited infrequently.

TABLE 3 | The top ten co-cited references related to the kynurenine pathway (KP) in mood disorders between 2000 and 2020.
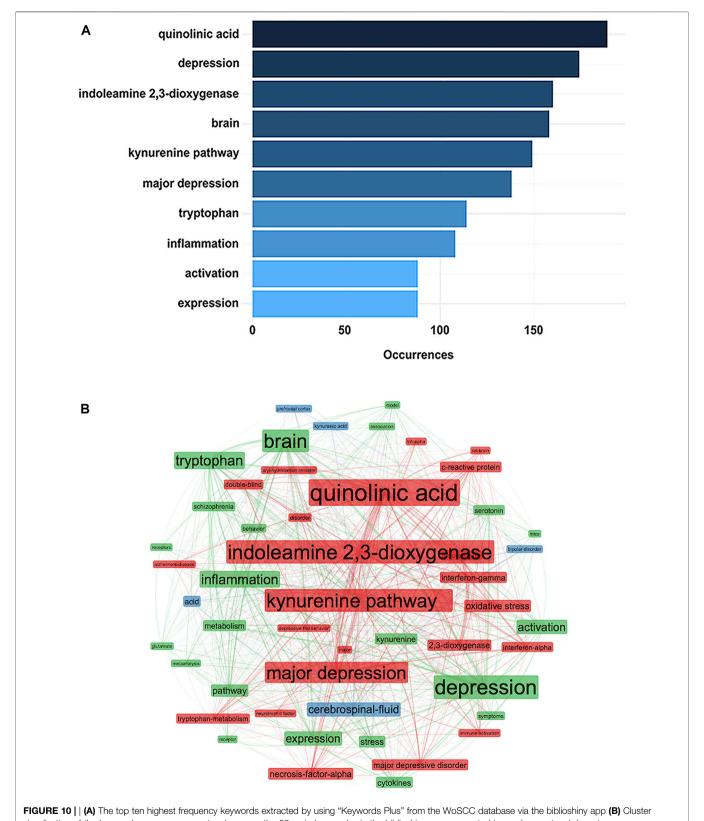
| Rank	References	Title	Source	Type	Total co-citations	In cluster
1	Schwarcz et al. (2012)	Kynurenines in the mammalian brain: When physiology meets pathology	Nature Reviews Neuroscience	Review	163	#0
2	Raison et al. (2010)	CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-alpha: Relationship to CNS immune responses and depression	Molecular Psychiatry	Article	127	#0
3	O'Connor et al. (2009b)	Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice	Molecular Psychiatry	Article	117	#2
4	Dantzer et al. (2008)	From inflammation to sickness and depression: When the immune system subjugates the brain	Nature Reviews Neuroscience	Review	108	#2
5	Dantzer et al. (2011)	Inflammation-associated depression: From serotonin to kynurenine	Psychoneuroendocrinology	Review	96	#0
6	Maes et al. (2011)	The new '5-HT' hypothesis of depression: Cell-mediated immune activation induces indoleamine 2,3-dioxygenase, which leads to lower plasma tryptophan and an increased synthesis of detrimental tryptophan catabolites (TRYCATs), both of which contribute to the onset of depression	Progress in Neuro-Psychopharmacology and Biological Psychiatry	Review	94	#0
7	Dowlati et al. (2010)	A meta-analysis of cytokines in major depression	Biological Psychiatry	Meta-analysis	89	#0
8	Steiner et al. (2011)	Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: Evidence for an immune-modulated glutamatergic neurotransmission?	Journal of Neuroinflammation	Article	86	#0
9	Myint et al. (2007)	Kynurenine pathway in major depression: Evidence of impaired neuroprotection	Journal of Affective Disorders	Article	83	#0
10	Bay-Richter et al. (2015)	A role for inflammatory metabolites as modulators of the glutamate N-methyl-D-aspartate receptor in depression and suicidality	Brain, Behavior, and Immunity	Article	83	#0
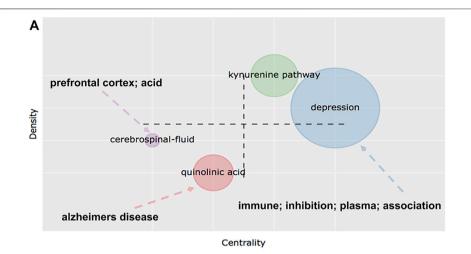
TABLE 4 | The top five representative references of cluster #9, labeled "gut-brain axis" in the co-citation network of references.

Rank	References	Title	Source	Type	Total co-citations
1	O'Mahony et al. (2015)	Serotonin, tryptophan metabolism and the brain-gut-microbiome axis	Behavioral Brain Research	Review	23
2	Kennedy et al. (2017)	Kynurenine pathway metabolism and the microbiota-gut-brain axis	Neuropharmacology	Review	6
3	Foster and McVey Neufeld (2013)	Gut-brain axis: how the microbiome influences anxiety and depression	Trends in Neurosciences	Review	6
4	Jiang et al. (2015)	Altered fecal microbiota composition in patients with major depressive disorder	Brain, Behavior, and Immunity	Article	5
5	Kelly et al. (2016)	Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat	Journal of Psychiatric Research	Article	5

such as Australia, the United Kingdom, and Bulgaria were also observed and were likely to be associated with the influential expert, Author "B" from Chulalongkorn University, who has a history of close long-term cooperation with researchers (e.g., Authors "E" and "J") from Deakin University (Australia), CRC Scotland and London (United Kingdom), Medical University of Plovdiv (Bulgaria), Federal University of Ceará (Brazil), and University of Toronto (Canada). Among the top ten active researchers in the field of the KP in mood disorders, Authors "B," "E," and "J" were also among the top ten most prolific authors in the gut microbiota research in the depression field, according to our previous published bibliometric analysis (Zhu et al., 2021).

In the current analysis of the reference co-citation network, the presence of the largest cluster #0, "kynurenine pathway," occurring in 2013, was unsurprising, because this enzymatic cascade involves a group of related-compounds, the kynurenines, originating from TRY. The next three largest clusters were "psychoneuroimmunology," "indoleamine 2,3-dioxygenase," and "proinflammatory cytokines," and the most recent one was "gut-brain axis," thus indicating the role of KP in bridging the gut microbiota and the host immune system. Most of the top ten co-cited references belonged to cluster #0, "kynurenine pathway," thus identifying important knowledge foundations in this field. For example, the most influential review, published by Schwarcz et al. (2012), and entitled

FIGURE 10 | | (A) The top ten highest frequency keywords extracted by using "Keywords Plus" from the WoSCC database via the biblioshiny app **(B)** Cluster visualization of the keyword co-occurrence network among the 50 main keywords via the biblioshiny app, presented in a sphere network layout.

FIGURE 11 | | (A) Thematic map of "Keywords Plus" created by the biblioshiny app **(B)** Keywords with the strongest currently ongoing citation bursts. The red bars indicate the duration of the burst and reflect keywords cited frequently; in contrast, the green bars indicate keywords cited infrequently. These keywords identified by Citespace were found within the clusters labeled "depression," "quinolinic acid," and "cerebrospinal-fluid" in the thematic map.

"Kynurenines in the mammalian brain: when physiology meets pathology," was co-cited more than 160 times. This review describes the metabolism and regulation of neuro-active kynurenines in the brain, and the communication pathways linking the peripheral and central KP, then explains how the dysregulation of the KP is associated with neurological and psychiatric diseases such as MDD and discusses the novel therapeutic interventions targeting the KP. The second highest co-cited reference was an article published by Raison et al. (2010), in which the authors found that the activation of IDO induced by peripheral administration of interferon-alpha in patients with hepatitis C conforms with cytokine responses in the brain, thus resulting in increased KYN and QUIN in cerebrospinal fluid, along with increased depressive symptoms. The next two highly co-cited references both belonged to cluster #2, "indoleamine 2,3-dioxygenase." One was an article published by O'Connor et al. (2009b), which reported that IDO is a critical molecular mediating the LPS-induced depressive-like behavior in mice, probably through an increase in degradation of TRY along an inflammatory pathway (i.e., the KP). The other was a review published by Dantzer et al. (2008), focusing on how peripheral inflammation acts on the brain and results in sickness behavior, and suggesting that pro-inflammatory cytokines may trigger the development of depression via multiple underlying molecular mechanisms, including activation of IDO.

Notably, the most recent research focus was in cluster #9, "gut-brain axis" (mean year 2015), thereby indicating that in the field of the KP in mood disorders, gut microbiota research was still in an early stage. The first representative reference with the strongest currently ongoing citation bursts in this cluster was a review published by O'Mahony et al. (2015) evaluating the evidence of the influence of the gut microbiota on TRY metabolism and the serotonergic system, and exploring the potential mechanisms, including direct and indirect microbial regulation of TRY utilization and 5-HT biosynthesis. The second highest co-cited reference in this cluster focused in detail on the microbial regulation of KP metabolism and reviewed the critical points of gut microbiota control in KP metabolism in both pharmacokinetic and pharmacodynamic aspects (Kennedy et al., 2017). A review published by Foster and McVey Neufeld (2013) had the third highest number of co-citations; in this review, the authors discuss the relationship between stress and microbiota, and how the altered microbiota affect stress-related disorders, including anxiety and depression, thus improving understanding of the microbiota-gut-brain axis. The next representative references in this cluster were two articles: one was published by Jiang et al. (2015), in which the authors found altered fecal microbiota composition in patients with MDD, observed as increased levels of *Enterobacteriaceae* and *Alistipes*, but decreased levels of *Faecalibacterium*; the other was

published by Kelly et al. (2016), wherein depression was found to be characterized by reduced richness and diversity in the gut microbiota, and depressed behavior, as well as the alterations in TRY metabolism, were reproduced via fecal microbiota transplantation technology in which the microbiota from depressed patients were transferred to microbiota-depleted rats. These representative references with the highest co-citations in this cluster reflected the important knowledge foundations of gut microbiota research in the field of the KP in mood disorders.

Among the hot topics, the main focus was on QUIN, emerging in 2006, according to the keyword co-occurrence analysis, and the three most relevant topics belonged to the red cluster: IDO, the KP, and major depression. A recent meta-analysis has suggested that KYNA and the KYNA:QUIN ratio decrease, and KYN is preferentially metabolized to the potentially neurotoxic QUIN instead of the neuroprotective KYNA in mood disorders (Marx et al., 2020). Thus, QUIN has been proposed as a potential target and biomarker for mood disorders; e.g., to evaluate the antidepressant effect of ketamine (Verdonk et al., 2019). Among the emerging topics in this field, an overlap analysis identified Alzheimer's disease (AD), the prefrontal cortex (PFC), and acid as research frontiers, which were in the blue cluster within the keyword co-occurrence network, together with the topics cerebrospinal fluid and BD in this cluster.

AD, one of the most common neurodegenerative disorders, is characterized by progressive memory and mental function loss along with neuropsychiatric symptoms such as depression and anxiety (Erkkinen et al., 2018); it has a prevalence of 10–30% in people over 65 years of age (Eratne et al., 2018). Growing evidence suggests that the KP may play a crucial role in the development of neuropsychiatric symptoms in AD in response to neuroinflammation (Maddison and Giorgini, 2015). A study by Souza et al. (2016) has demonstrated that the elevated pro-inflammatory cytokines lead to the elevated IDO activity, thus subsequently increasing KYN production and the KYN:TRY ratio, whereas decreasing neurotrophic factors in the PFC and hippocampus contribute to the amyloid-beta 1–42-induced neuroinflammation and behavioral abnormalities in mice, thus strongly suggesting a critical role of IDO in mediating the emotional disturbances in AD. Future research may focus on the mutual effects of the gut microbiota on AD and KP rate-limiting enzymes such as IDO (Dehhaghi et al., 2019). Recent studies have proposed perspectives and the potential role of gut microbiota modulation in AD (Garcez et al., 2019); for example, gut microbiota-derived vitamins may be used as possible interventions for psychiatric treatment in AD (Rudzki et al., 2021).

The PFC was another emerging topic identified with the strongest citation bursts. A previous study by Liu et al. (2017) has suggested imbalanced KP metabolism in the PFC, as reflected by a decrease in prefrontal KYNA in Flinders Sensitive Line rats, which might possibly be associated with the induction of the KP enzymes by pro-inflammatory cytokines. However, a study by Clark et al. (2016) has indicated that depression is associated with unexpectedly decreased KP metabolism and cytokine expression in the ventrolateral PFC, which is part of the orbitofrontal cortex

region associated with higher emotional function, thus indicating that the brain KP regulation may be region-specific. A recent study has shown that IDO1 expression in mice is upregulated by LPS in the PFC but not in the hippocampus, and microinjection of 1-MT (a potent IDO1 antagonist) or microRNA-874-3p into the PFC downregulates LPS-induced IDO1 expression and ameliorates LPS-induced depression-like behavior, thus revealing that microRNA-874-3p is a novel potential target for the treatment of MDD (Suento et al., 2020). Future research may examine the influence of the gut microbiota on enzymes involved in TRY metabolism along the KP in PFC. For example, a recent study by Xie et al. (2020) has reported that oral treatment with *Lactobacillus reuteri* 3 has anti-depressive effects through increasing the expression of enzymes involved in 5-HT biosynthesis, but inhibiting that of the KP enzymes, including IDO in the colon and PFC in mice with depression-like symptoms induced by chronic social defeat stress.

Limitations

Despite following certain bibliometric principles and comprehensive analysis strategies, our present study has some inevitable limitations. First, only English articles and reviews published within a particular period of time from the WoSCC and Scopus databases were used, thus potentially leading to language and publication biases. For example, although the retrieval time scope was sufficiently long to reflect the research trends in our field of focus, some critical, seminal original papers that gave rise to this field may be missing. These papers, such as those first describing the compounds involved in the KP, formed the basis of the research of the authors cited; thus, historical background information may need to be added in these types of bibliometric analysis. Additionally, in some cases, the identified researchers might not have published original discoveries but instead published large numbers of reviews, which were then cited frequently. This is a well-recognized anomaly in the citation analysis system that may yield misleading results regarding the contributions to the field. Second, only some specific terms referring to the main KP metabolites were included in our retrieval strategy, and consequently, the publications retrieved may contain possible false positives and false negatives, given that no search query is 100% perfect (Sweileh et al., 2017). Third, to date, there is a lack of adherence to internationally accepted ethical standards in bibliometric analysis, and the limitations of analytic tools may provide a subjective view of individual work and contributions. Therefore, we emphasize that the information generated and discussed has no relationship to the nature, originality or importance of the work, because it includes information consisting largely, or sometimes entirely, of reviews of original work by other people. Despite these limitations, given the sufficiently large number of collected documents in the present analysis, we believe that our findings provide a more comprehensive picture of research on the KP in mood disorders, especially involving gut microbiota research, which may help provide an instructive perspective on the current research and direct future research in this field.

CONCLUSION

This is the first bibliometric analysis to comprehensively evaluate the general aspects of, and future trends in, the field of the KP in mood disorders, particularly the gut microbiota research in this field. Overall, we found rapid growth in publications in this field since 2006, which was generally favored by high-IF journals in the field of neuropsychiatry. More widespread global collaboration may be necessary, particularly for China. The most important and most recent research focuses were the "kynurenine pathway" and "gut-brain axis," respectively, reflecting the research foundations in this field. Journal co-citation analysis showed a strong tendency toward co-citation of research in the field of psychiatry. Reference co-citation analysis revealed the key role of the KP in bridging the gut microbiota and the host immune system, and gut microbiota research has become the most recent focal point in the field of the KP in mood disorders. The largest research hotspot has become "quinolinic acid," appearing in 2006. Future research should focus on the topics of "Alzheimer's disease," "prefrontal cortex," and "acid," which were identified as research frontiers in this field. These timely analysis results provide a new glimpse into research on the KP in mood disorders, particularly focusing on the microbiota-gut-brain axis in this field, and may help researchers choose suitable journals for publication, find potential collaborators, and understand hotspots and frontiers, thus promoting the development of this field.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DS and YW together conceived and designed the study. XZ wrote the original draft preparation. JH and HL were responsible for data collection. XN, LL, and MZ performed the data analyses. CQ conducted the data interpretation. SH, TX, SL, and XL provided the figures. ZW, SD, YT, and YL reviewed and edited the manuscript mainly.

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What Is Our Understanding of the Influence of Gut Microbiota on the Pathophysiology of Parkinson's Disease?

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Microbiota have increasingly become implicated in predisposition to human diseases, including neurodegenerative disorders such as Parkinson's disease (PD). Traditionally, a central nervous system (CNS)-centric approach to understanding PD has predominated; however, an association of the gut with PD has existed since Parkinson himself reported the disease. The gut–brain axis refers to the bidirectional communication between the gastrointestinal tract (GIT) and the brain. Gut microbiota dysbiosis, reported in PD patients, may extend this to a microbiota–gut–brain axis. To date, mainly the bacteriome has been investigated. The change in abundance of bacterial products which accompanies dysbiosis is hypothesised to influence PD pathophysiology *via* multiple mechanisms which broadly centre on inflammation, a cause of alpha-synuclein (a-syn) misfolding. Two main routes are hypothesised by which gut microbiota can influence PD pathophysiology, the neural and humoral routes. The neural route involves a-syn misfolding peripherally in the enteric nerves which can then be transported to the brain *via* the vagus nerve. The humoral route involves transportation of bacterial products and proinflammatory cytokines from the gut *via* the circulation which can cause central a-syn misfolding by inducing neuroinflammation. This article will assess whether the current literature supports gut bacteria influencing PD pathophysiology *via* both routes.

Keywords: Parkinson's, microbiota, microbiome, bacteria, gut, dysbiosis, neurodegenerative, gut–brain

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. Insoluble intracytoplasmic protein aggregates, primarily consisting of misfolded fibrillar alpha-synuclein (a-syn), are PD neuropathological hallmarks. These aggregates [Lewy bodies (LBs) and Lewy neurites (LNs)], are believed to cause the catecholaminergic (CA) and dopaminergic (DA) neuronal loss which manifests as motor dysfunction (parkinsonism). Since PD motor symptoms are not evident until approximately 60–70% of DA neurons in the substantia nigra pars compacta (SNpc) are lost (Dauer and Przedborski, 2003), the gut–brain hypothesis may allow earlier interventions to be made before the motor system becomes affected.

The microorganisms in the gut are involved in gastrointestinal (GI) homoeostasis, such as maintaining the integrity of the gut epithelial barrier (Miraglia and Colla, 2019), and their abnormal colonisation and function (dysbiosis) can lead to peripheral and/or systemic inflammation (Chen et al., 2019) which may facilitate PD pathophysiology by neural and humoral routes.

EVIDENCE FOR GUT INVOLVEMENT IN PARKINSON'S DISEASE

Gastrointestinal symptoms have been shown to precede motor symptoms by Parkinson himself (Parkinson, 2002). Non-motor symptoms are thought to predate motor symptoms by approximately a decade and increase the risk of developing PD (Abbott et al., 2001; Adams-Carr et al., 2016). Prolonged colonic transit time is present in up to 80% of PD patients (Jost, 1997) with significantly higher constipation incidence also reported compared to healthy controls (HCs) (Edwards et al., 1991; Chen et al., 2015). Gut microbiota have been implicated since they can aid in host nutrient metabolism and modulate gastrointestinal motility (Miraglia and Colla, 2019).

Pathophysiological Evidence

The characteristic Lewy bodies (LBs) have also been observed in the enteric nervous system (ENS) of PD patients (Wakabayashi et al., 1988; Shannon et al., 2012b; Gold et al., 2013). Braak et al. (2003) defined the caudo-rostral axis in the brain along which a-syn pathology progresses and identified LBs and LNs in the dorsal motor nucleus of the vagus (DMV) in PD post mortem brain samples. This implicated the vagus nerve, and later the ENS (Braak et al., 2006), in the spreading of PD pathology. Braak's hypothesis states that retrograde transport of misfolded a-syn occurs from projection neurons in the ENS to the central nervous system (CNS), *via* the vagus nerve. The initial a-syn misfolding was posited to be induced in the enteric nerves by an exogenous, neurotropic pathogen (Lionnet et al., 2018). Indeed, other studies have corroborated this hypothesis (Shannon et al., 2012a; Stokholm et al., 2016).

NEURAL AND HUMORAL ROUTES FOR A-SYNUCLEIN MISFOLDING AND AGGREGATION

Dysbiosis may explain the association between PD and risk factors known to influence microbiome composition, such as pesticides and diet (Gorecki et al., 2019; Gubert et al., 2020). Studies in PD, discussed below, propose that changes in bacterial products accompanying dysbiosis could contribute toward the observed inflammation in PD patients (Devos et al., 2013; Chen et al., 2019). Indeed, functional increases in intestinal epithelial barrier permeability (Davies et al., 1996; Kelly et al., 2014) and decreased expression of intestinal barrier tight junctions (Edelblum and Turner, 2009; Clairembault et al., 2015; Perez-Pardo et al., 2019) in PD have been attributed to inflammation. This increased permeability would allow translocation of bacteria

and their products into the lamina propria, triggering further inflammation. Both neural and humoral routes converge on inflammation which, *via* oxidative stress, forms one way in which a-syn misfolding can occur (Hashimoto et al., 1999; Lema Tomé et al., 2013). Local inflammation facilitates peripheral a-syn misfolding which propagates to the brain *via* the neural route. Systemic inflammation induces neuroinflammation in the brain (Mogi et al., 1994) *via* the humoral route which causes a-syn misfolding. A-syn can induce further oxidative stress, forming a positive feedback loop which leads to neurodegeneration in the brain (Dias et al., 2013; Chen et al., 2019).

Neural Route

Braak's hypothesis forms the basis of the neural route. Dysbiosis in the gut lumen leads to a-syn aggregation in enteric nerves. Indirectly, inflammation increases intestinal barrier permeability and hence mucosal inflammation, from translocation of bacteria and their products, which facilitates a-syn misfolding (Forsyth et al., 2011; Lema Tomé et al., 2013; Kelly et al., 2014). Directly, enteroendocrine cells (EECs) may propagate misfolded a-syn from the lumen, along their neuropods, to enteric nerves *via* functional synapses (Chandra et al., 2017). This may explain how Braak's luminal exogenous pathogen could directly cause a-syn aggregation in the enteric nerves without violating the gut epithelium. Peripheral misfolded a-syn is proposed to be transported in a prion-like way between neurons, passing from the enteric nerves to the vagus nerve to the brain, where a-syn forms intracytoplasmic aggregates (Visanji et al., 2013). Exogenous a-syn fibrils enter neurons (Volpicelli-Daley et al., 2011), seeding intracellular a-syn aggregation both *in vitro* (Luk et al., 2009) and *in vivo* (Kordower et al., 2011; Holmqvist et al., 2014; Okuzumi et al., 2018), and upon exiting these neurons restarts the process (Lee et al., 2005).

Various animal models have supported Braak's hypothesis. Vagus nerve-mediated translocation of a-syn aggregations from the gut to the brain was observed after injections of human recombinant a-syn from PD patients into rats' intestinal walls (Holmqvist et al., 2014), or the peritoneal cavity of a-synuclein overexpressing (ASO) mice (Breid et al., 2016). Furthermore, injection of preformed fibrils (PFFs) into mouse gastrointestinal tracts (GITs) led to DA neuronal loss in the SNpc and motor symptoms after 7 months, with a-syn accumulation in anatomical locations associated with more advanced Braak's stages (Kim et al., 2019). In this study, truncal vagotomy in the PFF-injected mice prevented a-syn spreading to the brain and protected against loss of DA neurons. Decreased risk of PD with truncal vagotomy has also been observed in patients (Svensson et al., 2015; Liu et al., 2017).

Humoral Route: Dysbiosis and Inflammation

Bacterial products and proinflammatory cytokines in the systemic circulation trigger neuroinflammation, inducing central a-syn misfolding through oxidative stress (Hashimoto et al., 1999; Lema Tomé et al., 2013). Although mechanistically distinct to the neural route, the direction of pathology transfer remains the

same. The first study to link dysbiosis with PD demonstrated significant differences in faecal bacterial taxa between PD patients and HCs (Scheperjans et al., 2015). Many more case–control studies, using faecal samples, have followed (**Table 1**). Although heterogeneity between results exists, the consensus is that the dysbiosis observed represents a shift toward a proinflammatory profile.

Short-chain fatty acids (SCFAs) are produced by GI bacteria when anaerobically fermenting dietary fibres. These SCFAs (in particular butyrate, propionate and acetate) have anti-inflammatory effects both locally and systemically (Millard et al., 2002; Dalile et al., 2019). Reductions in the butyrate-producing families Lachnospiraceae and Prevotellaceae and bacterial genera such as *Blautia*, *Roseburia*, *Coprococcus*, and *Faecalibacterium prausnitzii* are most commonly found (**Table 1**). Decreased faecal SCFA concentrations in PD have been documented (Unger et al., 2016), which could increase local inflammation and in turn peripheral a-syn misfolding, facilitating the neural route. Since SCFAs can maintain the integrity of the intestinal barrier (Wang et al., 2012), their reduction could increase gut barrier permeability, facilitating the passage of other bacterial products and proinflammatory cytokines into the circulation, thereby engaging the humoral system (Dalile et al., 2019). Moreover, in normal physiology unmetabolised SCFAs can reach the systemic circulation and cross the blood–brain barrier (BBB) (Mitchell et al., 2011), following the humoral route, meaning that a lack of SCFAs could directly contribute to neuroinflammation. However, reports of SCFA-producing bacteria abundance are contradictory: *Prevotella* is decreased across studies whilst *Akkermansia* is increased, despite both containing mucin-degrading species (**Table 1**). This same pattern has been identified in multiple sclerosis (Freedman et al., 2018). Decreased *Prevotella* levels may reflect a lack of mucin synthesis, linked to increased barrier permeability (Bullich et al., 2019). Indeed, butyrate stimulates mucin synthesis (Brown et al., 2011) and putative-butyrate-producing (pBP) bacteria, such as *F. prausnitzi* and *Roseburia*, are consistently decreased (**Table 1**). *Akkermansia* may function as a double-edged sword: although mucin degradation is pro-inflammatory, decreased mucin levels could negatively feedback and increase other bacteria's mucin synthesis (Bullich et al., 2019). Moreover, *Akkermansia* converts mucin degradation products into SCFAs (Derrien et al., 2004). Therefore, without pBP bacteria decreases, increased *Akkermansia* could be anti-inflammatory; however, decreased pBP bacteria abundance could cause net mucin degradation and increased barrier permeability.

Molecular H2 is another bacterial fermentation product which could be affected by dysbiosis. H2 has anti-inflammatory and antioxidant properties (Ostojic, 2018). Reduced intestinal H2 production in PD, through decreases of *Clostridium* and *Prevotella* and species such as *Bacteroides fragilis* (**Table 1**), may compromise the function of tissues which use it (such as DA neurons) (Ostojic, 2018). Indeed, motor symptoms in rodent lesion models of PD were prevented by H2S inhalation and systemic administration of NaHS (an H2S donor), and DA neuronal loss was reduced (Hu et al., 2010; Kida et al., 2011). Therefore, a decrease in H2 might predispose to DA neuronal loss and hence PD pathology *via* the humoral route.

The bacterial endotoxin, LPS, is also implicated in PD pathogenesis and may arise from the enrichment of Gram-negative-rich phyla such as Proteobacteria and Verrucomicrobia (**Table 1**). Increased TLR4 (LPS-specific receptor) expression in PD colonic biopsies (Perez-Pardo et al., 2019) and decreased serum LPS-binding protein (LBP) concentrations in PD (Forsyth et al., 2011; Hasegawa et al., 2015), also indicated LPS involvement. Functional evidence comes from rotenone-treated TLR4-KO mice which, compared to rotenone-treated WT mice, had reduced inflammation (intestinal and of the brain) and dysfunction (intestinal and motor) (Perez-Pardo et al., 2019). LPS can subvert the intestinal epithelial barrier both indirectly, through induced proinflammatory cytokines, and directly (Forsyth et al., 2011; Pawłowska and Sobieszczańska, 2017). LPS-induced inflammation in the lamina propria facilitates the neural route and, by entering the systemic circulation, LPS can directly participate in the humoral route. Moreover, LPS can disrupt the BBB (Kortekaas et al., 2005; Banks and Erickson, 2010), and in the brain can activate microglial CD14/TLR4/LBP complexes (Rivest, 2003). This creates a positive feedback cycle whereby microglia release proinflammatory cytokines, causing neuroinflammation which results in neuronal death and release of a-syn which then binds to TLR4 and/or TLR2 to further activate microglia and astroglia (Fellner et al., 2013; Kim et al., 2013). This process can mediate DA neuronal loss in the SN *via* oxidative stress (Qin et al., 2007; Dias et al., 2013).

Bacterial amyloids are increasingly being implicated in PD pathology *via* the neural route or exacerbating existing pathology *via* the humoral route. Extracellular amyloid fibres, such as curli, are produced by bacterial species including *Escherichia coli* (Römling et al., 1998; Hufnagel et al., 2013). Since bacterial amyloids can cross-seed amyloids from other bacterial species to induce aggregation, they may also cross-seed human a-syn in enteric nerves for propagation *via* the vagus nerve (Santos et al., 2019). Evidence for this comes from a study which exposed aged rats with human-a-syn-expressing-*Caenorhabditis elegans* to curliated *E. coli*: rats had increased a-syn inclusions in the gut and brain, accompanied by neuroinflammation (Chen et al., 2016). Furthermore, administration of curliated *E. coli* to ASO mice produced motor defects and GI dysfunction in addition to increasing gut and brain a-syn aggregation (Sampson et al., 2020).

A role for bacterial amyloids in PD would presumably require a significant increase in amyloid-synthesising bacteria. Although none of the studies summarised in **Table 1** investigated amyloid-synthesising bacteria, consistent enrichment of *E. coli* is observed, though without changes in other amyloid-producing bacteria such as *Streptococcus mutans*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*. This potential discrepancy may be resolved by hypotheses that bacterial amyloids from different bacterial species induce cross-seeding in a strain-specific manner, i.e., solely on a-syn (Friedland and Chapman, 2017), meaning that even small quantities of bacterial amyloids could initiate pathology.

Bacterial amyloids and human oligomeric a-syn are recognised by the host immune system *via* the gut mucosal TLR2/TLR1 heterocomplex (Tükel et al., 2010;

TABLE 1 | Table presenting results of statistically significant ($p < 0.05$) changes in abundance of bacterial taxa in faecal samples between Parkinson's disease patients (PD) and healthy controls (HCs).

Studys	Increased in PD patient faeces	Decreased in PD patient faeces
Scheperjans et al. (2015)	Family: Lactobacillaceae, Verrucomicrobiaceae, Bradyrhizobiaceae, Ruminococcaceae	Family: Prevotellaceae, Clostridiales i.s. IV
Keshavarzian et al. (2015)	Phylum: Bacteroidetes, Proteobacteria, Verrucomicrobia Family: Bacteroidaceae, Clostridiaceae, Verrucomicrobiaceae Genus: *Akkermansia*, *Oscillospira*, *Bacteroides*	Phylum: Firmicutes Family: Lachnospiraceae, Coprobacillaceae Genus: *Blautia*, *Coprococcus*, *Dorea*, *Roseburia*
Hasegawa et al. (2015)	Genus: *Lactobacillus*	Species: *Bacteroides fragilis*, *Clostridium coccoides*, *Clostridium leptum*
Unger et al. (2016)	Family: Enterobacteriaceae Genus: *Bifidobacterium*	Phylum: Bacteroidetes Family: Lactobacillaceae, Enterococcaceae Species: *Faecalibacterium prausnitzii*
Bedarf et al. (2017)	Phylum: Firmicutes, Verrucomicrobiaceae Genus: Unclassified *Firmicutes*, *Akkermansia*	Family: Erysipelotrichaceae, Prevotellaceae Genus: *Eubacterium*, *Prevotella*
Hill-Burns et al. (2017)	Family: Bifidobacteriaceae, Christensenellaceae, Lactobacillaceae, Tissierellaceae, Verrucomicrobiaceae Genus: *Akkermansia*, *Lactobacillus*, *Bifidobacterium*	Family: Lachnospiraceae, Pasteurellaceae
Hopfner et al. (2017)	Family: Lactobacillaceae*, Barnesiellaceae, Enterococcaceae	n/a
Petrov et al. (2017)	Genus: *Bifidobacterium*, *Catabacter*, *Christensenella*, *Lactobacillus*, *Oscillospira*	Genus: *Bacteroides*, *Dorea*, *Faecalibacterium*, *Prevotella*
Li et al. (2017)	Phylum: Proteobacteria, Actinobacteria Family: Enterobacteriaceae, Veillonellaceae, Erysipelotrichaceae, Coriobacteriaceae, Streptococcaceae, Moraxellaceae, and Enterococcaceae Genus: *Acidaminococcus*, *Acinetobacter*, *Enterococcus*, *Escherichia–Shigella*, *Megamonas*, *Megasphaera*, *Proteus*, *Streptococcus*	Phylum: Bacteroidetes Genus: *Blautia*, *Faecalibacterium*, *Ruminococcus*
Heintz-Buschart et al. (2018)	Phylum: Verrucomicrobia Class: Verrucomicrobiae Order: Verrucomicrobiales Genus: *Akkermansia*	n/a
Lin et al. (2018)	Family: Eubacteriaceae, Bifidobacteriaceae, Aerococcaceae, Desulfovibrionaceae	Phylum: Firmicutes, Tenericutes, Euryarchaeota Family: Streptococcaceae, Methylobacteriaceae, Comamonadaceae, Halomonadaceae, Hyphomonadaceae, Brucellaceae, Xanthomonadaceae, Lachnospiraceae, Actinomycetaceae, Sphingomonadaceae, Pasteurellaceae, Micrococcaceae, Intrasporangiaceae, Methanobacteriaceae, Idiomarinaceae, Brevibacteriaceae, Gemellaceae
Qian et al. (2018)	Genus: *Clostridium IV*, *Sphingomonas*, *Holdemania*, *Clostridium XVIII*, *Butyricicoccus*, *Anaerotruncus*, *Aquabacterium*	n/a
Barichella et al. (2019)	Phylum: Proteobacteria, Verrucomicrobia Family: Enterobacteriaceae, Verrucomicrobiaceae, Bifidobacteriaceae, Christensenellaceae, Coriobacteriaceae, Lactobacillaceae Genus: *Akkermansia*	Family: Lachnospiraceae
Li et al. (2019)	Family: Ruminococcaceae, Verrucomicrobiaceae, Porphyromonadaceae, Hydrogenoanaerobacterium, Lachnospiraceae NK4A	Family: Bacteroides, Prevotellaceae
Pietrucci et al. (2019)	Family: Lactobacillaceae, Enterobacteriaceae, Enterococcaceae	Family: Lachnospiraceae
Vidal-Martinez et al. (2020)	Family: Verrucomicrobiaceae Genus: *Akkermansia*	n/a
Ren et al. (2020)	PD-MCI (mild cognitive impairment) Vs. PD-NC (normal cognition) and HC: Genus: *Blautia, Ruminococcus* PD-NC vs. PD-MCI and HC: Family: Rikenellaceae Genus: *Alistipes*, *Barnesiella*, *Butyricimonas*, *Odoribacter*	n/a
Zhang et al. (2020)	Phylum: Firmicutes, Actinobacteria, Verrucomicrobia Genus: *Oscillospira*, *Akkermansia*	Phylum: Bacteroidetes, Fusobacteria Genus: *Fusobacterium*

Further statistical analysis rendered change non-statistically significant.

Nishimori et al., 2012; Hufnagel et al., 2013; Kim et al., 2013; Daniele et al., 2015). This would initiate a local and central immune response against endogenous a-syn (Lindestam Arlehamn et al., 2020), creating a proinflammatory environment which could facilitate a-syn misfolding (Hufnagel et al., 2013; Friedland and Chapman, 2017; Miraglia and Colla, 2019).
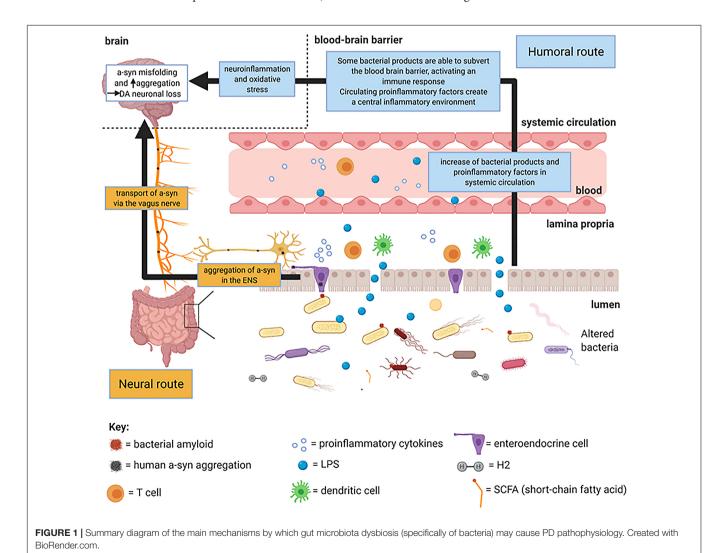
HOW USEFUL ARE THESE STUDIES?

Some common criticisms can be applied to these studies. Methodological differences, some of which are highlighted in **Supplementary Table 1**, may explain some of the heterogeneity between results. Many studies did not identify potential confounders in statistical analyses which may have resulted in false positive outcomes. For example, dietary differences between PD and HC groups, which could account for differential microbial composition (Graf et al., 2015), were not assessed. Sample handling methods (not listed), such as the time period between collection and freezing of samples, also varied greatly but were not considered as potential confounders (Haikal

et al., 2019). Future studies need to agree on a standardised protocol, with more stringent inclusion/exclusion criteria, to increase the reproducibility and hence the reliability of the reported results.

Cause or Consequence?

It is difficult to discern whether dysbiosis is a cause or consequence of PD. Although two longitudinal studies have been conducted in PD patients, neither have reported significant changes in microbial composition with progression (Minato et al., 2017; Aho et al., 2019). Support for a causative role comes from the association between PD risk and inflammatory conditions where dysbiosis is also reported, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Lai et al., 2014; Lin et al., 2016; Mertsalmi et al., 2017). Evidence for the ENS controlling microbial composition, with the CNS modulating these ENS signals (Rolig et al., 2017), suggests that dysbiosis is a consequence of PD. This substantiates the hypothesis that loss of central DA neurons initiates DMV degeneration which results in GI inflammation

FIGURE 1 | Summary diagram of the main mechanisms by which gut microbiota dysbiosis (specifically of bacteria) may cause PD pathophysiology. Created with BioRender.com.

and hence dysbiosis (Ulusoy et al., 2017; Rolli-Derkinderen et al., 2020). However, the decreased PD risk after vagotomy would suggest pathology originating from a region innervated by the vagus nerve, such as the GIT, refuting the CNS-centric hypothesis (Svensson et al., 2015; Liu et al., 2017; Kim et al., 2019). These opposing arguments could potentially be reconciled by an emerging theory which suggests the existence of different subtypes of PD: "PNS-first" or "CNS-first" (Borghammer and Van Den Berge, 2019). If a-syn pathology starts in the PNS in a subset of patients, early interventions to manipulate gut microbiota could be performed to halt the progression of neurodegeneration before motor system involvement. Faecal microbiota transplantation (FMT) studies in humans have produced varying results: whilst Xue et al. (2020) found improvements in clinical scoring scales (such as UPDRS and NMSS), Huang et al. (2019) did not but noted constipation alleviation. These studies' sample sizes and follow-up periods are too small to draw conclusions from, however, a larger clinical trial is currently ongoing (Santens, 2021 – NCT03808389). Probiotics administration in PD patients produced significant improvements (i.e., decreases in MDS-UPDRS score) (Tamtaji et al., 2019) which has been supported by DA neuron neuroprotective effects and decreased motor impairment observed after probiotic administration in a PD mouse model (Hsieh et al., 2020).

Seminal evidence for the role of GIT microbiota in PD pathogenesis comes from a study of germ-free (GF) ASO mice by Sampson et al. (2016). These mice had reduced a-syn pathology load, microglial activation and motor symptoms compared to specific pathogen-free (SPF) ASO mice, implicating gut microbiota in causing PD pathophysiology. Moreover, GF-ASO mice transplanted with faecal microbiota from human PD donors developed exacerbated motor symptoms compared to those given HCs (Sampson et al., 2016). This translates the associative evidence from **Table 1** into causal evidence.

SUMMARY OF GUT MICROBIAL MECHANISMS UNDERLYING PD PATHOPHYSIOLOGY

The routes linking gut microbiota to PD pathophysiology are illustrated in **Figure 1**. The change in SCFA- and H2-producing bacteria, which accompanies dysbiosis, initiates a local proinflammatory environment which triggers a-syn misfolding peripherally in the gut, thus facilitating the neural route. Bacterial amyloids may also induce peripheral a-syn misfolding independently of inflammation. Peripheral misfolded a-syn can be transported along the enteric nerves *via* the vagus nerve to the brainstem. Once in the brain, a-syn progresses along Braak's caudo-rostral axis. Local inflammation from dysbiosis can also increase the permeability of the gut brain barrier, allowing bacterial products to enter the lamina propria which can generate further inflammation. Some of these bacterial products (e.g., LPS)

and proinflammatory cytokines can enter the circulation from the lamina propria, acting *via* the humoral route by generating neuroinflammation which causes a-syn misfolding.

CONCLUSION

Microbiota undoubtedly play a role in PD pathophysiology. Associative evidence from case–control studies and functional evidence from animal models have provided support for microbiota causing PD *via* the neural and humoral routes. PD is primarily considered a disease of old age, despite pathogenesis preceding motor symptoms by years, with dysbiosis conceivably acting to exacerbate inflammation. Whilst previously gut dysbiosis was only considered as a consequence of PD, it is now also accepted that bacterial products may influence PD pathology through creating peripheral and systemic inflammatory environments, increasing both peripheral a-syn transport to the brain and neuroinflammation (Perez-Pardo et al., 2017). However, it is still not clear what initially triggers dysbiosis nor how inflammation would selectively cause a-syn aggregation rather than, for example, aggregation of Aβ in Alzheimer's disease since inflammation is an underlying feature of many neurodegenerative diseases. Other factors are most likely implicated, such as gene mutations (e.g., in clearance mechanisms for misfolded a-syn), since not every ageing person develops PD or a neurodegenerative disease (Tran et al., 2020).

Understanding the exact mechanisms by which dysbiosis could lead to the neuropathological hallmarks of PD will require case–control studies to shift from predominantly bacterial abundance measures to whole metagenome sequencing (**Supplementary Table 1**) which provides data on functional changes in the microbiota as well as the levels of other microbes such as yeasts and viruses (Scheperjans, 2016). Studies which look set to strengthen evidence for gut bacteria involvement are investigating the effects of antibiotic administration on PD risk, since antibiotics alter gut bacteria composition (Mertsalmi et al., 2020).

AUTHOR CONTRIBUTIONS

AH and PB made an equal contribution to the conceptualisation, drafting of the manuscript, scientific content proof-reading. AH drafted the final version of the document and constructed the accompanying figure and table. RW-M provided a significant contribution to clinical content and proof-reading. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins. 2021.708587/full#supplementary-material

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Impending Mental Health Issues During Coronavirus Disease 2019 – Time for Personalized Nutrition Based on the Gut Microbiota to Tide Over the Crisis?

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Coronavirus disease 2019 (COVID-19) is a major pandemic facing the world today caused by SARS-CoV-2 which has implications on our mental health as well. The uncertain future, fear of job loss, lockdown and negative news all around have taken a heavy toll on the mental health of individuals from across the world. Stress and anxiety can affect the COVID-19 patients even more. Recent study suggests COVID-19 infection may lead to post-traumatic stress disorder (PTSD). Certain prebiotics and probiotics have been shown to have anxiolytic effect through gut microbiota modulation. Incidentally, preliminary report also suggests a differential microbial profile in COVID-19 patients as compared to healthy individuals. Gut microbiota's role in anxiety and depression is well studied. The importance of the "gut-brain" axis has been implicated in overall mental health. It is known that diet, environmental factors and genetics play an important role in shaping gut microbiota. Trials may be initiated to study if personalized diet and supplementation based on individual's gut microbiome profile may improve the general mental well-being of people prone to anxiety during this pandemic. Also, COVID-19 patients may be provided personalized nutritional therapy based on their gut microbiota profile to see if PTSD and anxiety symptoms can be alleviated.

Keywords: COVID-19, mental health, gut microbiome, nutrition, precision medicine

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a raging pandemic causing a widespread disruption of normal life. Although, the first case was reported in the Hubei province of China in late 2019 yet it has spread to many countries in the world (Wang et al., 2020). Apart from the clinical symptoms that this disease manifest, it has now been reported that COVID-19 infection might lead to post-traumatic stress disorder (PTSD; Rogers et al., 2020). Even the healthcare workers who are at the forefront of managing this disease have reported an increased prevalence of anxiety, depression, and insomnia (Pappa et al., 2020). Not only COVID-19 is affecting the mental health of patients and the frontline healthcare workers, this pandemic is taking a heavy mental health toll on people from across the world (**Figure 1**). The uncertainties of the future, the job losses, the extended lockdown and the overall negative environment all around is causing a massive jump in the number of anxiety

and depression cases in the world (Taquet et al., 2021). In a recent research study in India, it was found that the adolescents who were quarantined and children experienced more psychological distress than the non-quarantined children and adolescents (Saurabh and Ranjan, 2020). Another international study covering 9565 people across 78 countries found people affected with low or moderate mental health during COVID-19, suggesting a major impact of the pandemic on mental well-being of majority of people across the world (Gloster et al., 2020).

Stress can trigger development of anxiety and depressive-like behavior (Yang et al., 2015). The current pandemic situation with lockdowns, quarantine processes, and misinformation can all add to the stress levels. The prevailing therapeutic options for treating mood disorders like anxiety and depression include the tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and the classical benzodiazepines (Busato et al., 2006). However, all of these drugs take a few days to weeks to show their affect and may not show positive effects in many and in few cases can cause adverse effects causing compliance issues (Balikci et al., 2014). Hence, it is imperative that alternatives that can be used alone or in conjunction with the current therapies should be studied and implemented. Herbal nootropics like Ashwagandha or *Withania somnifera*, certain prebiotics and probiotics have been shown to have anxiolytic effect *via* modulation of the gut microbiome (Peterson et al., 2019). Prebiotics are defined as non-digestible dietary fibers that can confer benefit to the host gut health by selectively stimulating growth of indigenous health promoting bacteria (Simpson and Campbell, 2015). Similarly, probiotics are defined as live non-pathogenic microorganisms like bacteria and yeast that exhibit beneficial health effects to the host when consumed in adequate amount (Cukrowska et al., 2009). Alterations in bacterial community metabolism as a consequence of medicinal herb-driven prebiotic may contribute to therapeutic efficacy (Peterson et al., 2019). Differential gut microbiota profile has been found in patients with general anxiety disorder (GAD) and major depressive disorder (MDD) (Jiang et al., 2018; Winter et al., 2018). Although, studies in animal models suggest a causal role of the gut microbiota in accentuating such mental conditions, more research and studies are needed to completely understand the mechanisms. COVID-19, with its impending effect on the psychological health of both the patients and millions of individuals worldwide, provides the necessary impetus to the scientific and the medical community to delve deeper into the role of the gut microbiota in depression and anxiety. This article is an effort to drive in the fact that gut microbiota-based nutritional supplementation solutions need to be looked into in greater depth as the current crisis has left open a deep gap in the health infrastructure especially dealing with mental health issues. In the subsequent paragraphs, I would try to highlight the current understanding with respect to the role of the gut microbiota in mental health conditions such as anxiety and depression and also the possibility of personalized nutrition solutions based on the gut microbiota profile of an individual to address the issues of mental well-being in COVID-19 times. This approach may be suitable for both the patients who are affected by the disease (COVID-19) and the general population who are vulnerable to anxiety and depression especially due to the conditions in this current pandemic.

BRAIN, GUT, AND THE MICROBIOME – CURRENT UNDERSTANDING

There is a complex communication between the microorganisms in the gastrointestinal tract and the peripheral and the central nervous system (CNS). The gut consists of the enteric nervous system (ENS) that can act independently of the brain and the spinal cord (Furness, 2012). The gut microbiota are the major component of the "gut-brain axis" that includes various pathways that enable communication between the intestine or the gut and the CNS (Misiak et al., 2020). The human intestinal microbiota consists of 10^{14} microorganisms including bacteria, fungi, viruses, and archaea (Gill et al., 2006). The intestinal bacteria in healthy individuals is dominated by four phyla belonging to Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes (Villanueva-Millán et al., 2015). The gut microbiota of adult humans is dominated by members of the Firmicutes and the Bacteroidetes phyla (Hall et al., 2017). The intestinal microbiota plays a key role in health and wellness through its protective, and metabolic actions (Cani, 2018).

"Gut dysbiosis" or alterations of gut microbiota have been shown to be associated with various diseases and disorders like inflammatory bowel disease (IBD; Khan et al., 2019), type 2 diabetes (Gurung et al., 2020), cardiovascular disease (Tang et al., 2017), and even mental disorders like depression and anxiety (Zalar et al., 2018; Peirce and Alviña, 2019). Incidentally, preliminary report also suggests a differential microbial profile in COVID-19 patients as compared to healthy individuals (Gu et al., 2020; Zuo et al., 2020). Hence, it is important to study the effect of such dysbiosis in mental health conditions of COVID-19 patients. This is mainly because gut dysbiosis has been implicated in depression and anxiety (Clapp et al., 2017). More so, when COVID-19 patients and healthcare providers have shown bouts of depression, anxiety, and stress (Pfefferbaum and North, 2020; Rogers et al., 2020).

The bidirectional "gut-brain axis" communication is known to involve neural (Vagus nerve and ENS), immune (cytokine), and endocrine [cortisol and hypothalamic–pituitary–adrenal (HPA) axis] pathways (Mörkl et al., 2020). Studies in germ-free (GF) mice have shown that gut microbiota are essential for development of neuronal circuits, anxiety behavior, and social responses (Das et al., 2017).

The Hypothalamic–Pituitary–Adrenal Axis – Modulation by the Gut Microbiota

The intestinal microbiota may determine the stress responsivity by modulating the HPA axis (Peirce and Alviña, 2019). The HPA axis gets initiated in the hypothalamus region of the brain by the synthesis of corticotrophin-releasing hormone (CRH). CRH then stimulates the production of the adrenocorticotrophic hormone (ACTH) in the pituitary gland which then leads to
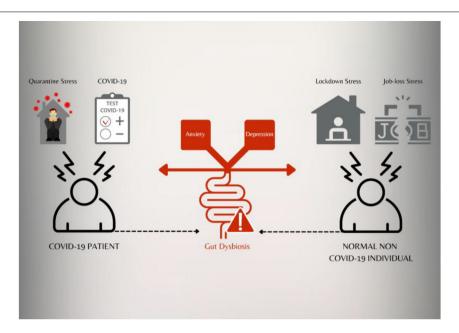
FIGURE 1 | Coronavirus disease 2019 itself and various stressors may impact the gut microbiota which can lead to gut dysbiosis. Gut dysbiosis may be implicated in anxiety and depression both in COVID-19 patients and healthy individuals exposed to various stressors.

release of the glucocorticoids from the adrenal cortex. Higher stress levels are known triggers of anxiety and depression (Rea et al., 2016). Research and studies on GF mice have shed a lot of light on the role of the gut microbiota in influencing the HPA axis. A pivotal study reported that GF mice have an overreactive HPA axis which leads to increased concentrations of ACTH and corticosterone following a stressful stimulus (Sudo et al., 2004). Other studies have shown a differential gene expression pattern in GF mice as compared to the control mice especially in the regions of hippocampus and cortex, which most likely influence the difference in HPA axis activity (Heijtz et al., 2011; Bellavance and Rivest, 2014). It has been shown that various stressors or stressful stimuli may impact the abundance of *Lactobacilli*, *Bacteroides*, and *Clostridium* in animal models affecting barrier integrity as well (Misiak et al., 2020). Studies have shown that probiotics based on *Bifidobacterium* and *Lactobacillus* species restore stress-induced HPA axis dysfunction and improve depression- and anxiety-like symptoms (Eutamene et al., 2007; Desbonnet et al., 2010). The intestinal microbiota can influence the HPA axis by increase in levels of cytokines, release of LPS and peptidoglycan (cell wall components of the bacteria) and by short chain fatty acid (SCFA) production (Misiak et al., 2020). Interestingly, it has been found that abnormal activation of the HPA axis impacts microbial colonization (Pellissier and Bonaz, 2017). Also, evidence from animal model studies points to the fact that stress-related HPA axis response may increase the intestinal permeability (Vicario et al., 2012). Although, less is known about a cross-talk between the HPA axis and the gut microbiota in major depression, a previous study showed transplantation of the gut microbiota from patients with depression to GF mice was associated with the development of anxiety- and depression-like phenotype. This was accompanied by a down-regulation

of the Stat5a gene in the hippocampus region of the brain (Luo et al., 2018). Interestingly, the Stat5a gene is known to regulate the HPA axis response. Furthermore, a murine model of depression has been reported to overproduce CRH indicating a hyper active HPA axis (Park et al., 2013). Although many preclinical studies have included GF and antibiotic treated mice, it is suggested that such treatment or microbiota free environment might lead to certain changes at the cellular/organ level and hence there are certain caveats that need to be kept in mind (Kennedy et al., 2018).Taken together, the evidence suggests the role of the gut microbiota in influencing the HPA axis that might lead to development of the "anxiety-depression" state. Conversely, stress mediated HPA activation can also affect the intestinal microbiota which again can lead to dysregulation of HPA axis and other pathways leading to the above disease conditions. Considering the various stressors in the current COVID-19 pandemic, it is tempting to speculate that this might be impacting the HPA axis and hence the role of the gut microbiota in such a scenario becomes important to study.

Inflammation and the Immune System – Possible Role of the Gut Microbiota

The interactions of the host with the microbes are complex and is also bidirectional. The intestinal microbiota are thought to regulate the development and function of the innate and adaptive immune system (Negi et al., 2019a). Intestinal micro-organisms secrete antimicrobial peptides such as bacteriocins, compete for the nutrients and the habitat site thereby aiding in the state of homeostasis (Moens and Veldhoen, 2012). GF mice studies have provided important insights into the role of the resident microbiota in host immune system development. For

example, GF mice have been shown to have an under-developed mucosal immune system. Also, GF mice have been found to have lesser number of regulatory T cells (Tregs) with reduced anti-inflammatory activities (Strauch et al., 2005). In the CNS, the microglia are a kind of immune cells that post activation can release lot of cytokines and chemokines, regulate neurotransmitters and can undergo morphological changes (Rea et al., 2016). Stress induces glucocorticoid (cortisol in humans) secretion and it is interesting to note that glucocorticoid receptors are expressed abundantly on microglia throughout the brain (Sierra et al., 2008). Cytokines release post microglia activation have been found to have a role in behavioral phenotype in stress models (Kreisel et al., 2014). The gut microbiota and immune homeostasis are intertwined and this relationship is also a domain of great interest and research. It is suggested that decrease in gut diversity and change in the normal gut microbiota profile may alter normal immune function (Dhar and Mohanty, 2020). Also, signals derived from the intestinal microorganisms can tune the immune cells for pro and anti-inflammatory responses thereby affecting the susceptibility to various diseases (Negi et al., 2019b). It is documented that the immune gut homeostasis is controlled by the intestinal microbiota by the fine tuning of the regulatory balance of pro-inflammatory responses such as Th17 versus anti-inflammatory Tregs (Round and Mazmanian, 2010). The role of inflammation in depression is well documented in various studies. In fact, genetic variants in various immune related genes have been implicated in depression (Barnes et al., 2017). Studies have also reported correlation between higher pro-inflammatory cytokines and depression in humans (Lamers et al., 2013). It is suggested that breach in gut barrier integrity may lead to the translocation of bacteria

and bacterial antigens (such as lipopolysaccharides) into the blood stream causing chronic low-grade inflammation (Mörkl et al., 2020). Stress increases intestinal permeability in several animal models (Yu et al., 2013). This may lead to endotoxins and other harmful bacteria to seep into the circulation causing an immune reaction and inflammation. Many animal studies have shown that administration of endotoxins peripherally causes global expression of pro-inflammatory cytokines in the brain (Peirce and Alviña, 2019). The other mechanisms through which peripheral inflammation spread to the brain and cause neuro-inflammation can be by sending inflammatory signals to the brain by afferent nerves, activated immune cells migrating to the brain and cytokines crossing the blood brain barrier (Peirce and Alviña, 2019).

The Vagus Nerve and the Gut Microbiota – Is There a Connection?

The Vagus nerve innervates a large proportion of the body's digestive tract and is known to be responsive to a number of endogenous chemicals emanating in the digestive tract (Bonaz et al., 2018). The Vagus nerve is known to relay signals from the brain to the viscera. Interestingly, approximately 80% of Vagus nerve fibers are afferent, relaying sensory information from the viscera, including the digestive tract, to the brain to maintain homeostasis (Winter et al., 2018). Microbiota secretions can activate Vagal afferents which then signal to the hypothalamic regions of the brain (Forsythe et al., 2014). Evidence suggests that for probiotics to mediate beneficial effects in anxiety and depression, intact Vagus nerve is required. Example, a study revealed that mice with inflammation in the intestine that

TABLE 1 | Select studies with nutritional supplements that modulate the gut microbiota in treating COVID-19.

Study	Evaluation	Clinical trial identifier
Modulation of gut microbiota to enhance health and immunity of vulnerable individuals during COVID-19 pandemic	Double-blinded, randomized, active-placebo controlled study for evaluation of the efficacy of modulating the gut microbiota with a specific probiotic composition (3 bifidobacteria, 10 billion cfu per sachet) in COVID-19 patients with comorbidity like type 2 diabetes and elderly	NCT04884776
An exploratory, open label, clinical study to evaluate the physiologic effects of KB109 in adult patients with mild-to-moderate COVID-19 on gut microbiota structure and function in the outpatient setting	Evaluation of the modulatory effect of the glycan KB109 in mild to moderate COVID-19 patients	(Haran et al., 2021); NCT04486482
Modulation of gut microbiota with NBT-NM108 as an early treatment for suspected or confirmed symptomatic COVID-19 patients	Open labeled, randomized, and controlled clinical trial for evaluation of a novel botanical based fixed combination drug – NBT-NM 108 in modulating the gut microbiota and treat early stage COVID-19 patients	NCT04540406
The intestinal microbiota as a therapeutic target in hospitalized patients with COVID-19 infection	Prospective case-control pilot study for evaluation of a specific probiotic (1 billion cfu) mix to improve outcome in COVID-19 patients	NCT04390477
Multicentric study to assess the effect of consumption of *Lactobacillus coryniformis* K8 on healthcare personnel exposed to COVID-19	Preventive study to evaluate the effect of consumption of the probiotic *Lactobacillus coryniformis* K8 (3 billion cfu) in incidence and severity of COVID-19 in health workers exposed to the virus	NCT04366180
Evaluation of the efficacy of probiotics to reduce the duration and symptoms of COVID-19 (PROVID-19 study): a randomized, double-blind, controlled trial	Randomized controlled trial to evaluate the efficacy of probiotics (2 strains 10×10^9 UFC) to reduce the duration and symptoms of COVID-19	NCT04621071
Efficacy of probiotics in treatment of hospitalized patients with novel coronavirus infection	A randomized controlled open-label study for evaluation of probiotics *L. rhamnosus* PDV1705 (1 billion cfu*)*, *Bifidobacterium bifidum* PDV 0903 (1 billion cfu), *B. longum* PDV 1911 (1 billion cfu), *B. longum* PDV 2301 (1 billion cfu) in treatment of patients hospitalized with COVID-19	NCT04854941
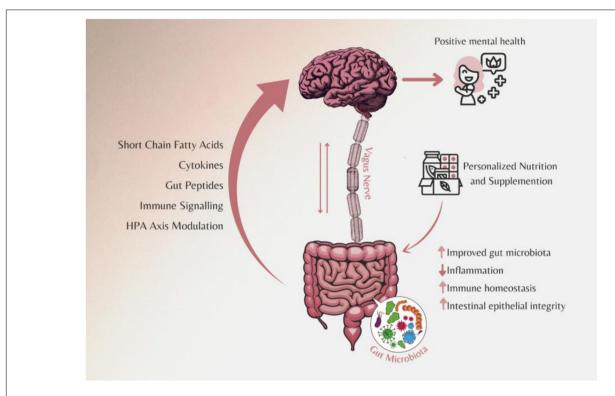
FIGURE 2 | Personalized nutritional strategies including diet, prebiotics, and probiotics combination based on the individual's gut microbiome can improve mental health conditions.

normally exhibited anxiety-like behavior showed less anxiety symptoms when treated with *Bifidobacterium longum*; however, this anxiolytic effect was not observed in mice in which the Vagus nerve was severed (Bercik et al., 2011). Similar observation was found with the probiotic *Lactobacillus rhamnosus* in mice (Bravo et al., 2011). Incidentally, the anxiolytic property of the probiotic *B. longum* has also been observed in humans (Allen et al., 2016). It is also possible that pathogenic microbes might modulate Vagal afferents causing subsequent pathologic changes in the CNS, which may then lead to anxiety/depression like diseases (Winter et al., 2018). Similarly, there are multiple studies conducted in mice which suggest the role of the vagus nerve in depressive behavior (Pu et al., 2021; Wang et al., 2021). Taken together, the evidences point out to the possible role the gut microbiota may play in modulating the Vagus nerve thereby influencing the mental diseases like depression and anxiety.

DIET AND PROBIOTICS/PREBIOTICS – NEED A PERSONALIZED APPROACH

Intestinal microbiota is considered malleable and can be modulated by diet, medication, lifestyle, environment, etc. The type of food that we eat is known to play an important role in shaping the composition of the gut microbiota. Diet is found to influence the specific compositional patterns of the gut microbiota based on the nutritional components of the food like, e.g., the different composition of the microbiota

with animal fat and protein-based diets versus vegetable-based diets (De Filippis et al., 2016). Systemic stress and chronic inflammation can also differentially affect the gut microbiota thereby proving that environmental factors along with diet can modulate the composition of the gut microbiome (Earley et al., 2015). Dietary fats, particularly trans, and saturated fats, are known to transiently increase intestinal inflammation (Okada et al., 2013). This in turn alters gut microbial population by increasing pathogenic and decreasing commensal taxa (Das et al., 2017). High fat, low fiber diet is also known to decrease gut microbiome diversity (Simpson and Campbell, 2015). Therefore, it is important to have a balanced diet rich in diverse and plant-based products which would likely lead to a more diverse, balanced and resilient microbiome composition in the gut. This would eventually have an impact on the mental health of the individual as well. In the context of COVID-19, it was found that specific formulation of probiotics containing various strains of *Streptococcus thermophilus* DSM 32345, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helvetics* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, *Lactobacillus brevis* DSM 27961, *Bifidobacterium lactis* DSM 32246, *B. lactis* DSM 32247 reduced the risk of progression to severe COVID-19 in patients treated with the special probiotic formulation as compared to the patients not given the oral bacteriotherapy (d'Ettorre et al., 2020). Although the psychological parameters were not evaluated in this study yet this study provided the proof of concept of the role of the probiotics in improving the outcome in COVID-19

patients. Few more studies are being conducted to find the effect of nutritional supplements known to modulate the gut microbiota in treating COVID-19 (**Table 1**). In COVID-19 context therefore, one of the ways by which stressors can be neutralized is by following a diet and supplement intake based on the individual's gut microflora.

The term "Psychobiotics" now define all microbiota targeting interventions including prebiotics and probiotics that can influence bacteria-brain relationship (Sarkar et al., 2016). Many preclinical studies have investigated the role of prebiotics and probiotics in mental health. A study has reported that a combination of prebiotics, fructooligosachharide (FOS) and galactooligosaccharide (GOS) attenuated anxiety related behavior in mice (Rea et al., 2016). Similarly, administration of GOS and polydextrose to rats prevented anxiety and depression like behavior (Mika et al., 2017). Many preclinical studies have illustrated positive role of probiotics in anxiety and depression. Colonizing GF mice with *Bifidobacterium infantis* stabilizes their overreactive HPA axis in response to restraint stress and returns their stress hormone levels to normal as observed in control mice (Sudo et al., 2004). Resilience to stress is another aspect where probiotics have been shown to have a positive effect in preclinical studies. It was found that *Bifidobacterium* might play a role in resilience in mice subjected to chronic social defeat stress (Yang et al., 2017). Probiotics administration has been shown to improve the intestinal integrity thereby decreasing its permeability and reducing endotoxemia. For example, mice undergoing water avoidance stress display increased intestinal permeability however treating them with *Lactobacillus farciminis* improved gut barrier integrity and conferred epithelial and mucosal barrier strengthening (Da Silva et al., 2014). Such effects of probiotics in gut barrier integrity strengthening has been observed in humans as well. For instance, a mix of *L. rhamnosus* and *Lactobacillus reuteri* was found to reduce small intestinal permeability in children with eczema (Rosenfeldt et al., 2004). Thus, certain probiotics decrease intestinal epithelial permeability and thereby lowering the risk of endotoxemia and uncontrolled inflammation. This may have positive effects on anxiety and depression. Many probiotics have also found to have positive effects on neuroinflammation. *L. farciminis* administration to mice suppressed stress-induced neuroinflammation during partial restraint stress (Ait-Belgnaoui et al., 2014). Lot of probiotics such as *Bifidobacterium breve*, *Lactobacillus helveticus* NS8, *L. rhamnosus*, and *B. longum* have shown anxiolytic effects in preclinical models (Peirce and Alviña, 2019). Similar benefit of probiotics has been observed in many human studies. For example, *L. helveticus* and *B. longum* probiotic mix given to healthy human volunteers for 30 days reduced psychological distress in comparison to a control group (Messaoudi et al., 2011). Significant reduction in depression scores were found in one study where the patients were administered *B. longum* (Pinto-Sanchez et al., 2017).

Although many studies with probiotics have shown promising results in clinical trials, still some discrepancy has been observed which might be due to different strains being used. For an effective treatment with such "psychobiotics," it is also important to combine this with a balanced diet that would provide adequate micro and macro nutrients along with fibers. Hence, personalized nutrition with a mix of probiotics, prebiotics, and diet based on the individual's gut microbiota may be more effective in dealing with such conditions (**Figure 2**).

CONCLUSION AND FUTURE PERSPECTIVE

Coronavirus disease 2019 has impacted everyone in the world. The world is grappling with not only the infectious nature of the disease but there is an even bigger danger looming in the background and that is the impending mental health crisis. Various meta-analysis of COVID-19 patients, frontline healthcare workers, etc., have pointed to the fact that psychological ailments like anxiety, depression, and PTSD affect them and this poses a challenge for the healthcare community (Pappa et al., 2020; Rogers et al., 2020). This along with the fact that every individual who is not infected by SARS-Cov2 virus in this planet is also affected mentally indirectly by this disease, shows the importance of tackling this crisis. Relying on the current therapies, although effective for some and with many side effects, may not be the right approach. COVID-19 has given an opportunity to the scientific and medical community to address the mental health domain by utilizing and improving on the knowledge of the gut microbiome that might provide newer strategies to counter such ailments. As evidenced, stress may lead to intestinal dysbiosis and increased gut permeability. This can lead to peripheral inflammation that can lead to neuronal inflammation in the brain. Empirical data both preclinical and clinical, suggest important role, gut microbiota might play in the mental well-being. However, there are few challenges that also need to be addressed. More studies to delineate the causal role of the microbiota in mental health need to be performed. Secondly, the role of fungi, phages should also be looked into, as much of the focus has been on bacteria. There have been few conflicting results in human trials with respect to probiotics use in alleviating depression and anxiety (Mörkl et al., 2020). This may be due to different strain of the probiotics being used in the study. Also, it is a possibility that probiotics might not work in the same way in all individuals. Host genetics, diet and colonization potential of the probiotics may also play a significant role. Hence one size might not fit all with respect to specific probiotics and prebiotics in countering anxiety and depression. Additional bigger trials in diverse population are needed to define efficacy, treatment duration, adverse effects, and dosage. Future trials might also include some aspects of genotyping to probe the effect of certain genes in probiotic colonization and their efficacy. In the current COVID-19 context, trials with personalized nutrition and supplements based on individual's gut microflora may be initiated to check if that can improve the mental well-being of the patients both during and post recovery. The fact that there is a high burden of depression symptoms in adults especially with lower income category of society during COVID-19 pandemic suggests an overhaul of the ways by which this disease needs to be treated (Ettman et al., 2020). Overall, personalized gut microbiome based nutritional strategies, if adopted by people

affected by stress and anxiety due to the prevailing environment of COVID-19 and COVID-19 patients themselves, can improve the mental well-being and might act as an alternate mode to assist the mental healthcare infrastructure which is so inadequate in developing countries.

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.

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Clostridium butyricum RH2 Alleviates Chronic Foot Shock Stress-Induced Behavioral Deficits in Rats via PAI-1

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Recent investigations have demonstrated that the chronic stress-induced behavioral disorders can be ameliorated by probiotics including *Clostridium butyricum* (C. butyricum) via the gut-brain-axis. However, the molecular mechanisms underlying the beneficial effects of C. butyricum on brain remain largely unknown. Here, we investigated whether chronic foot shock stress (CFSS) paradigm used for a hypertensive animal model could induce mood disorders such as anxiety, depression and cognitive impairments. Then, we assessed the impact of *C. butyricum* RH2 on the behavior disorders and neurobiological alterations in the hippocampus. Male Sprague-Dawley (SD) rats received intermittent electric shocks for consecutive 14 days and were treated with *C. butyricum* RH2 for 17 days. Anxiety- or depression-like behaviors were evaluated by open field test (OFT), and elevated plus maze (EPM). The Morris water maze test (MWM) was used to evaluate the cognitive functions. CFSS intervention led to mild anxiety- or depression-like behavior or cognitive impairment and *C. butyricum* RH2 treatment reversed the CFSS-induced symptoms. The serum ACTH or CORT was increased following CFSS but was completely reversed by *C. butyricum* RH2 treatment. In the hippocampus of CFSS rats, the expressions of BDNF and TrkB were downregulated but proBDNF and P75[NTR] were upregulated. These expression changes were partially reversed by *C. butyricum* RH2, suggesting a mode of action on BDNF and proBDNF balance. CFSS exposure resulted in downregulation of tissue-type plasminogen activator (tPA) but upregulation of plasminogen activator inhibitor 1(PAI-1), which could contribute to the decrease in BDNF by reduced conversion from proBDNF to BDNF in the hippocampus. *C. butyricum* RH2 treatment reversed the upregulated PAI-1 but not the downregulated tPA, which was in parallel with the amelioration of behavioral abnormalities, suggesting a novel tPA independent mechanism for PAI-1 action. Our results demonstrate for the first time that *C. butyricum* RH2 attenuates stress-induced behavior disorders via inhibiting the expression of brain PAI-1.

Keywords: *Clostridium* butyricum RH2, stress, mood disorders, gut-brain axis, hippocampus, BDNF

1 INTRODUCTION

Humans live in a stressful environment and are constantly exposed to unavoidable stressors. Different stressors can provoke a series of behavioral, emotional or physiological responses in animals (Gold, 2015). The stress-induced maladaptive responses are determined by the nature (physical or psychological), intensity and duration of the stressor. Electric foot shocks have been widely used for the development of various animal models of human disorders such as hypertension, anxiety, depression and post-traumatic stress disorder (PTSD) by introducing subtle variations in current intensity, duration, number of shock exposures and post-exposure treatment (Bali and Jaggi, 2015; Kim et al., 2017; Schöner et al., 2017). In the animal model of stress-induced hypertension, rats received intermittent electric shocks (Wu et al., 2020), but behavioral changes of rats exposed to this chronic foot shock stress (CFSS) paradigm remains unknown.

Chronic stress not only induces behavioral disorders, but also results in gut dysbiosis in animals and humans (Bharwani et al., 2016; Gao et al., 2018; Tian et al., 2020). Mounting evidence suggests that the gut microbiome is involved in mood disorders (Cruz-Pereira et al., 2020; Rutsch et al., 2020). Analysis of gut microflora in depressed patients or rodents has revealed a significant difference from that in healthy controls (Jiang et al., 2015; Zheng et al., 2016). Fecal microbiota transplantation from depressed patients induced depression-like behavior in the microbiota-deficient rodents and the depressed recipient animals exhibited disturbances of gut microbiota (Kelly et al., 2016; Zheng et al., 2016). Mice given the microbiota from the chronically stressed mice showed higher levels of anxiety- and depression-like behavior compared to the controls (Bruce-Keller et al., 2015; Li et al., 2019). Vice versa, fecal microbiota transplantation from the health donors to the stressed recipient rats ameliorated stress-induced depression-like behaviors (Rao et al., 2021). Based on these findings that gut dysbiosis is causally linked to the deleterious effects of stress (Cryan et al., 2020), restoration of gut microbiota homeostasis by probiotic intervention has gained a great attention in recent years (Rutsch et al., 2020). The beneficial effects of probiotics on the chronic stress-induced behavioral disorders, cognitive impairments, neurochemical abnormalities by reshaping the disturbed gut microbe in rodents have been shown by supplementation of Bifidobacterium breve CCFM1025(Tian et al., 2020), Bifidobacterium breve M2CF22M7 (Tian et al., 2019a), Bifidobacterium longum (Han and Kim, 2019), Bifidobacterium longum CCFM687 (Tian et al., 2019b), Faecalibacterium prausnitzii ATCC 27766 (Hao et al., 2019), Lactobacillus helveticus NS8 (Liang et al., 2015), Lactobacillus casei (Gu et al., 2020), Lactobacillus kefiranofaciens ZW3 (Sun et al., 2019), respectively. Clostridium butyricum (C. butyricum) is a butyric acid-producing Gram-positive anaerobe in the gut. The C. butyricum is a well-known probiotic and has been widely investigated for a potential usage in a wide range of human diseases (Stoeva et al., 2021). Recently, experimental studies have revealed that the administration of C. butyricum -specific

strains including Miyairi 588 or WZMC1018 significantly improved depressive-like behavior in the chronically stressed mice through the gut-brain axis (Sun et al., 2018; Tian et al., 2019a). The mechanisms underlying the role of C. butyricum in alleviating the stress-induced psychiatric disorders include an increase of the 5-HT levels and BDNF expression in the hippocampus, suppression of pro-inflammatory cytokines and inhibition of hippocampal microglial activation (Sun et al., 2018; Tian et al., 2019a). In addition, C. butyricum can stimulate the secretion of intestinal GLP-1 which may stimulate the up-regulated GLP-1R in the hippocampus of the stressed mice (Sun et al., 2018). However, the antidepressive mechanism of C. butyricum remains largely unknown.

Brain derived neurotrophic factor (BDNF) plays an important role in learning and memory formation. Decreased expression of BDNF and upregulated proBDNF (precursor to BDNF) in brain have been implicated in the stress-induced mood disorders (Bai et al., 2016; Sun et al., 2018; Yang et al., 2020; Lin et al., 2021). BDNF can be converted from proBDNF by plasmin which is cleaved from plasminogen by tissue-type plasminogen activator (tPA). Plasminogen activator inhibitor 1(PAI-1) can inhibit tissue-type plasminogen activator (tPA), resulting in the accumulation of proBDNF. PAI-1 is widely expressed in the brain. The expression of PAI-1 was increased in the prefrontal cortex and hippocampus of the chronically stressed rats (Tang et al., 2015), suggesting that PAI-1 up-regulation may play a key role in the pathological process of depression (Tang et al., 2015; Jiang et al., 2016; Han et al., 2019).
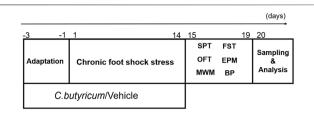
C. butyricum RH2 has been found to exert anti-inflammatory effect through correction of gut microbiota dysbiosis induced by an antibiotic (Li et al., 2021). However, the beneficial role of C. butyricum RH2 in alleviating the stress-induced behavioral disorders or cognitive impairment and the underlying mechanisms has not yet been explored.

In the current study, we investigated whether the CFSS paradigm used for hypertensive animal model led to the development of behavioral disorders associated with anxiety, depression and cognitive impairments. Furthermore, we assessed the beneficial effects of C. butyricum RH2 on the behavior disorders and also examined whether C. butyricum RH2 exerted anxiolytic effects through decreasing the expression of PAI-1 in hippocampus of CFSS rats, aiming to explore a novel therapeutic approach for alleviation of the stress-induced mood impairments.

2 MATERIALS AND METHODS

2.1 Animal Preparation

Male adult Sprague-Dawley (SD) rats (200–250 g) used in this study were purchased from the Shanghai Jiesijie Experimental Animal Co., Ltd. (SCXK (hu) 2018–0004). The rats were housed in a temperature-controlled room (22 ± 1°C) under a 12 h light-dark cycle with food and water ad libitum. All procedures were approved by the Animal Care and Use Committee of Jiangsu Vocational College of Medicine (Ethics review form NO.2019201).

FIGURE 1 | The schematic diagram of the experiments. SD rats were pretreated with *C. butyricum* RH2 or vehicle for 3 days before receiving chronic foot shock stress for 14 days, and the rats treated with *C. butyricum* RH2 or vehicle for 17 days in total. Between days 15 and 20, the rats were given behavioral tests (15th–16th day:SPT; 17th–19th day:FST, OFT, EPM; 15th–20th day:MWM). Blood and brain specimens were collected on the 20th day for further measurement and analysis. SPT, sucrose preference test; FST, forced swim test; OFT, open field test; EPM, elevated plus maze test; MWM, Morris water maze; BP, blood pressure.

Rats were randomly assigned into four groups: sham, stress, stress + *C. butyricum* RH2 (C.b) and positive control (Reserpine) groups, with 7 rats in each group. After 1 week of acclimatization, Stress + *C. butyricum* RH2 group rats were given *C. butyricum* RH2 (1 × 10^9 CFU/ml/day/rat) by gastric gavage (*C. butyricum* RH2 was kindly shared by Chongqing Taiping Pharmaceutical Co., LTD., P. R. China) once a day for consecutive17 days (3 days before the stress and 14 days during the stress). *C. butyricum* RH2 solution was freshly prepared before administration. Freeze-dried bacteria powder of C. butyricum RH2 containing 4 × 10^9 colony forming units (CFU)/g was adjusted in 4 ml sterilized saline to a final concentration of 1 × 10^9 CFU/ml. The dose of *C. butyricum* RH2 used in the present study was based on previous studies (Sun et al., 2018; Li et al., 2021). Rats of stress group were given the same volume of saline by gastric gavage. On day 3, rats of stress + *C. butyricum* RH2 group and stress group were individually placed in a cage (22 cm × 22 cm×28 cm) with a grid floor and exposed to electric foot-shock combined with buzzer noise stress administered for 2 h twice a day, with a 4-h interval between the sessions, for consecutive 14 days (Wu et al., 2020). Control rats were also individually placed in a similar cage but without receiving stressors (electric shocks and noises). Then, behavioral tests were performed. After the behavioral experiments, blood and brain specimens of the experimental rats were taken out for further analysis. A single and repeated administration (once daily for 14 days) of reserpine ((Sigma-Aldrich, USA, 0.2 mg/kg i. p.) as the positive control group for depressive disorders (Antkiewicz-Michaluk et al., 2014).

Food intake was recorded every day throughout the experiment in each group of rats. Food consumed was recorded by subtracting the residual food from the food given (more than daily eaten) on the previous day. Body weight was monitored three times starting at the onset, on the 7th day and 14th day of CFSS. Body weight gain (Δ body weight) was calculated by subtracting the starting body weight from body weight on the 14th day.

The details of the experimental design conducted in this study are shown in **Figure 1**.

2.2 Behavioral Assessments

Behavioral alterations of CFSS rats were tested by the well established methods including sucrose preference test (SPT), forced swim test (FST), open field test (OPT), elevated plus maze test (EPM), and Morris water maze (MWM). The devices were provided by Anhui Zhenghua Biological Instrument Equipment Co. LTD. All behavioral tests were videotaped and data were analyzed by ANY-maze animal behavior analysis system developed by Stoeling, United States.

2.2.1 Sucrose Preference Test

SPT was used for the measurement of anhedonic-like behavior. Before the experiment, rats were trained to drink sucrose solution (2% w/v) for 24 h. After being deprived of food and water for 12 h, each rat was given a bottle of sucrose solution and a bottle of drinking water for free intake. Two hours later, consumption of sucrose solution and drinking water were measured respectively. The animals' sucrose preference (SP) was calculated as following formula: SP = sucrose solution consumed/ (sucrose solution consumed + water consumed) × 100%.

2.2.2 Forced Swim Test

FST was used for the measurement of behavioral despair (giving up hope of escape). Rats were placed individually in a glass cylinder (50 cm in height and 20 cm in diameter) filled with 35 cm depth of water (23 ± 1°C). Before the experiment, the rats were put into the behavior laboratory to adapt to the environment for 1 h. During the test, each rat was gently placed in water and adapted for 2 min. Then, the sum of the time that the rat remained motionless in the water in the following 4 min was recorded. After the test, the rat was dried with a dry towel and placed in a warm place. After 20 min, it was moved back to its original cage. After 24 and 48 h, the rat received the second and the third test session, respectively. The average immobility time of the three trials was analyzed.

2.2.3 Open Field Test

OFT was used to measure the spontaneous exploratory behavior and curiosity of animals to new environments. The apparatus for OFT was composed of a 50 × 50 × 40 cm^3 open box. A camera attached to the recording system was placed above the box. Real-time images of rats were analyzed by the system to obtain the behavior data of the rats in open field. The bottom of the open box square was divided into nine squares of equal size, and the middle one was defined as the central area. Before the experiment, the rat was put into the behavior laboratory to adapt to the environment for 1 h. In a quiet environment, the rat was put into the central square at the bottom of the open field and left to move freely for 5 min. The following indexes were observed and recorded: the total movement distance, the movement distance in central area and average movement speed. The box was cleaned with 75% alcohol and dried between the tests. The environment remained quiet during the entire procedure.

2.2.4 Elevated Plus Maze Test

EPM is often used to assess an animal's anxiety or exploratory behavior in a new environment. The experimental device was

elevated 70 cm from the ground, and was composed of two open arms (122.3 cm) and two closed arms (110.5 cm) with the central area (12×12 cm^2). It was perpendicular to each other in a cross shape of "+." A camera was placed above the joint between the open arm and the closed arm. The experiment was performed in a dim light environment. Before the experiment, the rats were put into the behavior laboratory to adapt to the environment for 1 h. At the beginning of the experiment, the rat was placed at the central junction with the head facing an open arm, and allowed to move freely for 5 min. When all four legs of the rat entered the open/closed arm, one entry was recorded. The percentage of times the rats entered the open arm and the percentage of time they stayed in the open arm were calculated to evaluate the emotional state of the rats. Between the experiments, each rat was cleaned of residues in the box to remove the smell of the previous rat, so as not to affect the behavior of the next rat.

2.2.5 Morris Water Maze

The Morris water maze test was performed to assess the memory and spatial learning functions of animals. A hidden platform with a diameter of 12 cm was maintained in II quadrant of a circular basin, 1–2 cm below the surface of water ($23 \pm 1°$C). The place navigation task lasted for 5 days. Rats were placed into the water from four entry points facing the wall every day, and the time of finding the platform (escape latency) was recorded. In addition to observing and recording the escape latency, the swimming track, total swimming distance and average swimming speed in the water were also observed and recorded. After the positioning navigation experiment, spatial probe test was performed to assess memory consolidation by removing the platform. Each rat was placed into the water from four different quadrants. The crossing number of the former platform position and time spent crossing the former platform quadrant were recorded in a period of 60 s.

2.3 Western Blot

The protocol of western blot was the same as that previously reported [Wu et al., 2020]. Briefly, 200 μl of pre-cooled extraction reagent was added to the rat Hip sample. The extraction reagent was pre-added with 2 μl of protease inhibitor, 10% PMSF, and phosphatase inhibitor. Protein concentration was measured by the BCA method. 10% separation gel and 60 V constant voltage were first used, then 120 V constant voltage was used for electrophoresis until the bromophenol blue color reached the bottom of the separation gel. Subsequently, proteins were transferred onto a PVDF membrane that was washed with TBST solution 3 times, 5 min each time. The membrane was then blocked with 5% milk in PBS-Tween 20 for 1 h, and incubated overnight at 4°C with mouse anti-GFAP (3670s, Cell Signaling Technology, United States; 1:1,000), rabbit anti-BDNF (ab108319, Abcam, United Kingdom; 1:1,000), rabbit anti-TrkB (ab33655, Abcam, United Kingdom; 1:1,000), rabbit anti-p75[NTR] (ab52987, Abcam, United Kingdom; 1:1,000), anti-Pro-BDNF (sc-65513, SANTA CRUZ, USA; 1:200), mouse anti-β-Actin (AF0003, Beyotime, China; 1:1,000) antibodies, rabbit anti-PAI-1 (ab66705, Abcam, United Kingdom; 1:1,000) and rabbit anti-tPA (10147-1-AP, Proteintech, China, 1:1,000), respectively. Then, the membrane was washed 3 times (10 min each time),

incubated with anti-rabbit or anti-mouse secondary antibodies at room temperature for 60 min, washed, immersed in ECL solution for about 1 min, and photographed with a gel imaging system. The bands were quantified by ImageJ software.

2.4 Immunofluorescence Staining

The rat brain samples were fixed (4% paraformaldehyde, PFA), gradient sugar deposition (10, 20, 30% sucrose), and then coronal sections (35 mm in thickness) of the Hip were sliced using a freezing microtome (CM 1850; Leica, Germany). Sections were incubated in rabbit anti-PAI-1 (ab66705, Abcam, United Kingdom; 1:200) and rabbit anti-tPA (10147-1-AP, Proteintech, China, 1:50), 4°C overnight. After the sections were washed with 0.01 PBS, they were incubated with goat anti-mouse IgG H&L (Alexa Fluor® 488) (ab150113, Abcam, United Kingdom; 1:200) or goat anti-rabbit IgG H&L (Cy3®) preadsorbed (ab6939, Abcam,United Kingdom; 1:200) as secondary antibody at room temperature for 2 h. A laser scanning confocal microscope (LSM 900, Zeiss, Germany) was used to observe the positive cells.

2.5 Enzyme-Linked Immunosorbent Assay

After the rats were decapitated, blood samples were collected, centrifuged, and the serum was stored at −20°C. The serum norepinephrine (NE), adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels were measured using an ELISA kit (Weiao, Shanghai, China). All samples and standards were measured in duplicate.
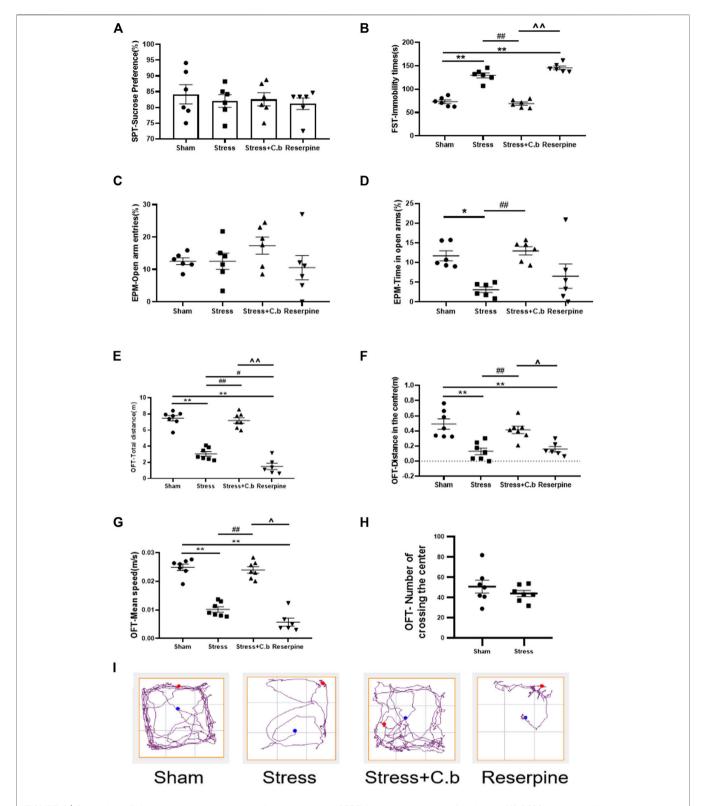
2.6 Statistical Analysis

Data are presented as mean±the standard error of the mean (SEM). All statistical analysis was performed using GraphPad Prism 8.0, compared by one-way ANOVA followed by the Tukey post hoc test. $p < 0.05$ was considered statistically significant.
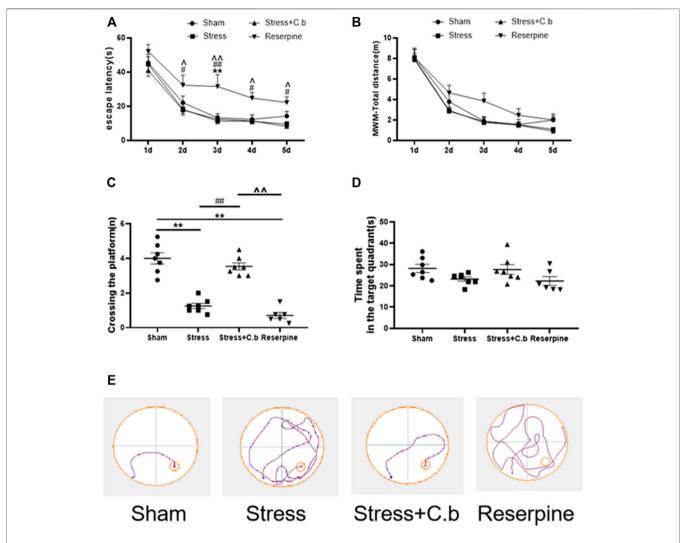
3 RESULTS

3.1 C. butyricum RH2 Treatment Improved CFSS-Induced Anxiety- and Depression-like Behaviors

The effects of chronic administration of C. butyricum RH2 on anxiety-like behaviors were evaluated in open field test (OFT), and elevated plus maze (EPM). Compared to rats in the sham group, anxiety-like behaviors were significantly induced in rats subjected to CFSS, as reflected by a decrease in the percentage time spent in open arm in the EPM (**Figure 2D**, $p < 0.05$), and the reduction in total distance, central distance or mean speed in the OFT (**Figures 2E–G**, $p < 0.01$). The number of events crossing the center area in the OFT was not significantly different between CFSS rats and control rats (**Figure 2H**, $p > 0.05$), suggesting that CFSS did not affect the locomotor activity. Rats exposed to CFSS also exhibited significant depression-like behaviors, as reflected by the increased immobility time in the forced swim test (FST) (**Figure 2B**, $p < 0.01$). However, CFSS exposure was without effects on the percentage of open arm entries in the EPM test (**Figure 2C**, $p > 0.05$) and sucrose consumption in the sucrose preference test (SPT) (**Figure 2A**,

FIGURE 2 | C. butyricum RH2 treatment improved chronic foot shock stress (CFSS)-induced behavioral deficits in rats. **(A)** CFSS treatment did not induce a decrease in the sucrose consumption. **(B)** C. butyricum RH2 improved the increased immobility time induced by CFSS. **(C)** CFSS treatment did not change open arm entries. **(D)** C. butyricum RH2 prolonged CFSS-induced decrease in time spent in the open arm time. **(E–G)** C. butyricum RH2 lengthened the central area distance, the total distance and the average speed in CFSS rats. **(H)** The number of crossing the center area in the OFT was not significantly different between CFSS rats and control rats ($p > 0.05$). **(I)** Typical track in OFT. The data are expressed as mean ± SEM (n = 7 in sham, stress, stress + C. butyricum group; n = 6 in reserpine group). *$p < 0.05$, **$p < 0.01$ versus sham group; #$p < 0.05$, ##$p < 0.01$ versus stress group; ^$p < 0.05$, ^^$p < 0.01$ versus stress + C. butyricum group.
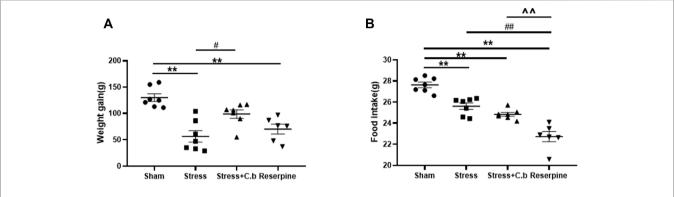
FIGURE 3 | C. butyricum treatment ameliorated the cognitive deficits in the CFSS rats by reversing the decreased number of crossing in target quadrant **(C)** but revealed no effects on the escape latency **(A)**, the total distance **(B)** and time spent in the target-quadrant **(D)**. **(E)** The representative locus plot in the MWM test. The data are expressed as mean ± SEM (n = 7 in sham, stress, stress + C. butyricum group; n = 6 in reserpine group). *$p < 0.05$, **$p < 0.01$ versus sham group; #$p < 0.05$, ##$p < 0.01$ versus stress group; ^$p < 0.05$, ^^$p < 0.01$ versus stress + C. butyricum group.
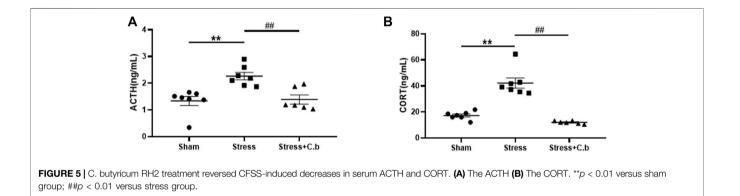
$p > 0.05$). Seventeen days of C. butyricum RH2 treatment reversed the CFSS-induced anxiety-like symptoms (**Figure 2D**, $p < 0.01$; **Figures 2E–G**, $p < 0.01$; **Figure 2H**, $p < 0.01$) and the depression-like behaviors (**Figure 2B**, $p < 0.01$). Reserpine was used as positive control and reserpine treatment induced anxiety-and depression-like behaviors as expected, which is in line with CFSS exposure. Typical tracks in OFT in sham-, stress-, stress + C. butyricum RH2—and reserpine treated animals are shown in **Figure 2I**, respectively.

3.2 C. butyricum RH2 Treatment Ameliorated CFSS-Induced Cognitive Deficits

The Morris water maze test was carried out for 5 days to evaluate the effects of 17 days C. butyricum RH2 treatment on the cognitive functions. During the five consecutive training days,

all rats learned to find the submerged platform and the escape latency were gradually shortened. Two-way ANOVA of the escape latency revealed no significant difference between sham, CFSS and CFSS + C. butyricum groups (**Figure 3A**, $p > 0.05$) but a significantly longer escape latency in reserpine group (**Figure 3A**, $p < 0.05$ or 0.01, respectively). Two-way ANOVA of the total escape distance revealed no significant difference between any of the groups (**Figure 3B**, $p > 0.05$). In the probe trails, the number of platform crossings in CFSS treated rats was significantly decreased compared to that in sham rats and the decrease was significantly reversed by C. butyricum RH2 treatment (**Figure 3C**, $p < 0.01$). The time spent in the target quadrant was not significantly different between the groups regardless of a trend of decline in CFSS or reserpine groups (**Figure 3D**, $p > 0.0.5$). The representative locus plots of **Figures 3A,B** are shown in **Figure 3E**.

FIGURE 4 | C. butyricum RH2 treatment reversed CFSS-induced decrease in weight gain but not the decrease in food intake. **(A)** The weight gain **(B)** The food intake. $**p < 0.01$ versus sham group; #$p < 0.05$, ##$p < 0.01$ versus stress group; $^{\wedge}p < 0.01$ versus stress + C. butyricum group.

FIGURE 5 | C. butyricum RH2 treatment reversed CFSS-induced decreases in serum ACTH and CORT. **(A)** The ACTH **(B)** The CORT. $**p < 0.01$ versus sham group; ##$p < 0.01$ versus stress group.

3.3 Effects of C. butyricum RH2 Treatment on Weight Gain and Food Intake of CFSS Rats
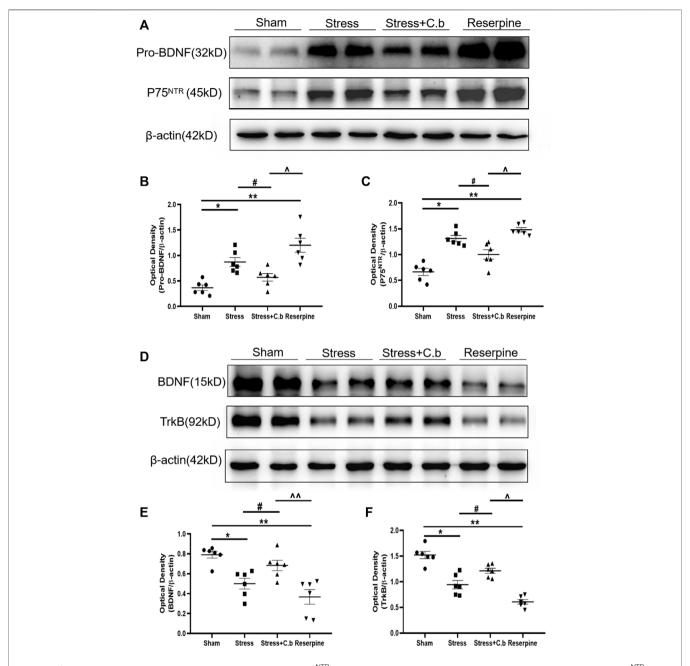
Body weight gain was significantly different between groups as shown in **Figure 4A** ($p < 0.01$). After 2 weeks of CFSS exposure, the body weight gain of rats was found to be significantly reduced compared to controls ($p < 0.01$). The decrease was significantly reversed by C. butyricum RH2 treatment for 17 days ($p < 0.05$). The measurement of food intake exhibited a significant difference between the groups as well (**Figure 4B**, $p < 0.01$). In comparison with sham rats, however, C. butyricum RH2 treatment could not reverse the decrease of food intake ($p > 0.05$). Reserpine remarkably reduced body weight gain and food intake of rats.

3.4 Effects of C. butyricum RH2 Treatment on Serum ACTH or CORT

Measurements using ELISA revealed that the serum levels of both ACTH and CORT were significantly increased (**Figures 5A–B**, $p < 0.01$) in CFSS rats compared to the control rats. After 17 days of C. butyricum RH2 treatment, the increase of the serum ACTH or CORT levels in CFSS rats was reversed (**Figures 5A–B**, $p < 0.01$).

3.5 Effects of C. butyricum RH2 Treatment on Expression of BDNF, proBDNF, TrkB and P75^{NTR} in Hippocampus

The mechanisms for CFSS induced neurological changes and the C. butyricum RH2 action were further sought. As shown by western blotting analysis in **Figures 6A–C**, CFSS significantly increased protein levels of proBDNF and P75^{NTR} (receptor for pro-BDNF) in the hippocampus ($p < 0.05$), whereas C. butyricum RH2 treatment reversed these changes ($p < 0.05$), i.e. decreasing the upregulated expression levels of proBDNF and P75^{NTR}. However, their respective protein levels in the CFSS + C. butyricum group were still significantly higher than those in the sham group. These data, indicate that C. butyricum RH2 treatment only partially reversed CFSS-induced upregulation of proBDNF and P75^{NTR}. On the contrary, CFSS significantly decreased protein levels of BDNF and TrkB (receptor for BDNF) in the hippocampus (**Figures 6D–F**, $p < 0.05$), whereas C. butyricum RH2 treatment partially reversed CFSS-induced downregulation of both BDNF and TrkB (**Figure 6B**, $p < 0.05$). These data together were consistent with a mechanism of changed balance between proBDNF and BDNF in the action of C. butyricum RH2. Finally, these CFSS-induced changes in protein levels of
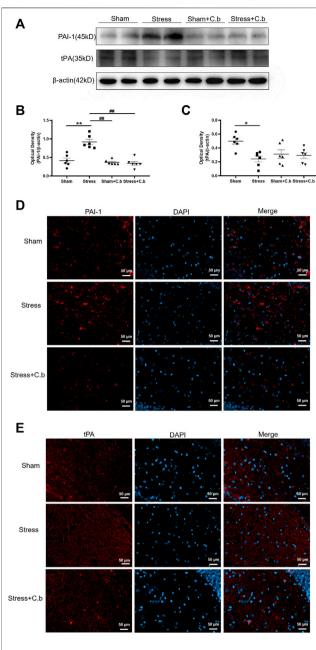
FIGURE 6 | Effects of C. butyricum RH2 on the expression of proBDNF, P75[NTR], BDNF and TrkB in the hippocampus of CFSS rats. **(A–C)** proBDNF and P75[NTR] were upregulated after CFSS but the increase was partially reversed by C. butyricum RH2. **(D–F)** BDNF and TrkB were downregulated after CFSS but the decrease was partially reversed by C. butyricum RH2. *$p < 0.05$, **$p < 0.01$ versus sham group; #$p < 0.05$, ##$p < 0.01$ versus stress group; ^$p < 0.05$, ^^$p < 0.01$ versus stress + C. butyricum group.

proBDNF, P75[NTR], BDNF, TrkB were also seen in reserpine intervention, emphasizing stress-specific effects.

3.7 Effects of C. butyricum RH2 Treatment on Expression of PAI or t-PA

To explore the molecular basis underlying the effects of C. butyricum RH2 treatment on the expression of proBDNF and BDNF as seen in **Figure 6**, the protein levels of tPA (a proteolytic enzyme for cleavage of proBDNF to mBDNF) and PAI-1 (the tPA inhibitor) were examined respectively. Western blot analysis of PAI-1 immunoreactivity revealed a low protein level of PAI-1 in sham rats but the protein level of PAI-1 was significantly increased after CFSS exposure ($p < 0.01$, **Figures 7A,B**). The CFSS-induced increase of PAI-1 was completely reversed by C. butyricum RH2 treatment ($p < 0.01$, **Figures 7A,B**) whereas C. butyricum RH2 alone had no effects on PAI-1 expression in sham rats ($p > 0.05$, **Figures 7A,B**). In contrast to PAI-1 changes, the

FIGURE 7 | C. butyricum RH2 treatment reversed CFSS-induced upregulation of PAI-1 in the hippocampus. **(A–C)** Western blot analysis of expression of PAI-1 and tPA in the hippocampus of CFSS rats before and after C. butyricum RH2 treatment. **(D–E)** The immunoreactive spots of PAI-1 and tPA protein in the hippocampus of CFSS rats before and after C. butyricum RH2 treatment. *$p < 0.05$, **$p < 0.01$ versus sham group; #$p < 0.05$, ##$p < 0.01$ versus stress group; ʹ$p < 0.05$, ʹ$p < 0.01$ versus stress + C. butyricum group.

expression of tPA protein was significantly downregulated by CFSS exposure ($p < 0.05$, **Figures 7A,C**). However, the CFSS-induced decrease of tPA protein expression was not reversed by C. butyricum RH2 treatment ($p > 0.05$, **Figures 7A,C**). The immunoreactive spots of PAI-1 and tPA protein examined by LCSM in hippocampal sections exhibited the same changes as

that detected by the western blot analysis, respectively (**Figures 7D,E**). These data suggest a key role for PAI-1 in the reversal action of C. butyricum RH2 on CFSS induced pathological changes, but it is tPA-independent.

4 DISCUSSION

In the present study, we demonstrated that the CFSS paradigm induced not only a physiological response as indicated by the elevation of blood pressure (Wu et al., 2020a; Wu et al., 2020b) but also behavioral changes, as reflected by mild anxiety or depression-like behavior. The present data support the idea that the CFSS is a complex stressor with both physical and emotional components (Bali and Jaggi, 2015). Moreover, CFSS resulted in mild cognitive impairment as evaluated by the Morris water maze test. However, mild electric foot shocks of 0.15 mA intensity for 0.5 s induced cognitive enhancement in mice (Bali et al., 2013). In the CFSS paradigm, the output voltage instead of current was used. The amplitude of the output voltage was adjusted just enough to cause the rat's forelimbs to cling to the side wall of the cage in an upright position. These results suggest that varying shock parameters including current/voltage intensity, duration, number, and interstimulus interval provoke differential stress responses in animals (Bali and Jaggi, 2015).

The CFSS paradigm has been employed to induce a hypertensive model in rodents (Wu et al., 2020). Since the electric foot shock induced stress has been a well established animal model of mood disorders, especially PTSD (Bali and Jaggi, 2015; Kim et al., 2017; Schoner et al., 2017), we question whether the CFSS paradigm influences psychological behavioral alterations. In this study, CFSS exposure induced a significant decrease in food consumption and body weight gain. The decreased food intake and body weight gain was a well described symptom of exposure to chronic stress (Yau and Potenza, 2013; González-Torres and Dos Santos, 2019). Results of EPM, and OFT exhibited anxiety-like behavior in CFSS rats, but the percentage of open arm entries in EPM test was not significantly decreased in the stressed rats. The lack of the influence of CFSS on the percentage of open arm entries suggests that CFSS exposure did not produce a stable anxiety-like behavior in terms of EPM test. The SPT and FST were used to detect depression-like behavior. The decrease in sucrose consumption in SPT indicates anhedonia, a symptom of depression. In this study, CFSS exposure did not induce a significant decrease in sucrose consumption/preference. These findings were in accordance with previous studies, suggesting that simple tests of sucrose consumption may not be valid as a hedonic measure in the chronic stress-induced depression models (Matthews et al., 1995; Forbes et al., 1996). While no inter-group differences in sucrose consumption, the increased immobility time in FST was induced in rats exposed to CFSS. This reflects depression-like behaviors because the behavioral outcome of the normalized FST has proven to be a reliable marker of depression (Cryan et al., 2005). In the Morris water maze test, CFSS subjected rats displayed significantly fewer platform crossings than the controls. But the escape latency, the total escape distance, and

the time spent in the target quadrant were not significantly different. The current evidence of the Morris water maze test suggests a slight cognitive impairment induced by CFSS. Taken together with the behavioral variations described above, the CFSS paradigm used for the hypertensive animal model has mild effects on mood behavior and cognition but could not be employed as a valid depression model. Unlike the widely used depression models including chronic unpredictable mild stress (CUMS) or chronic mild stress (CMS) with more than 4 weeks of duration in their paradigms, the duration of the CFSS paradigm is 2 weeks. A 2-week duration in CFSS paradigm is enough to induce a stable sympathetic excitation which in turn induced a sustained elevation of blood pressure (Wu et al., 2020), but might not be enough to produce a stable depression model.

Considering stress-induced behavioral impairment is at least partially a consequence of gut microbiota alteration, supplementation with probiotics has been considered as a potential but promising therapy to counteract the detrimental effects of stress by restoring the microbiota (Foster et al., 2017; Nishida et al., 2019; Tian et al., 2020; Westfall et al., 2021). Our observation revealed that *C. butyricum* RH2 treatment ameliorated the CFSS-induced anxiety/depression-like symptoms, cognitive decline and the decrease in body weight gain but not in food intake. The beneficial behavioral effects of *C. butyricum* RH2 are in line with the previous studies in CUMS mice treated with the other *C. butyricum*-specific strains (Tian et al..2020; Sun et al., 2018). The probiotic C. butyricum provides benefit to the microbial ecosystem of the gut by increasing probiotics and decreasing pathogens (Zhao et al., 2020), inducing a higher abundance of *Clostridium* at the genus level which was positively correlated with the attenuation of the stress-induced behavioral disorders (Tian et al., 2019b) and producing more butyrate as the C. butyricum genome contains butyrate producing gene buk (butyrate kinase) (Louis et al., 2004; Stoeva et al., 2021). Our previous studies revealed that the CFSS paradigm altered gut microbiota characterized by decreases in the richness or diversity and the species composition of gut microbiota (Wu et al., 2020a). The *C. butyricum* RH2 has been proven to restore the disturbance of α-diversity and β-diversity of gut microbiota in ceftriaxone-treated mice (Li et al., 2021). Moreover, our unpublished data showed that the CFSS paradigm decreased number of butyric acid-producing bacteria. The abundance of the bacteria was significantly lower in CFSS rats than in control rats. Of the reduced bacterial units—210 OTUs, 25 were related to the butyrate producing ability. Furthermore, the butyric acid of feces was significantly lower in CFSS rats than in control rats. *C. butyricum* commonly exists in the gut of humans and animals, producing butyric acid (Detman et al., 2019), which has been implicated in depression (van de Wouw et al., 2018). Exposure to psychosocial stress decreased the relative abundance of *Clostridium spp.* in the caecum of mice (Bailey et al., 2011). *C. butyricum* is one of the commonly used butyrate-producing bacteria in clinical settings. In the present study, supplementation of *C. butyricum* RH2 reversed the decrease in butyric acid-producing bacteria and butyric acid in CFSS rats (unpublished data), which might mediate the beneficial role of C.

butyricum RH2 in amelioration of mood deficits of CFSS rats. Our current investigation adds additional evidence that the probiotics supplementation can ameliorate stress related mood changes by rectifying dysbiosis and restoration of the normal balance in the gut microbiome.

It is well documented that chronic stress results in hyperactivation of the HPA axis. Increasing evidence suggests that the imbalances of the HPA axis could be the pathogenesis of the stress-induced behavioral aberrations (Lin et al., 2016; Huo et al., 2017; Kinlein et al., 2019). Recent studies have demonstrated that gut microbiota modulates stress-related behavior disorders via the HPA axis (Frankiensztajn et al., 2020; Misiak et al., 2020). This is supported by previous studies showing that probiotics supplementation attenuates the stress response by modulating the HPA axis activity (Ait-Belgnaoui et al., 2018). We previously reported that CFSS paradigm led to overactivation of the HPA axis and the exacerbated HPA axis was causally linked to CFSS-induced gut dysbiosis (Wu et al., 2020b). In the present study, the increased serum levels of ACTH and CORT in CFSS rats were almost completely reversed by *C. butyricum* RH2 intervention. The normalization of hormones ACTH and CORT by *C. butyricum* RH2 was accompanied by alleviation of behavioral disorders, providing evidence that beneficial effects of *C. butyricum* RH2 on stress-induced maladaptive changes may be achieved by improving the HPA axis dysfunctions via reshaping the gut microbiota.

Neurotrophin BDNF, by binding with high affinity to the tropomycin receptor kinase B (TrkB) receptor, is vital for synaptic formation, synaptic plasticity in the brain. The numerous studies suggest that BDNF-TrkB signaling plays an important role in learning, memory formation, and the pathophysiology of mood disorders (Vicario-Abejón et al., 2002; Autry and Monteggia, 2012; Duman et al., 2021). Clinical studies in major depressive disorder patients have shown that BDNF and TrkB levels were downregulated in the hippocampus and frontal cortex (Pandey et al., 2008; Thompson et al., 2011; Autry and Monteggia, 2012). An increase in brain BDNF levels by direct infusion or transgenic overexpression of BDNF-TrkB signaling in the hippocampus produces antidepressant-like behavioral effects (Shirayama et al., 2002; Hoshaw et al., 2005; Govindarajan et al., 2006). These data suggest a causal link between BDNF in the hippocampus and depression-like behavior. The central reduction in BDNF and its receptor TrkB has also been found in depressive animals subjected to various stressors (Phillips, 2017; Sun et al., 2018; Duman et al., 2021). Consistently, the expression of BDNF and TrkB was significantly downregulated in the hippocampus of CFSS rats in the present study. The selective deletion of BDNF in the hippocampus does not induce an increase in depression-like behavior per se but attenuates the responses of the antidepressant (Adachi et al., 2008), suggesting that the downregulated endogenous BDNF-TrkB signaling in the hippocampus of CFSS rats may not contribute to CFSS-induced anxiety/depression-like behaviors but to the susceptibility to mood disorders (Adachi et al., 2008). Gut dysbiosis downregulates the expression of BDNF and TrkB in the hippocampus (Bercik

et al., 2011; Bistoletti et al., 2019). Therefore, the decreased BDNF and TrkB in the hippocampus of CFSS rats may be attributable to the gut dysbiosis induced by CFSS paradigm (Wu et al., 2020a). C. butyricum supplementation has been found to restore the decreased brain BDNF levels after stress (Sun et al., 2018) or sepsis (Liu et al., 2020). Given the role of C. butyricum RH2 in reshaping gut microbiota (Li et al., 2021), we assessed the effect of C. butyricum RH2 treatment on the expression of BDNF in the hippocampus of CFSS rats. Our results reveal that C. butyricum RH2 administration partially reversed the decrease in BDNF, which was in parallel with the amelioration of behavioral abnormalities. This demonstrates the involvement of upregulation of BDNF in the anxiolytics effect of C. butyricum RH2, at least in part.

BDNF is the cleaved form of proBDNF. Being a precursor to BDNF, proBDNF also binds specifically to the p75 neurotrophin receptor (p75^{NTR}) to exert opposite biological effects to BDNF (Greenberg et al., 2009). In contrast to the downregulation of BDNF, the expression of proBDNF and p75^{NTR} was increased in the hippocampus of CFSS rats, which is in line with previous studies in other stressed rodents (Bai et al., 2016; Yang et al., 2020; Lin et al., 2021). Mounting evidence from patients and animals suggests a close association of proBDNF and p75^{NTR} with depression. In major depressive disorder patients, serum proBDNF and p75^{NTR} were significantly increased (Zhou et al., 2013). In animal models of depression, proBDNF and p75^{NTR} were upregulated in the brain areas such as hippocampus. Intracerebroventricular injection of proBDNF triggered depression-like behavior in normal rats (Bai et al., 2016). The upregulated proBDNF/ P75^{NTR} in the brain has been implicated in the pathogenesis of stress-induced mood disorders (Bai et al., 2016; Yang et al., 2020; Lin et al., 2021). Suppression of proBDNF/ p75^{NTR} signaling alleviated depressive and anxiety-like behaviors in chronically stressed mice (Yang et al., 2017; Lin et al., 2021). In CFSS rats, CFSS-induced upregulation of proBDNF/p75^{NTR} signaling was partially reversed by C. butyricum RH2 treatment, which was associated with the attenuation of CFSS-related mood disorders. In line with previous studies (Tian et al., 2020; Yang et al., 2020), these results indicated that CFSS elicited the imbalance between the proBDNF/p75^{NTR} and BDNF/TrkB signaling pathways. The C. butyricum RH2 corrected this imbalance in part, accounting to some extent for the beneficial effect of C. butyricum RH2 on mood disorders after CFSS. However, the molecular mechanisms by which C. butyricum RH2 normalized the imbalance remain to be explored.

CFSS resulted in the upregulation of proBDNF and downregulation of BDNF in the hippocampus, both of which could potentially synergistically elicit depression- and anxiety-like behaviors. To increase conversion of proBDNF to BDNF within the hippocampus would counter the deleterious effects of CFSS. In accordance, we speculated that C. butyricum RH2 restored CFSS-induced changes in proBDNF and BDNF by enhancing pro-BDNF cleavage into mature BDNF. Extracellular proBDNF can be converted to mature BDNF by

plasmin (Pang et al., 2004). Plasmin is cleaved from plasminogen by tissue-type plasminogen activator (tPA) (Melchor and Strickland, 2005). The downregulation of tPA or decrease in tPA activity may decrease proBDNF cleavage, resulting in an increase of proBDNF levels and decrease of BDNF levels. Plasminogen activator inhibitor 1(PAI-1) is the major endogenous inhibitor for tPA (Melchor and Strickland, 2005). PAI-1 may decrease proBDNF cleavage and increase proBDNF levels by inhibiting the tPA/plasminogen system. Thus, the role of PAI-1 in the proBDNF and BDNF balance via tPA regulation under CFSS was explored. In this study, the protein level of PAI-1 in the hippocampus of CFSS rats was greatly increased while tPA was significantly decreased, accounting for the upregulation of proBDNF and downregulation of BDNF. Peripheral PAI-1 expression is increased by corticosterone treatment and PAI-1 deficiency significantly reduces GC-induced deleterious effects such as insulin resistant, bone loss (Tamura et al., 2015). This means that the CFSS-induced increase in CORT might be involved in the modulation of PAI-1 expression in the hippocampus, but further investigation is required. Importantly, the increase in PAI-1 in CFSS rats was completely reversed by C. butyricum RH2 treatment but the decrease in tPA was not affected. As a result, C. butyricum RH2 exerted anxiolytic effect more likely by the inhibition of PAI-1 expression in the hippocampus after stress, at least in part. The up-regulation of PAI-1 has been implicated in the stress-induced behavioral disorders in animals and patients (Tang et al., 2015; Jiang et al., 2016; Han et al., 2019). Collectively, it was proposed that the CFSS-induced PAI-1 expression might be involved in CFSS-provoked mood abnormalities through inhibition of tPA cleavage activity, resulting in compromised maturation of BDNF and accumulated proBDNF. Additionally, PAI-1 may also cause depression by a mechanism independent of tPA and BDNF pathway but associated with impaired serotonin and dopamine metabolism (Party et al., 2019). Whether the disruption of serotonin and dopamine metabolism mediates the effects of PAI-1 in CFSS rats needs to be examined in more details in future studies. These findings suggest that targeting PAI-1 could be an innovative strategy for the development of new drugs to counter the effects of stress.

Gut dysbiosis has been implicated in modulating peripheral PAI-1 levels. The consumption of probiotic yogurt resulted in a significant reduction in the level of PAI 1 in patients with metabolic syndrome (Rezazadeh et al., 2019). A study by Gomez-Arango demonstrates a close association of the decrease of butyrate-producing bacteria and butyrate production in the gut microbiota with the increase in circulating PAI-1 levels in obese pregnant women with higher blood pressure (Gomez-Arango et al., 2016). However, few studies have been carried out to link the gut microbiota to PAI-1 in the brain. In this study, C. butyricum RH2 supplementation by gastric gavage reversed the increase of PAI-1 expression in the hippocampus of CFSS rats, providing the first evidence that brain PAI-1 expression can be modulated by gut microbiome. Further investigations are needed to determine the causality and to explore the potential

mechanisms whereby *C. butyricum* RH2 may exert direct or indirect actions on PAI-1 expression in the hippocampus via butyrate or other metabolites.

In conclusion, a 2-week CFSS led to partial anxiety- or depression-like behaviors and also mild cognitive impairment. *C. butyricum* RH2 treatment reversed the CFSS-induced symptoms. This action may be mediated by changing BDNF and proBDNF imbalance and also be attributable to the inhibition of PAI-1 expression in the brain independent of BDNF and probDNF pathway.

4.1 Limitations

Effects of *C. butyricum* RH2 treatment on the CFSS-induced gut dysbiosis remain to be investigated. Furthermore, correlation of *C. butyricum* RH2-induced downregulation of PAI-1 in the hippocampus of CFSS rats with relevant behavior amelioration needs to be analyzed.

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 animal study was reviewed and approved by the Animal Care and Use Committee of Jiangsu Vocational College of Medicine.

AUTHOR CONTRIBUTIONS

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Effect of Probiotic Supplements on Oxidative Stress Biomarkers in First-Episode Bipolar Disorder Patients: A Randomized, Placebo-Controlled Trial

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Background: Currently no study has examined the effects of probiotic administration on the symptoms of anxiety, depression, and mania, as well as their correlations with the biomarkers of oxidative stress in patients with bipolar disorder (BPD). The aim of this study is to determine the effects of probiotic supplementation on plasma oxidative stress-related biomarkers and different domains of clinical symptom in patients suffering from BPD.

Methods: Eighty first-episode drug-naive patients with BPD were recruited. The subjects were randomized to receive psychotropic drugs supplementing with either probiotic or placebo and scheduled to evaluate with follow-ups for clinical symptom improvements and changes in the oxidative stress biomarkers. The Hamilton Depression Rating Scale, Hamilton Anxiety Rating Scale, and Young Mania Rating Scale were used to assess the clinical symptomatology. The panel of plasma oxidative stress biomarkers were determined by ultra-performance liquid chromatography–mass spectrometry (UPLC–MS/MS) at baseline and for 3 months of follow-up, i.e., at post-treatment month 1, 2, and 3.

Results: After 3 months of intervention, decreased levels of plasma lysophosphatidylcholines (LPCs) were found in both placebo and probiotic groups. However, six other oxidative stress biomarkers (i.e., creatine, inosine, hypoxanthine, choline, uric acid, allantoic acid) increased in BPD patients after the two types of therapies. In addition, a positive correlation between changes of LPC (18:0) and YMRS scale was found in BPD patients and this association only existed in the probiotic group. Additionally, the mania symptom greatly alleviated (pretreatment–posttreatment, odds ratio = 0.09, 95%CI = 0.01, 0.64, p= 0.016) in patients who received probiotic supplements as compared with the placebo group.

Conclusion: The changes in plasma biomarkers of oxidative stress in patients with BPD have a potential to be trait-like markers, and serve as prognostic indexes for bipolar

patients. Daily intakes of probiotics have advantageous effects on BPD patients with certain clinical symptoms, especially manic symptoms. The treatment may be a promising adjunctive therapeutic strategy for BPD patients in manic episode.

Keywords: probiotics, purinergic metabolism, oxidative stress, mania, bipolar disorder
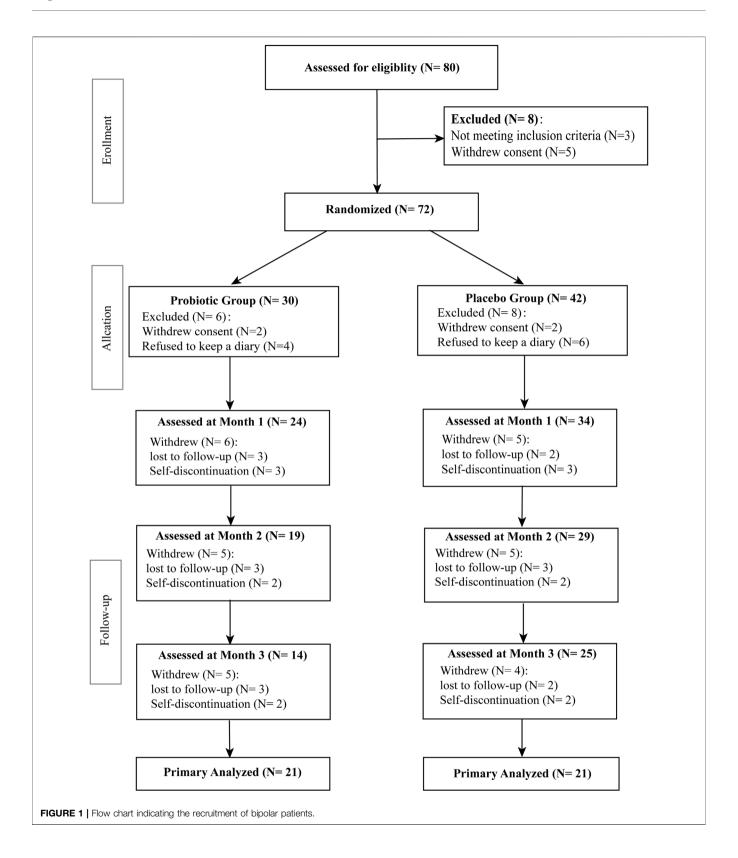
INTRODUCTION

Bipolar disorder (BPD) is a complex and chronic mental disorder, affecting approximately 1–2% of the general population at some point in their lifetime (Fagiolini et al., 2013). Bipolar and related disorders including manic episode, depression episode, and cyclothymic disorder are characterized by recurring depressive and hypomanic states (Carvalho et al., 2020). Recent evidence suggests that dysfunction of the purinergic system may play an important role in pathophysiology and treatment of BPD (Machado-Vieira et al., 2008; McQuillin et al., 2009). In addition, the activity of purinergic metabolic cycle was associated with increased oxidative stress in BPD (Albert et al., 2014). Meanwhile, purines affect the activity of other neurotransmitters, including GABAergic, dopaminergic, serotonergic and glutamatergic systems. It is worth noting that these factors are involved in the pathophysiology of BPD (Machado-Vieira et al., 2002). Purinergic system biomarkers include the purine nucleotides adenosine monophosphate (AMP), adenosine triphosphate (ATP), xanthines, uric acid, and related metabolites (Ali-Sisto et al., 2016). Adenosine is metabolized into inosine, hypoxanthine, and xanthine by cabolization with the enzymes adenosine deaminase and xanthine oxidase. In addition, oxidized phosphatidylcholine (PC) has been considered as a prominent biomarker of oxidative stress (Nakanishi et al., 2009). In support, lysophosphatidylcholines (LPCs) converted from PC during oxidation of low-density lipoprotein (LDL) increased in schizophrenia (Cai et al., 2012) and BPD (Schwarz et al., 2008) as compared to healthy controls, indicating elevated level of oxidative stress. Among the biomarkers, uric acid, a pivotal antioxidant in human body, is the key end product of nitrogen metabolism. Interestingly, a meta-analysis indicated that the plasma uric acid level in BPD patients was higher than that of healthy controls, especially in manic/mixed phases (Bartoli et al., 2016). The therapeutic effects on BPD symptoms may be associated with changes of plasma uric acid. After 8 weeks of treatment, non-remission subjects were reported to show higher level of serum uric acid concentration than remission subjects (Chen et al., 2019). Thus, it is possible that higher vulnerability to bipolar disorder is characterized by increased uric acid levels as a trait marker, which even increased more during mania (Albert et al., 2014). Increased uric acid levels implies accelerated purinergic transformation and reduced adenosinergic transmission (Burnstock, 2008). Activation of adenosine A1 receptors contributes to inhibition of neurotransmitter release and cellular excitability. The detailed mechanism remains to be illucidated.

Normally, gut microbiota modulates functions of the nervous system, immune and endocrine system (Tognini, 2017; Zeng et al., 2021). Disturbances in gut microbiota are also related to many mental diseases such as BPD, suggesting that the altered bacterial genus may cause oxidative stress and inflammation in patients (Coello et al., 2019). At the same time, specific species of the gut microbiota in turn regulate host purine metabolism, affecting disease status (Chiaro et al., 2017; Yamauchi et al., 2020). A vast number of studies have demonstrated that probiotics play an essential role in healthy population and clinical patients. Probiotics improves depressive symptoms, biomarkers of inflammation, and oxidative stress through their effects on neuronal circuits and the central nervous system mediated by the gut-brain axis (Foster and McVey Neufeld, 2013). A recent study has found the probiotics containing *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* regulate depressive symptoms in BPD patients (Akkasheh et al., 2016). However, a recent systematic review conducted by Fusar-Poli and others (Fusar-Poli et al., 2019), has shown that the efficacy of adjunctive nutraceuticals in BPD is inconsistent, though they seem to be largely devoid of relevant side effects. On top of that, improved mood status and oxidative stress by decreasing inflammation and oxidative stress markers and increased nitric oxide bioavailability were observed after 3 months of supplementation with probiotics for Alzheimer's patients (Ton et al., 2020). Although probiotic administration containing *Lactobacillus rhamnosus* strain GG and *Bifidobacterium* had no apparent therapeutic effects on the psychopathology, it may help prevent common somatic symptom associated with schizophrenia after 14 weeks (Dickerson et al., 2014). Other than psychiatric disorders, probiotic supplementation has therapeutic effects on mental health of petrochemical workers (Mohammadi et al., 2016). By comparison, there is now relatively robust evidence in animal studies to prove that probiotics can change behavior and improve depressive symptoms through regulating critical neurotransmitters, reducing overall inflammation, as well as its antioxidant and free radical scavenging abilities (Wallace and Milev, 2017). However, the benefits of probiotics on symptom relief and reduction of oxidative stress in patients with BPD has not been assessed to date.

This article aims to evaluate the therapeutic effects of probiotics on different symptoms of BPD patients in a randomized, placebo-controlled trial, and clarify underlying mechanisms of purine metabolism in BPD pathophysiology. We measured the plasma levels of nine metabolites linked to oxidative stress and purine metabolism [i.e., creatine, inosine, hypoxanthine, choline, uric acid, allantoic acid and LPCs (16:0, 18:1, and 18:0)] to investigate: 1) the changes of plasma levels of oxidative stress biomarkers in BPD patients, 2) the efficacy of probiotics on allieviating symptoms of BPD patients, and 3) the relationship between mitigation of BPD symptoms and changes of biomarkers after administrating probiotics.

Assessed for eligiblity (N= 80)

Erollment

Excluded (N= 8):
Not meeting inclusion criteria (N=3)
Withdrew consent (N=5)

Randomized (N= 72)

Allcation

Probiotic Group (N= 30)
Excluded (N= 6):
Withdrew consent (N=2)
Refused to keep a diary (N=4)

Placebo Group (N= 42)
Excluded (N= 8):
Withdrew consent (N=2)
Refused to keep a diary (N=6)

Assessed at Month 1 (N= 24)

Withdrew (N= 6):
lost to follow-up (N= 3)
Self-discontinuation (N= 3)

Assessed at Month 1 (N= 34)

Withdrew (N= 5):
lost to follow-up (N= 2)
Self-discontinuation (N= 3)

Follow-up

Assessed at Month 2 (N= 19)
Withdrew (N= 5):
lost to follow-up (N= 3)
Self-discontinuation (N= 2)

Assessed at Month 2 (N= 29)
Withdrew (N= 5):
lost to follow-up (N= 3)
Self-discontinuation (N= 2)

Assessed at Month 3 (N= 14)
Withdrew (N= 5):
lost to follow-up (N= 3)
Self-discontinuation (N= 2)

Assessed at Month 3 (N= 25)
Withdrew (N= 4):
lost to follow-up (N= 2)
Self-discontinuation (N= 2)

Primary Analyzed (N= 21)

Primary Analyzed (N= 21)

FIGURE 1 | Flow chart indicating the recruitment of bipolar patients.

MATERIALS AND METHODS

Participants

This study was approved by the Medical Ethics Committee of the Second Xiangya Hospital, Central South University (2018-067). All participants gave signed and informed consent before all research procedures started. This study was registered in Chinese Clinical Trial Registry (ChiCTR1900021379).

Eighty patients suffering from BPD were recruited from March 2019 to November 2019 in the Second Xiangya Hospital of Central South University. The subjects were recruited from outpatients or inpatients who visited the hospital spontaneously. Based on the medical history and treatment provided by the patients and their families, as well as past medical records, it was confirmed that the patients had not received any drug treatment for BPD before enrollment. The inclusion criteria were as follows: 1) subjects aged 16–50 years; 2) diagnosed with BPD for a current manic or major depressive episode or mixed phase by two independent senior psychiatrists according to the DSM-5 (American Psychiatric Association, 2013); 3) no previous and current use of any psychotropic drugs was confirmed by medical records and patient self-reports. All participants were Han Chinese.

All subjects had no record of organic brain diseases, active or chronic inflammatory disease, cardiovascular disease, gout and renal disease. Patients' exclusion criteria were comorbid mental disorders, intellectual disability, serious somatic diseases, autoimmune diseases and pregnancy or breast feeding. Subjects prescribed with drugs such as acetylsalicylic acid, thiazide diuretics, vitamin E, steroids, antiepileptic drugs or other drugs that may influence oxidative stress-related markers were also excluded. Seventy-two BPD patients finally met all inclusion criteria and none of the exclusion criteria were randomly assigned to the two intervention groups. The flow chart of the study design is shown in **Figure 1**.

Intervention

This trial adopted second-level blind design. The first level of blind codes was the group corresponding to each subject number (such as group A and group B), and the second level of blind codes was the treatment plan for each group (such as test group and placebo group). The probiotic and placebo were produced by Shanghai Xinyi Pharmaceutical General Factory, and were numbered with groups A and B, respectively. Patients were assigned to receive either probiotic or placebo (without any restriction and stratification) by a computer-generated random number to ensure approximately equal numbers of patients within two groups. The treatment allocation was determined after all evaluations and study randomization. Patients were randomized to receive either probiotic (Live combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus* capsules. Every capsule contains at least $1.0*10^7CF$ live probiotics for each capsule) plus psychotropic drugs or placebo plus psychotropic drugs for 3 months. Patients in the probiotic group were asked to receive three capsules after the meal twice a day at a fixed time, totaling six capsules daily. Patients in the placebo group were administrated with the placebo containing starch only. The

appearance of the treatment products was indistinguishable in shape, package, size, color, taste, and smell to keep the treatment hidden to participants and researchers after allocation. Participants' adherence to probiotic and psychotropic drugs treatment for each visit was defined as taking more than 80% and less than 120% of the pre-required medication dose specified for that interval. If participants did not comply, both patients and caregivers were informed the importance of taking prescribed doses of study drugs.

Assessment

The team of clinicians and researchers was blinded to the information about the treatment group. Three rating scales were used for comprehensive assessment of symptom. The Hamilton Depression Rating Scale (HAMD) 17-item is a rating three- or five-point scale and is widely used to measure the severity of depressed symptoms (Hamilton, 1960). Hamilton Anxiety Rating Scale (HAMA) 14-item is a rating four-point scale used to measure the severity of anxiety symptoms (Hamilton, 1959). Young Manic Rating Scale (YMRS) is a 11-item multiple-choice diagnostic questionnaire used to measure the severity of manic symptoms (Young et al., 1978). Before conducting the study, five psychiatrists who had worked in clinical practice for more than 6 years simultaneously received training courses on how to use HAMD, HAMA and YMRS to ensure consistency and reliability of the scores. Repeated assessments of the study were performed to ensure that the correlation coefficient remained greater than 0.8. (Chen et al., 2019). All patients who received the treatment were scheduled for clinical assessment through each visit at baseline, months 1, 2 and 3. The baseline evaluation comprised demographics, anthropometric measures (body weight and height), physical examination, and comprehensive medical history. Severities of mood symptoms were measured in all subjects by HAMD, HAMA, and YMRS. During each follow-up, physical examination, anthropometry tests and three scales scores were repeated. A per-protocol analysis of the evaluable population was conducted to assess the clinical and metabolite outcomes. Only those patients who completed the study according to the intervention protocol specified at randomization and had complete metabolite measurements were included. The remaining patients were excluded from the analysis. The blood samples were collected from patients after fasting for 12 hours, using EDTA-coated vacuum tubes for plasma isolation. Plasma samples were obtained at baseline and post-treatment month 1, 2, and 3.

Plasma samples were immediately stored under −80°C until analysis. Concentrations of relevant biomarkers were measured using the ultraperformance liquid chromatography–mass spectrometry (UPLC–MS/MS) method of our research group (Cai et al., 2019).

Statistical Analysis

The Kolmogrov–Smrinov test was used to test the normal distribution and homogeneity of variables. The independent *t*-test was employed to compare the variables at the baseline between the two groups. The two-way ANOVA (treatment and time as factors) analysis was conducted to compare the oxidative

TABLE 1 | Baseline characteristics of the study participants.

Variable	Probiotic Group	Placebo Group	p
Patients	21	21	-
Age (years)	22.29 ± 5.13	20.86 ± 2.90	0.5604
Height (cm)	161.90 ± 5.57	164.81 ± 8.80	0.2839
Body weight (kg)	56.16 ± 7.38	58.38 ± 12.17	0.9752
BMI (kg/m^2)	21.41 ± 2.61	21.37 ± 3.39	0.9648
Illness phase, n (%)			
Major depressive episode	14 (66.67)	15 (71.43)	-
Manic episode	4 (19.05)	4 (19.05)	-
Mixed phase	3 (14.29)	2 (9.52)	-
Psychiatric medication, n (%)			
Mood stabilizers	17 (80.95)	21 (100.00)	-
Antidepressants	2 (9.52)	1 (4.76)	-
Antipsychotic medications n (%)	14 (66.67)	18 (85.71)	-
Score of HAMD	20.10 ± 8.57	18.19 ± 10.04	0.5223
Score of HAMA	21.86 ± 8.16	19.38 ± 10.32	0.4050
Score of YMRS	10.29 ± 7.18	10.38 ± 7.28	0.9670

BMI, body mass index; HAMD, hamilton depression rating scale; HAMA, hamilton anxiety rating scale; YMRS, young manic rating scale; Data are mean ± SD; p-values were calculated using unpaired t-tests.

stress-related biomarkers concentrations, HAMD, HAMA and YMRS scores between the baseline and each time point in each patient group. The Pearson correlation analysis was performed to explore the relationship between oxidative stress-related markers levels and three scales scores. The ordinal logistic regression model analysis was carried out to assess the effects of probiotic supplementation on biomarkers of oxidative stress, YMRS scores, HAMD scores, and HAMA scores. The odds ratio indicated probiotics supplementation is a protective factor for the improvement of HAMD, HAMA, and YMRS. The treatment response was classified into two categories: poor response (Scale total score reduction from baseline<50%), and good response (Scale total score reduction from baseline≥50%) (Melander et al., 2003; Allgulander et al., 2004; Sachs et al., 2006; Hedayati et al., 2016). Results are expressed as mean ± SD. A two-tailed level of 0.05 was considered as statistically significant. All data were analyzed using SPSS (version 26.0).

RESULTS

Demographic and Clinical Characteristics
Demographic and clinical characteristics of the subjects are shown in **Table 1**. No significant changes were observed in age (p = 0.5604), height (p = 0.2839), body weight (p = 0.9752), BMI (p = 0.9648), HAMD score (p = 0.5223), HAMA score (p = 0.4050), or YMRS score (p = 0.9670) within probiotic group and placebo group. As presented in **Table 1**, 17 patients were given mood stabilizers, 2 were given antidepressants and 14 were given antipsychotic medications in the probiotic group, whereas 21 were given mood stabilizers, 1 was given antidepressants and 18 were given antipsychotic medications in the placebo group. In probiotic group, 14 patients were in a depressive episode, 4 patients were in a manic episode, and three patients were in a mixed

episode phase, while for the placebo control group, 15 patients were in a depressive episode, 4 patients were in a manic episode, and 2 patients were in a mixed episode phase. During the trial period, 38 patients withdrew from the trial and 42 patients completed the trial (**Figure 1**). Of 42 patients who completed the trial, 21 patients were randomly assigned to each group. There is no significant difference between the two groups (p > 0.05) in adverse events related to psychotropic drugs, including dizziness, somnolence, and nausea. These mild adverse reactions were alleviated or stabilized after treatment for 1–2 weeks. Meanwhile, no significant adverse effects occurred in subjects of the both groups.

Comparison of Oxidative Stress Biomarkers and Scales in Probiotic and Placebo Group After Treatment
Figure 2 displays an intuitive summary of the treatment effects over time. After 3 months of probiotic supplements concurrently used with antipsychotics medication, patients had significant increases in inosine (p = 0.0004), hypoxanthine (p< 0.0001) (**Figures 2B,C**), choline (p < 0.0001), and uric acid (p< 0.0001) (**Figures 2D,E**), while significant decreases in LPC (16:0) (p < 0.0001), LPC (18:1) (p< 0.0001), and LPC (18:0) (p < 0.0001) as compared to the baseline levels (**Figures 2G–I**), both in the probiotic and placebo groups. Additionally, patients had significantly lower HAMD (p = 0.0003), HAMA (p = 0.0001), and YMRS (p = 0.0150) scores as compared with their baselines (**Figure 2J**–L). However, there is no significant difference in improvements of oxidative stress biomarkers and symptomatology between probiotic and placebo groups.

In **Table 2**, it is indicated that probiotic supplementation is a protective factor for BPD patients. By comparing the probiotic groups with the placebo groups, the odds ratio was 0.62 for the rate of change in HAMD
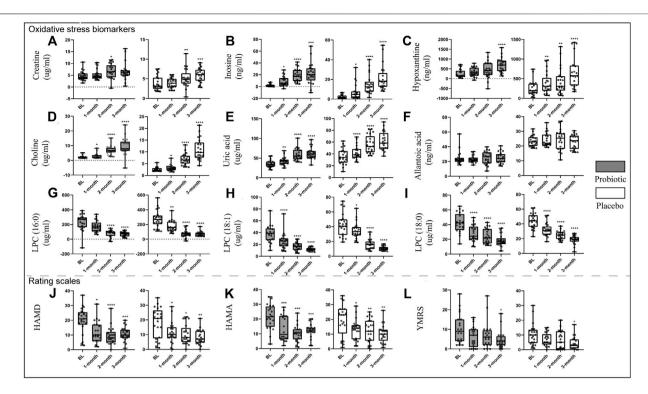
FIGURE 2 | Changes in oxidative stress-related biomarkers [(A), creatine; (B), inosine; (C), hypoxanthine; (D), choline; (E), uric acid; (F), allantoic acid; (G), LPC (16:0); (H), LPC (18:1); (I), LPC (18:0)] and scales [(J), HAMD; (K), HAMA; (L), YMRS] after treatment in both groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Abbreviations: LPC, Lysophosphatidylcholines; HAMD, hamilton depression rating scale; HAMA, hamilton anxiety rating scale; YMRS, young manic rating scale.

TABLE 2 | Outcome measures in a study of probiotic and placebo therapy in bipolar patients.

	Total sample (N = 42)		Probiotic Group (N = 21)		Placebo Group (N = 21)		Analysis		
Binary outcome measures	N	%	N	%	N	%	Odds ratio	95% CI	P
HAMD							0.62	0.26, 1.47	0.276
Rate of Change≥50%	26	61.90	13	61.90	12	57.14			
Rate of Change<50%	17	40.48	8	38.10	9	42.86			
HAMA									
Rate of Change≥50%	18	42.86	9	42.86	9	42.86	2.21	0.71, 6.90	0.173
Rate of Change<50%	24	57.14	12	57.14	12	57.14			
YMRS							0.09	0.01, 0.64	0.016
Rate of Change≥50%	26	61.90	14	66.66	12	57.14			
Rate of Change<50%	16	38.10	7	33.33	9	42.86			
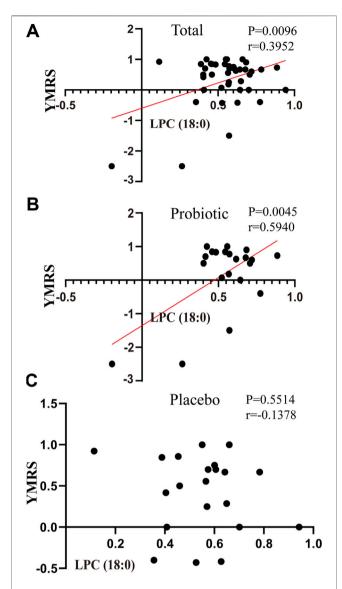
HAMD, hamilton depression rating scale; HAMA, hamilton anxiety rating scale; YMRS, young manic rating scale.
Rate of change ≥50%, Good response; Rate of change <50%, Poor response.

(pretreatment–posttreatment, 95% confidence interval [CI] 0.26, 1.47, p = 0.276), and 2.21 for the rate of change in HAMA (pretreatment–posttreatment, 95% confidence interval [CI] 0.71, 6.90, p = 0.173), without significance. The odds ratio for the rate change in YMRS between probiotic and placebo groups was 0.09 (pretreatment–posttreatment, 95% confidence interval [CI] 0.01, 0.64, p = 0.016), indicating that probiotic

supplementation is a protective factor for the alleviation of manic symptoms.

Correlations Between Oxidative Stress Biomarkers and Clinical Assessments

As shown in **Figure 3**, Pearson correlation analysis showed a significant correlation between LPC (18:0) concentrations and

FIGURE 3 | Correlations between the changes in oxidative stress-related markers and scales in placebo and probiotic groups after treatment. **(A)** the association between YMRS and LPC (18:0) in bipolar patients as a whole; **(B)** the association between YMRS and LPC (18:0) in the probiotic group; **(C)** the association between YMRS and LPC (18:0) in the placebo group. Abbreviations: LPC, Lysophosphatidylcholines; YMRS, young manic rating scale.

YMRS scores (pretreatment–posttreatment, r = 0.3952, p= 0.0096) in all BPD patients who completed the trail (**Figure 3A**). Specifically, a significant correlation between LPC (18:0) and YMRS index was seen in the probiotic group (pretreatment–posttreatment, r = 0.5940, p = 0.0045; **Figure 3B**), but not in the placebo group (pretreatment–posttreatment, r = -0.1378, p= 0.5514; **Figure 3C**).

DISCUSSION

The present study aims to assess oxidative stress biomarkers in BPD patients co-administrated with psychotropic drugs and

probiotic/placebo. In the present study, decreased plasma LPC (16:0, 18:1, 18:0) levels in both placebo and probiotic groups were found. The other six oxidative stress biomarkers (i.e., creatine, inosine, hypoxanthine, choline, uric acid, allantoic acid) increased in BPD patients. Previous studies reported that the gut microbiota regulated purine metabolism (Chiaro et al., 2017). Of interest, purine metabolism relating to oxidative stress also plays a vital role in pathophysiology of BPD (Albert et al., 2014). Phospholipids belong to the most vulnerable class of biological targets of oxidative stress (Del Rio et al., 2005). The LPCs were converted from the oxidization of PC as markers of oxidative stress. Interestingly, the disturbance of lipid metabolism in patients with first-episode schizophrenia includes elevations of LPCs (16:0, 18:1, and 18:0), indicating an association with increase of oxidative stress (Cai et al., 2012). Uric acid, a purinergic metabolite produced by the xanthine oxidoreductase from xanthine or hypoxanthine, is an important nitrogenous end product of purinergic metabolism (ATP and adenosine) as well. Previous studies reported inconsistent data on blood levels of uric acid in BPD patients (Salvadore et al., 2010; Albert et al., 2014; Yang et al., 2018). Nevertheless, we observed increased uric acid levels in both treatment groups. Overall, changed plasma oxidative stress biomarkers, especially LPCs and uric acid, may serve as potential trait-like markers of prognostic values for BPD.

With regard to the role of gut microbiota in regulation of the immune system (Kamada et al., 2013), it is not surprising that imbalance of the intestinal ecosystem can increase inflammation and oxidative stress in the host (Nguyen et al., 2018), which may play a key role in the pathophysiology of BPD (Brown et al., 2014; Fernandes et al., 2016; Dickerson et al., 2018). The association of therapeutic outcome and the gut microbiota in patients with BPD was further explored, suggesting that the fractional representation of *Faecalibacterium* associated with better self-reported health outcomes (Evans et al., 2017). The association of increased fractional representation of *Faecalibacterium* with reduced depressive symptoms and improved physical health was also observed. Moreover, a recent study by Coello and others showed that *Flavonifractor*, a bacterial genus, may be associated with the pathogenesis of BPD by inducing host oxidative stress and inflammation (Coello et al., 2019). These results indicate that gut microbiota may be a new therapeutic target for BPD patients. Theoretically, supplementation of probiotics may mitigate oxidative stress and show a better outcome than placebo group. However, the add-on improvement did not occur in our study. Therefore, the possible influence of antipsychotics on the gut microbiota should be taken into account. Recently, the effects of the atypical antipsychotic drugs on the gut microbiome of 117 BPD patients have been examined in a cross-sectional design (Flowers et al., 2017). As compared with non-antipsychotic treated BPD patients, the BPD patients treated with antipsychotics were younger but had increased body mass index, and decreased species richness of the gut microbiota, suggesting that antipsychotic drugs have a negative impact in regulation of
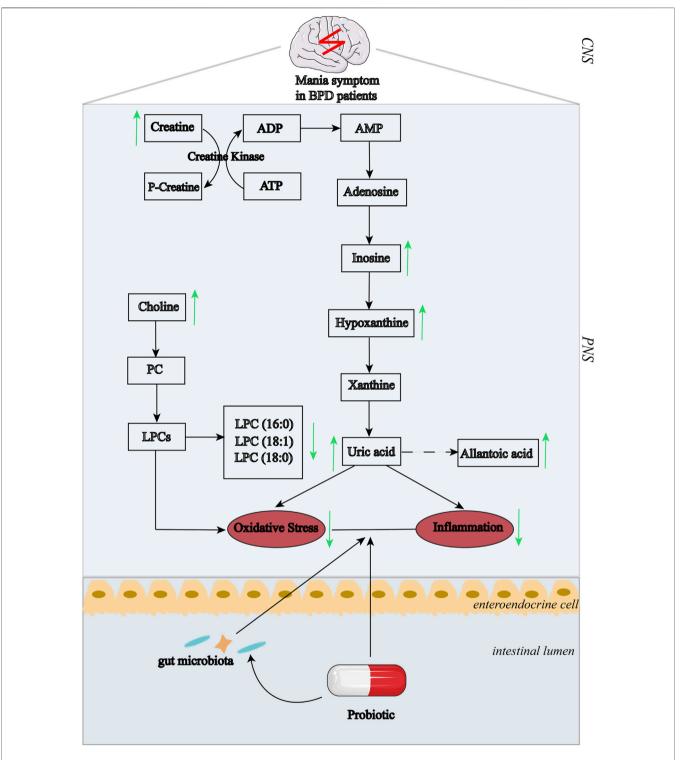
FIGURE 4 | Summary of the associations between pathways of purinergic metabolism and BPD symptoms after probiotic treatment. The changing trend in response to treatment:↑, increase; ↓, decrease. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PC, phosphatidylcholine; LPCs, lysophosphatidylcholines; CNS, central nervous system; PNS, peripheral nervous system.

gut microbiota. Thus, the beneficial effects of probiotics may be partially neutralized by the co-administrated psychotropic drugs in our study.

The other purpose of the present study was to evaluate the effects of probiotic treatment on clinical symptoms of BPD patients. The results revealed that mania symptom in patients

who received probiotic supplements alleviated more than that of the placebo group (**Table 2**). A significant reduction in mania symptoms observed in those BPD patients taking the probiotics reported by Dickerson and others was consistent with our findings (Dickerson et al., 2018). Their studies have shown that supplementation of probiotic prevents rehospitalization in patients with BPD, due to their anti-inflammatory effects through modulating the immune response (Dinan et al., 2013; Dickerson et al., 2018).

In addition, we also investigated the relationship between the improvement of BPD symptoms and changes of oxidative stress biomarkers after administrating probiotics. YMRS has primarily been used to evaluate the symptoms of mania in clinical trials. It is the main measure in the Systematic Treatment Enhancement Program for Bipolar Disorder study, which is the largest study to date on the effectiveness of treatment for BPD (Young et al., 1978). Notably, the improvement of YMRS scores presents a positive relationship with LPC (18:0) reductions in the probiotic group instead of the placebo group (**Figure 3**). LPCs are known to be a pathological component of oxidized LDL. It has been found that BPD patients experiencing a manic episode had significantly lower total cholesterol, high-density lipoprotein (HDL), and LDL than euthymic patients. The BPD patients have significantly lower total cholesterol and LDL levels in (hypo)manic than depressed patients (Fusar-Poli et al., 2020), indicating a greater oxidative stress in manic episode. As such, the decreased oxidation of LDL was related to the alleviation of manic symptoms in BPD. Our results emphasize the efficacy of probiotic supplements in specifically alleviating manic symptoms in BPD patients.

The underlying mechanisms of the therapeutic effects of probiotics on BPD symptoms are still unclear. Nonetheless, as mentioned earlier, there is evidence that probiotics may have anxiolytic effects itself (Foster and McVey Neufeld, 2013) by keeping the homeostasis of host microbiome (Wang et al., 2017). The absence of conventional gut microbiota influences the development of behavior accompanied by neurochemical alterations (such as N-methyl-D-aspartate, serotonin) in the brain as well (Neufeld et al., 2011). Another possible mechanism is that probiotics are able to reduce stress-induced corticosterone and the severity of BPD by improving the function of GABAergic system. However, similar neurochemical and behavioral effects were not observed in vagotomized mice. Interestingly, these results indicated that the vagus may be an important modulatory constitutive communication pathway between the gut microbiota and brain (Bravo et al., 2011).

There were limitations in the current study. First, the sample size was relatively small, especially only included BPD patients from one district of China. Further studies should include more patients from different districts to validate the findings. Second, the dietary profiles were not recorded, which could possibly influence precise analyses of the effects of probiotic. Third, the study of potential differences between the subtypes in plasma oxidative stress biomarkers was prevented, since bipolar I and bipolar II were not distinguished. Fourth, the drop-out rate is relatively high. Fifth, intention-to-treat (ITT) analyses was not performed. Finally, the modulatory effects of different types of psychotropic drugs on the composition of intestinal microbiota have not been investigated.

In conclusion, significant differences in oxidative stress-related biomarkers were determined in all BPD patients.

Moreover, the probiotic supplementation specifically alleviated manic symptom as compared to the placebo group, possibly by regulating purine metabolism and reduction of oxidative stress status (**Figure 4**).

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 Medical Ethics Committee of the Second Xiangya Hospital, Central South University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HC, HW, CZ, and YQ designed the study and drafted the manuscript. HW and YQ performed or coordinated patient recruitment, psychometric assessment, and blood sample collection. SL, ZT, HX, and JC supervised patient recruitment and sample collection, and ascertained the psychometric data. XW, TC, SZ, and QC planned and performed all of the statistical analyses, and/or commented on/edited the drafts of this work. CZ supervised the whole work and revised the manuscript according to the comments from the other authors. All authors have approved the final manuscript. The authors alone are responsible for the views expressed in this article and they do not necessarily represent the views, decisions, or policies of the institutions with which they are affiliated.

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Frontiers in Pharmacology

Gut Microbial Dysbiosis and Cognitive Impairment in Bipolar Disorder: Current Evidence

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Recent studies have reported that the gut microbiota influences mood and cognitive function through the gut-brain axis, which is involved in the pathophysiology of neurocognitive and mental disorders, including Parkinson's disease, Alzheimer's disease, and schizophrenia. These disorders have similar pathophysiology to that of cognitive dysfunction in bipolar disorder (BD), including neuroinflammation and dysregulation of various neurotransmitters (i.e., serotonin and dopamine). There is also emerging evidence of alterations in the gut microbial composition of patients with BD, suggesting that gut microbial dysbiosis contributes to disease progression and cognitive impairment in BD. Therefore, microbiota-centered treatment might be an effective adjuvant therapy for BD-related cognitive impairment. Given that studies focusing on connections between the gut microbiota and BD-related cognitive impairment are lagging behind those on other neurocognitive disorders, this review sought to explore the potential mechanisms of how gut microbial dysbiosis affects cognitive function in BD and identify potential microbiota-centered treatment.

Keywords: bipolar disorder, gut microbiota, cognitive function, gut-brain axis, psychobiotic

1 INTRODUCTION

Bipolar disorder (BD) is a mood disorder characterized by recurring manic or hypomanic episodes alternating with depressive episodes, which presence increases the risk of multisystem complications, including cognitive impairment and metabolic disorders, seriously affecting the quality of life (Goldstein et al., 2011; Carvalho et al., 2020). Moreover, symptomatic remission constantly precedes the recovery of psychosocial function following a mood episode in BD patients, which is a trend mostly attributed to persistent neurocognitive impairment (Gitlin and Miklowitz, 2017). Thus, therapies focusing on cognitive improvement are greatly significant, considering the well-being and quality of life of BD patients (Bonnín et al., 2019).

Cognitive impairment in BD patients is characterized by defects in neurocognitive areas, including executive function, verbal and visual memory, working memory, attention, and reaction time (Cullen et al., 2016). Tatay-Manteiga et al. (2018) observed neurocognitive dysfunction across all stages of BD. Selective cognitive decline even persists during remission in BD patients (Ott et al., 2021). Cross-sectional evidence also suggests that neurocognition may worsen with chronic comorbidity progression in BD patients (Berk et al., 2017). Compared to patients with

TABLE 1 | Gut microbial dysbiosis can affect cognitive function in rodent models.

References	Model type	Effects on cognitive function	Mechanism
Bercik et al. (2011)	germ-free mice	reduced exploratory behavior	reduced hippocampal levels of BDNF
Gareau et al. (2011)	germ-free mice	memory dysfunction	gut microbial dysbiosis; dysfunction of HPA axis
Crumeyrolle-Arias et al. (2014)	germ-free mice	deficits in social interaction	decreased dopaminergic turnover rate in the frontal cortex, hippocampus, and striatum
Desbonnet et al. (2014)	germ-free mice	social impairments and decreased social preference	gut microbial dysbiosis; modulation of immune cell cytokines release, changes in vagal nerve activity, and neuroendocrine function
Hoban et al. (2016b)	germ-free mice	anxiety-related behaviors and impaired social cognition	hypermyelinated axons in the prefrontal cortex
Luczynski et al. (2016)	germ-free mice	increased maladaptive stress responsivity	expansion and dendritic morphological changes in the amygdala and hippocampal
Lu et al. (2018)	germ-free mice	deficits in spatial memory, learning memory, and social novelty	abnormal morphological development and maturation in the grey and white matter
Desbonnet et al. (2015)	antibiotic-treated mice	deficits in memory and social interaction	altered dynamic of the tryptophan metabolic pathway; reduced BDNF, oxytocin, and vasopressin expression
Fröhlich et al. (2016)	antibiotic-treated mice	deficits in novel object recognition	gut microbial dysbiosis; brain region-specific changes in the expression of cognition-relevant signaling molecules, notably BDNF, NMDA receptor subunit 2B, serotonin transporter, and neuropeptide Y system
Hoban et al. (2016a)	chronic antibiotic-treated mice	deficits in spatial memory	altered CNS serotonin concentration along with changes in the mRNA levels of corticotrophin-releasing hormone receptor 1 and glucocorticoid receptor
Möhle et al. (2016)	antibiotic-treated mice	decreased working memory	decreased hippocampal neurogenesis; reduced Ly6C(hi) monocytes
Ceylani et al. (2018)	antibiotic-treated mice	decreased locomotor activity and impaired recognition memory	lower levels of serum BDNF are not associated with cognitive impairment but with changes in affective-like behaviors
Zhan et al. (2018)	senescence-accelerated mouse prone 8	deficits in learning and memory of spatial orientation	gut microbial dysbiosis
Lee et al. (2020a)	fecal transplant gavages from aged mice	depressive-like behavior, impaired short-term memory, and impaired spatial memory	decreased fecal SCFAs, acetate, propionate, and butyrate
Lee et al. (2020b)	oral gavage of *Escherichia coli*	deficits in spatial learning and memory	gut microbial dysbiosis; release of lipopolysaccharide; stimulation of vagal-dependent gut-brain signaling
Pearson-Leary et al. (2020)	short-defeat latencies/vulnerable rats	increased depressive-type behaviors	inflammation in the ventral hippocampus; higher microglial density and IL-1β expression in the ventral hippocampus
Xie et al. (2020)	fecal transplant gavages from septic mice	learning impairments and anxiety-like behaviors	gut microbial dysbiosis; stimulation of vagal-dependent gut-brain signaling
Wang et al. (2021)	fecal transplant gavages from sleep deprivation patients in germ-free mice	deficits in attention and memory domain	metabolic dysbiosis; increased neuroinflammation and microglial activity in the hippocampus and medial prefrontal cortex
Hua et al. (2021)	spared nerve injury mice	deficits in spatial learning and memory	the increase of *Actinobacteria*, *Proteus*, and *Bifidobacterium*; disturbances of lipids and amino acid metabolism

schizophrenia (Barch and Sheffield, 2014) or neurodegenerative diseases, such as Alzheimer's disease (AD) (Simjanoski et al., 2021), BD patients exhibit a similar cognitive profile but with a milder degree of impairment, suggesting that there is overlapping pathophysiology underlying the cognitive impairment across these diseases. Currently, potential mechanisms of cognitive impairment in BD patients require further exploration. Neuroimaging studies have identified certain structural abnormalities associated with cognition, including a reduction in the prefrontal lobe (Abe et al., 2015) and hippocampal volume (Hoseth et al., 2016) and cortical thinning. A reduction in brain volume, especially in the hippocampus, is mostly attributed to excessive glucocorticoid exposure induced by oxidative stress (Alfarez et al., 2008) and abnormal neuroplasticity induced by inflammation and reduced brain-derived neurotrophic factor

(BDNF) concentrations (Mondelli et al., 2011). Multiple studies have also demonstrated that significant associations exist between cognitive impairment in BD and inflammation, oxidative stress, and metabolic disorders (Hoseth et al., 2016; Cuperfain et al., 2020).

With the emergence of microbiome research, gut microbiota and gut-brain hormones have also been linked to cognitive impairment in severe mental disorders (Bioque et al., 2021; Misiak et al., 2020). Gut microbiota may modulate the function of the central nervous system, thereby altering behavior and cognition (**Table 1**), while the brain can activate signaling pathways affecting immune and metabolic function and host behavior—influencing the population and composition of the gut microbiota. The 2-way crosstalk between the central nervous system and gut microbiota *via* various routes
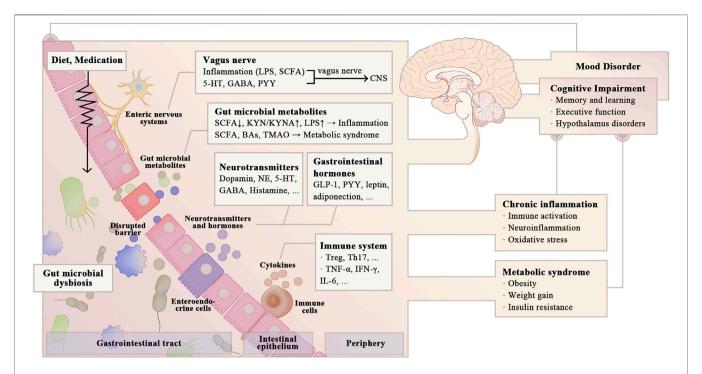
FIGURE 1 | Possible pathways for gut microbial dysbiosis to affect cognitive function in BD. Gut microbial dysbiosis is widely viewed in BD patients, which may have a negative effect on cognitive function (e.g., learning and memory, executive function, and cognitive flexibility). Alterations in the gut microbial composition in BD patients increase intestinal permeability, promoting the release of pro-inflammatory cytokines and microbial-derived metabolites into the circulatory system, causing systemic inflammation and metabolic dysbiosis. Gut microbiota also modulates energy metabolism and cognitive function by influencing the synthesis of neurotransmitters and gastrointestinal hormones, as well as the vagal-dependent gut-brain signaling. Psychiatric medications and changing diet patterns in BD patients have complex interactions with gut microbiota, thereby influencing cognitive function. LPS, lipopolysaccharides; SCFA, single-chain fatty acids; GABA, γ-aminobutyric acid; PYY, Peptide YY; KYN, kynurenine; KYNA, kynurenic acid; Ba, bile acid; TMAO, trimethylamine-N-oxide; NE; GLP-1, glucagon-like peptide-1; Treg, regulatory T cell; Th, helper T cell; INF, Interferon; IL, Interleukin.
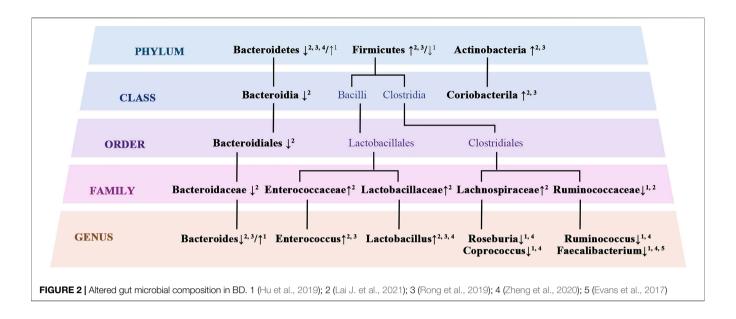
including the immune system, enteroendocrine signaling, the vagus nerve and the enteric nervous system, as well as multiple gut microbial metabolites is known as the brain-gut axis (Cryan et al., 2019). The gut microbiota might be involved in postnatal development and have long-term implications for brain function (Sharon et al., 2016). Increased intestinal permeability promotes the release of pro-inflammatory cytokines and microbial-derived metabolites [e.g., lipopolysaccharides (LPS) and lipoproteins] into the circulatory system, causing systemic inflammation and blood-brain barrier impairment (Braniste et al., 2014; Schirmer et al., 2016; Houser and Tansey, 2017; Zhao et al., 2017). Neurochemical signals activated by the gut microbiota can be transmitted from the enteric nervous system to the central nervous system *via* the vagus nerve (Forsythe et al., 2014; Fung et al., 2017). Gut microbial dysbiosis also alters the expression of 5-hydroxytryptamine (5-HT) receptors, neurotrophic factors (e.g., BDNF), and N-methyl-d-aspartic acid (NMDA) receptor subunits in the hippocampus (Bercik et al., 2011), as well as myelin formation in the prefrontal cortex (Hoban et al., 2016a), leading to impaired social cognition. Such extensive overlapping mechanisms suggest a significant role of the gut microbiota in the development of cognitive impairment in BD.

Studies explicitly linking the gut microbiota to cognitive impairment in BD remain limited in number compared to those focusing on other neurodegenerative and psychiatric disorders. Therefore, this review summarizes preclinical and clinical evidence to explore potential mechanisms by which the gut microbiota affects cognitive function in BD patients and identify potential microbiota-centered treatment. Studies linking the gut microbiota to cognitive impairment in neurodegenerative and mental disorders might have implications for BD patients. Several studies have addressed the involvement of microbial-derived metabolites [e.g., single-chain fatty acids (SCFAs), secondary bile acids (BAs), and LPS], neurotransmitters, and gastrointestinal hormones in cognitive function. Complex interactions with the gut microbiota may also explain some cognitive side effects of certain psychiatric medications (**Figure 1**). This review concludes by discussing potential cognitive treatment targeting the gut microbiota, which may improve the quality of life of BD patients.

2 Direct Effects of the Gut Microbiota and its Metabolites on Cognitive Function in BD Patients

Evidence of altered gut microbial composition in BD patients suggests that gut microbial dysbiosis contributes to disease progression and pathophysiology in BD patients (Evans et al., 2017; Lai et al., 2020; McIntyre et al., 2021). Although there are

FIGURE 2 | Altered gut microbial composition in BD. 1 (Hu et al., 2019); 2 (Lai J. et al., 2021); 3 (Rong et al., 2019); 4 (Zheng et al., 2020); 5 (Evans et al., 2017)

few studies discussing the relationship between gut microbiota and cognitive impairment in BD patients, some have found that specific gut microorganisms are associated with reduced cognitive function (Severance et al., 2016). The relationship between the gut microbiota and specific cognitive domains, including learning, memory, attention, processing speed, and executive function—all of which are also impaired in BD—has been demonstrated in animal and human studies on hepatic encephalopathy (Bajaj et al., 2012), diabetes mellitus (Zheng et al., 2021), and aging (Li et al., 2021). Gut microbial dysbiosis also contributes to cognitive impairment in various neurodegenerative and psychiatric disorders, including Parkinson's disease (Mulak and Bonaz, 2015; Sampson et al., 2016), AD (Jiang et al., 2017), and schizophrenia (Bioque et al., 2021), suggesting potential overlapping mechanisms between these conditions and BD.

2.1 Gut Microbial Alterations in BD Patients

Disruption of intestinal homeostasis affects the host's metabolism and immune responses, leading to systemic disorders ranging from metabolic syndrome to chronic inflammation, which is closely related to the development of cognitive impairment (Kesika et al., 2021; Liu et al., 2020; McGrattan et al., 2019; Zeng et al., 2021). Therefore, a quantitative analysis of the gut microbiota in BD patients may help to improve an understanding of the mechanisms behind the development of cognitive impairment in this population. The most dominant gut microbial phyla in healthy adults include Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia, with Firmicutes and Bacteroidetes accounting for nearly 80% of the total amount (Eckburg et al., 2005). In contrast, multiple studies have demonstrated alterations in gut microbial composition in BD patients compared to healthy controls (**Figure 2**). Lai et al. (2020) found that, in BD patients, counts of *Faecalibacterium prausnitzii*, *Bacteroides*, *Prevotella*, *Atopobium* cluster, *Enterobacter* spp., and *Clostridium* cluster

organisms were significantly increased, and the log10 (ratio of *Bifidobacteria* to *Enterobacteriaceae*) was decreased. Another two studies have shown an increase in organisms of the *Firmicutes* and *Bacteroides* phyla but a decrease in those of the *Bacteriodetes* phylum in BD patients, with the Firmicutes-to-Bacteriodetes ratio being accordingly increased (Rong et al., 2019; Lai WT. et al., 2021). An increased Firmicutes-to-Bacteriodetes ratio is a recognized indicator of obesity (Crovesy et al., 2020; Magne et al., 2020). However, bacteria of the *Ruminococcaceae* family and *Faecalibacterium* genus, which are butyrate-producing bacteria, were reduced in BD patients relative to healthy controls in other studies (Hu et al., 2019; Lai WT. et al., 2021; Sublette et al., 2021). In 4 of 5 studies, lower α-diversity was observed in BD patients compared to healthy controls (Sublette et al., 2021).

In conclusion, the most convergent taxonomic finding in BD patients is the reduction in butyrate-producing bacteria, gut microbiota known to impact cognitive function by producing SCFAs, including acetate and butyrate (Tanca et al., 2017). A decrease in butyrate-producing bacteria, such as those of the genus *Faecalibacterium*, was also observed in patients with AD and accompanied by neuroinflammation and impaired cognitive function (Ling et al., 2020; Marizzoni et al., 2020). Associations between cognitive impairment and decreased butyrate-producing bacteria have also been observed in patients with Parkinson's disease (Nuzum et al., 2020; Tan et al., 2021) and alcohol use disorders (Leclercq et al., 2020) and are likely mediated by butyrate. It has been demonstrated that butyrate levels in the central nervous system can be influenced by gut microbial composition; for example, *Clostridium butyricum*, as a probiotic bacteria, restored butyrate content in the brain and significantly alleviated cognitive impairment and histopathological changes in a mouse model of vascular dementia (Liu et al., 2015). *Clostridium butyricum* treatment also attenuated cognitive impairment and prevented microglia-mediated neuroinflammation in a manner mediated by butyrate in an AD mouse model (Sun et al., 2020). Prolonged treatment

with sodium butyrate stimulates neurogenesis and improves memory and associative learning (Kim et al., 2009; Govindarajan et al., 2011). On the one hand, butyrate inhibits histone deacetylase, which plays an important role in intestinal barrier regulation and intestinal energy metabolism, thereby affecting cognitive function (Leonel and Alvarez-Leite, 2012; Tanca et al., 2017). Butyrate can also increase hippocampal neurogenesis and the expression of the neurotrophic factor BDNF, improving learning behavior and long-term memory (Levenson et al., 2004; Sleiman et al., 2016). Considering the neuroprotective and cognitive improvement effects of butyrate, inadequate concentrations of butyrate-producing bacteria may be involved in the pathophysiology of cognitive impairment in BD patients.

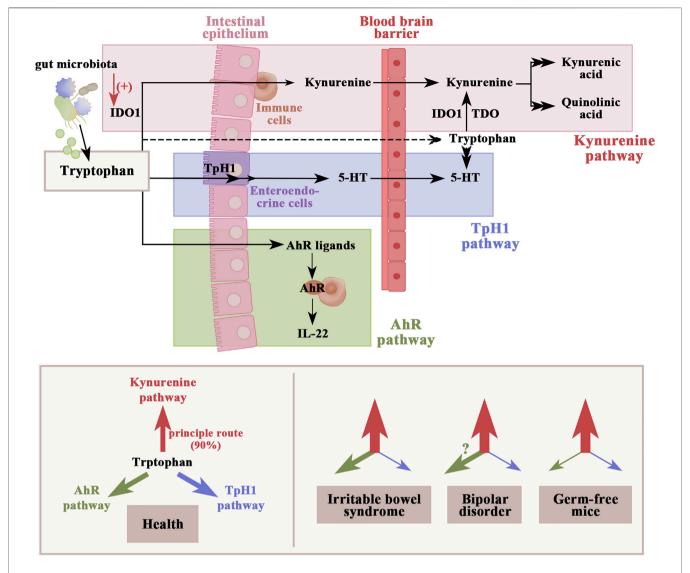## 2.2 Effects of Gut Microbial Metabolites on Cognitive Function in BD Patients

The interaction between butyric acid-producing bacteria and cognitive impairment suggests that microbiota-produced small-molecule metabolites mediate host-microbiome interactions (Donia and Fischbach, 2015). An analysis of brain transcriptomic data from BD patients has also revealed disturbances in gut microbial metabolites, such as tryptophan, SCFAs, and BAs (Moolamalla and Vinod, 2020). These overlapping metabolite profiles suggest potential routes for the involvement of the gut microbiota in cognitive impairment in BD.

2.2.1 Effects of Inflammatory Gut Microbial Metabolites

SCFAs (including acetate, propionate and butyrate), which are typical anti-inflammatory molecules produced by the gut microbiota, can exert anti-inflammatory effects by inhibiting interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α) expression through the *FFAR2* (GPR43) receptor (Pirozzi et al., 2018). Animal studies have provided evidence on the effects of SCFAs on cognitive function through immunological pathways. The effects of butyrate on cognitive function have been described above. Additionally, decreased absolute concentrations of acetate, propionic acid, and butyrate lead to blood-brain barrier dysfunction, microglial activation, and elevated cortical IL-1β, IL-6, and TNF-α expression levels in mice maintained on a high-salt diet, exhibiting a reduced number of organisms in the *Bacteroidetes* and *Proteobacteria* phyla and an increased number of those in the *Firmicutes* phylum, respectively (Hu et al., 2020). Mice with a deficiency of the SCFA receptor *FFAR2* showed global defects in microglia similar to those of germ-free mice, suggesting that the gut microbiota regulates microglia maturation and function (Borre et al., 2014; Erny et al., 2015). Microglia cells mediate neuroinflammation as the major innate immune cell population in the brain, playing an important role in the pathophysiology of BD (Maletic and Raison, 2014) and cognitive impairment (Feng et al., 2017; Zhao et al., 2019). However, to our knowledge, studies directly linking SCFAs to cognitive impairment in BD are still scarce. Indirectly, butyrate has been tested as a potential treatment for mood disorders, including BD and major depressive disorder, acting by

controlling epigenetic programming associated with cognitive and behavioral regulation as a histone deacetylase inhibitor (Machado-Vieira et al., 2011). Lithium carbonate, one of the most commonly used drugs for treating BD, may activate anti-inflammatory regulatory T-cell responses through an *FFAR2*-dependent mechanism by altering the SCFA-producing gut microbiota (e.g., through upregulation of the butyric acid-producing bacterium *Akkermansia muciniphila*) to change SCFA profiles (Huang et al., 2022). With the support of some clinical studies, given the positive effects of lithium carbonate on cognitive functions, including memory and attention (Dias et al., 2012), disruption of the SCFA profile may impair cognitive function in BD patients through systemic inflammation. Marizzoni et al. (2020) found that both cognitive function and endothelial dysfunction in older adults are positively correlated with the pro-inflammatory cytokines acetate and valerate but negatively correlated with the levels of butyric acid and IL-10. The gut microbiota with reduced SCFA production can also trigger an intestinal inflammatory response and progression of Parkinson's disease (Unger et al., 2016; Vascellari et al., 2020; Aho et al., 2021). Such clinical evidence suggests that SCFAs are involved in the development of cognitive impairment through systemic inflammation caused by endothelial dysfunction.

Gut-derived tryptophan metabolites are also one of the microbiome-dependent signals regulating inflammatory responses in the host. In the gastrointestinal tract, tryptophan metabolism follows three major pathways, including the kynurenine pathway, the aryl hydrocarbon receptor pathway, and the serotonin production pathway (**Figure 3**). A review by Więdłocha et al., 2021) supposed that metabolites of tryptophan degradation along the kynurenine pathway not only have an adverse effect on several psychiatric disorders, including BD, schizophrenia, depression, dementia, and AD, but also toxicity on cognitive function. An experiment conducted by Leblhuber et al. (2018) revealed that elevation of the kynurenine pathway may be associated with reduced *Faecalibacterium prausnitzii* and activation of macrophages in AD patients with impaired cognitive function. Notably, BD patients also show reductions in *Faecalibacterium* populations (Hu et al., 2019). Several studies have determined that gut microbial dysbiosis activates indoleamine 2, 3-dioxygenase 1, the rate-limiting enzyme in the kynurenine pathway (Agus et al., 2018). Depletion of the gut microbiota in mice induces elevation of the kynurenine–kynurenic acid pathway, anxiety-like behavior, and cognitive deficits (Desbonnet et al., 2015). The kynurenine–kynurenic acid pathway is also elevated in germ-free mice transplanted with microbiota from schizophrenic patients, leading to impaired learning and memory functions (Zhu et al., 2020). In contrast, performances in both cognitive function (including object exploration and recognition, passive avoidance, and spatial discrimination, all depending on the integrity of hippocampal function) and synaptic plasticity were improved in a rodent model with reduced kynurenic acid synthesis (Potter et al., 2010). Aside from the kynurenine pathway, tryptophan can also be degraded into aryl hydrocarbon receptor agonists by the gut microbiota, which not only protect against increased gut permeability (Scott

FIGURE 3 | Dietary tryptophan metabolism follows three major pathways in the gastrointestinal tract: 1) the kynurenine pathway *via* IDO1, which can be activated by gut microbial dysbiosis; 2) production of serotonin in enteroendocrine cells and indirect regulation of tryptophan availability under the control of gut microbiota; and 3) the direct conversion to AhR ligands by gut microbiota. The figure shows an altered dynamic of the tryptophan metabolic pathway in IBS, BD, and germ-free mice based on the available clinical data. Weights of arrows indicate the strength of pathway activation. IDO1, indoleamine 2,3-dioxygenase 1; TDO, tryptophan 2,3-dioxygenase; TpH1, tryptophan hydroxylase 1; AhR, aryl hydrocarbon receptor.

et al., 2020) but also reduce neuroinflammation by inducing interferon-I signaling in astrocytes (Rothhammer et al., 2016). Therefore, interventions aimed specifically at reducing kynurenine pathway activation may constitute a promising strategy for cognitive improvement in BD patients.

Gut microbial dysbiosis also increases the production of LPS, a pro-inflammatory endotoxin, which diffuses into the blood along with increased gut permeability. LPS also contributes to the development of cognitive impairment through systemic inflammation, as evidenced by lower scores on the Boston naming test measuring visual confrontation naming, Verbal Fluency measuring executive function, and Word List Memory test measuring working memory (Chen et al., 2008). Additionally, LPS has adverse effects on both structure and function in the

brain, increasing the activity of the amygdala responsible for emotion control and affecting object-exploration behavior by impairing cognitive function (Haba et al., 2012). LPS also decreases BDNF expression in the hippocampus, inducing cognitive impairment (Dinel et al., 2014). Consistently, gut-derived LPS-related immune activation has been observed in BD patients (Rudzki and Szulc, 2018). In mice transplanted with intestinal flora from BD patients, elevated levels of inflammatory cytokines and *TRANK1* messenger RNA (*TRANK1* is an important risk gene of BD) in the hippocampus and prefrontal cortex may be associated with LPS stimulation of BV-2 microglia (Lai J. et al., 2021). Therefore, LPS leakage due to gut microbial dysbiosis may play an important role in cognitive impairment in BD.

Bidirectional crosstalk between the hypothalamic-pituitary-adrenal (HPA) axis and the gut-brain axis in severe mental illness has been demonstrated in several studies (Misiak et al., 2020; Bioque et al., 2021), in which pro-inflammatory gut microbial metabolites play a key role. SCFAs not only decrease the activity of microglia and limit local inflammatory processes, but also decrease the expression of genes encoding proteins involved in the HPA axis (van de Wouw et al., 2018). LPS can directly stimulate cortisol secretion by human adrenal cells by a cyclooxygenase-dependent mechanism (Vakharia and Hinson, 2005). Reduced SCFA production (Pirozzi et al., 2018) and LPS leakage (Chen et al., 2008) in BD patients can also trigger an intestinal inflammatory response, enhancing release of cytokines, including IL-1β, IL-6 and TNF-α, all of which are potent activators of the HPA axis (Turnbull and Rivier, 1995). Since HPA axis activation and increased basal cortisol are known to be responsible for cognitive impairment including worse performance in visuospatial associative memory, attention and executive function in BD patients (Tournikioti et al., 2018), gut microbial dysbiosis may impair cognitive function in BD patients by activating the HPA axis. A study by Aizawa et al. (2018) provide some insight into the association between gut microbial dysbiosis and the HPA axis dysregulation in BD, as a significant negative correlation between the count of *Bifidobacterium* and cortisol levels was found. Notably, HPA axis dysregulation in BD may in turn further exacerbate gut microbial dysbiosis and intestinal permeability (Vanuytsel et al., 2014; Yoshikawa et al., 2017), thus creating a vicious cycle leading to further cognitive impairment.

2.2.2 Effects of Gut Microbial Metabolites Associated With Metabolic Syndrome

Studies have shown that gut microbial metabolites (including SCFAs, BAs, and LPS) as signaling molecules regulate physiological processes ranging from appetite and intestinal motility to energy metabolism in the host (Heiss and Olofsson, 2018). Therefore, gut microbial dysbiosis may lead to metabolic disorders and contribute to metabolic comorbidities, including diabetes mellitus and insulin resistance, playing an important role in the pathophysiology of various psychiatric diseases, including BD. BD patients presenting with metabolic syndromes are often at high risk of cognitive impairment.

In addition to SCFAs, food-derived gut microbial metabolites include BAs, trimethylamine-N-oxide (TMAO), and glutamate. BAs are synthesized from cholesterol in the liver and further metabolized to secondary bile acids by the gut microbiota (Schmidt et al., 2010). Gut microbial dysbiosis disrupts the signaling of BAs binding to the nuclear farnesoid X receptor (FXR) and Takeda G protein-coupled membrane receptor 5 (TGR5) (Huang et al., 2016; Chiang and Ferrell, 2020), which is also found in the brain (He et al., 2021). Although the effect of BAs on cognitive function in BD remains unclear, an increased ratio of secondary cytotoxic BAs to primary BAs has been linked to AD and cognitive impairment (MahmoudianDehkordi et al., 2019). INT-777, a TGR5 agonist, ameliorates synaptic dysfunction and reverses Aβ1-42-induced cognitive impairment in the mouse model of acute neurotoxicity by

upregulating the expression of postsynaptic and presynaptic proteins (PSD95 and synaptophysin) and inhibiting apoptosis (Wu et al., 2018). BAs, such as tauroursodeoxycholic acid, have also been suggested as a potential treatment for AD (Zangerolamo et al., 2021). Thus, BAs may also be potential mediators between gut microbiota and cognitive impairment in BD patients.

TMAO has also been demonstrated to mediate gut microbiota-induced cognitive impairment. Administration of TMAO increases synaptic impairments by inhibiting the mammalian target of the rapamycin signaling pathway and decreases the expression levels of synaptic plasticity-related proteins, thereby exacerbating cognitive impairment (Li et al., 2018). A choline-induced AD mouse model exhibited increased TMAO synthesis, which is positively correlated with cognitive deterioration (Wang et al., 2020). Therefore, the role of TMAO in BD patients deserves further investigation.

3 THE ROLE OF GASTROINTESTINAL HORMONES AND NEUROTRANSMITTERS IN THE INTERACTION BETWEEN THE GUT MICROBIOTA AND COGNITIVE IMPAIRMENT IN BD

The brain–gut axis reflects a complex bidirectional communication network between the gut microbiota and the brain, which relies on various neurotransmitters, gastrointestinal hormones, cytokines, and growth factors (Quigley, 2017; Cryan et al., 2019). These neurotransmitters and gastrointestinal hormones are produced by entero-endocrine cells in response to gut microbial inducement (Heiss and Olofsson, 2018). The gut microbiota also regulates the metabolisms of amino acids essential for the synthesis of neurotransmitters and gastrointestinal hormones (Holzer, 2016). Thus, gastrointestinal hormones and neurotransmitters may be important mediators in the interaction between the gut microbiota and cognitive impairment in BD.

3.1 The Role of Gastrointestinal Hormones

Gut microbial dysbiosis increases the intestinal permeability and penetration of pro-inflammatory factors, such as LPS, leading to intestinal inflammation, which, in turn, promotes energy absorption and reduces satiety *via* gastrointestinal hormones (Gomes et al., 2018; Nagpal et al., 2018). These gastrointestinal hormones have receptors expressed in regions of the brain that regulate not only hunger and energy metabolism but also stress, behaviors, and cognitive function (Chan et al., 2003; Bucinskaite et al., 2009).

Glucagon-like peptide-1 (GLP-1), an insulinotropic hormone secreted by entero-endocrine L-cells, can stimulate glucose-dependent insulin secretion in response to carbohydrate uptake (Holt & Trapp, 2016). In rodent models, butyrate and propionate stimulate GLP-1 secretion from entero-endocrine L-cells *via FFAR2* (Tolhurst et al., 2012). Therefore, reduced concentrations of butyrate-producing bacteria in BD patients

may downregulate GLP-1 secretion. GLP-1 receptors are expressed in the cerebral cortex, hypothalamus (ventral medial nucleus and arcuate nucleus), and limbic system (amygdala and hippocampus), all of which are regions responsible for the regulation of emotion and cognition (Heppner et al., 2015). GLP-1 and GLP-1 receptor (GLP-1R) signaling have a neuroprotective role in the control of insulin resistance, synaptic plasticity, and neuroinflammation and improve cognitive function in learning, memory, executive function, and attention (Müller et al., 2019; Flintoff et al., 2021). Therefore, reduced GLP-1R signaling caused by reduced butyrate-producing bacteria in BD patients may impair synaptic plasticity and cognitive function, as demonstrated by several studies. Liraglutide, a GLP-1 agonist, reversed manic-like symptoms and impairment in working and recognition memory by improving hippocampal oxidation and BDNF levels in a D-amphetamine-induced BD model (Chaves Filho et al., 2020). Serum GLP-1 levels are significantly lower in BD patients compared to healthy controls and negatively correlate with previous mood episodes (Rosso et al., 2015). In non-diabetic BD patients, liraglutide had beneficial effects on several cognitive domains, including auditory verbal learning, working memory, and attention (Mansur et al., 2017). Therefore, it is likely that gut microbial dysbiosis affects cognitive function in BD patients *via* GLP-1R signaling.

Peptide YY (PYY) is another intestinal satiety hormone produced by entero-endocrine L-cells, which affects the central nervous system by inhibiting orexigenic neurons expressing neuropeptide Y (Wynne and Bloom, 2006). Although there is limited evidence directly linking PYY to BD, a reduction in peripheral PYY concentrations might underlie the lack of GABA inhibition associated with impaired cognitive function in BD, considering that both neuropeptide Y and γ-aminobutyric acid (GABA) are released by arcuate neuropeptide Y neurons (Acuna-Goycolea et al., 2005; Huber et al., 2018).

Adipokines, represented by adiponectin and leptin, are cytokines or hormones secreted by white adipocytes in response to increased circulating inflammatory factors (Aguilar-Valles et al., 2015). Gut microbial dysbiosis elicits the penetration of microbial components (e.g., LPS), compromising leptin signaling and leading to leptin resistance and sustained high leptin levels (Faggioni et al., 1997). Intracerebroventricular administration of LPS induces an elevation in leptin receptors in the hippocampus, accompanied by impaired learning and memory, suggesting that leptin signaling disturbances in the hippocampus are involved in the regulation of cognitive responses (Da Ré et al., 2020). Studies suggest that leptin levels may affect cognitive function in BD patients. The TNF-α antagonist, infliximab, reduces plasma leptin levels in BD patients by modulating soluble tumor necrosis factor receptor 2, contributing to better performance on non-literal memory tasks and increased overall cortical volume, both of which are negatively correlated with the leptin level (Mansur et al., 2020). Adiponectin is the most abundant plasma adipokine that regulates energy expenditure, and it improves insulin sensitivity and fatty acid oxidation as an anti-inflammatory factor (Berg et al., 2002; Kubota et al., 2007). In current studies, adiponectin has been shown to improve cognitive function mainly by regulating insulin sensitivity and inhibiting inflammation (Rizzo et al., 2020). Additionally, it alleviates isoflurane-induced cognitive impairment in aging models by activating the p38-mitogen-activated protein kinase pathway and promoting the proliferation of hippocampal precursor cells (Zhang et al., 2019). Studies on adiponectin levels in BD patients remain controversial since body mass index seems to be more strongly correlated with adiponectin levels than mood status in BD patients (Platzer et al., 2019). Therefore, it is difficult to identify the role of the gut microbiota in the interaction between adiponectin and cognitive function in BD patients, which may be more directly correlated with comorbidities in BD patients.

Cholecystokinin (CCK) is a satiety hormone secreted from I-cells of the proximal small intestines during digestion (Raybould, 2007). Vagal afferent neurons, primary sensory neurons to regulate meal size, exhibit decreased CCK sensitivity when the gastrointestinal tract is colonized with high-fat-type microbiome, leading to increased food intake and body weight (Kim et al., 2020). LPS leakage due to increased inflammatory microbiome including *Bacteroides* and *Prevotella* also impairs CCK-induced satiety, and promotes food intake and excessive weight gain in aging mice (Rubio et al., 2021). Therefore, decreased CCK signaling caused by gut microbial dysbiosis may exacerbate emotional and cognitive impairment associated with obesity and metabolic syndrome. Except for binding to local vagally expressed receptors, periphery CCK can also act on CCKB receptors, which is widely expressed in limbic system (including the hippocampus and the prefrontal cortex), and thus directly affect emotion and cognition (Ballaz et al., 2020). Despite multiple studies showing consistent cognitive enhancing effects by CCKB receptor activation (Taghzouti et al., 1999; Hebb et al., 2005; Plagman et al., 2019), its role is virtually unknown in BD. Only one study by Sears et al. (2013) found associations between suicide attempt and 12 SNPs of CCKB receptors in BD patients. Therefore, further investigation into the role of CCK in cognitive function in BD is warranted.

3.2 The Role of Neurotransmitters Regulated by the Gut Microbiota

Gut microbiota can produce most of the neurotransmitters or neuromodulators in the human brain, including dopamine (which can be produced by *Bacillus* and *Serratia*), norepinephrine (produced by *Escherichia*, *Bacillus*, and *Saccharomyces*), serotonin (produced by *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus*), or GABA (produced by *Lactobacillus* and *Bifidobacterium*) (Donia and Fischbach, 2015; Strandwitz, 2018). These neurotransmitters mediate gut-brain signals, communicating with various regions of the brain, including the frontal cortex, limbic system, and autonomic and neuroendocrine centers, regulating not only appetite-related sensations but also emotion, cognition, and behavior (Holzer, 2016).

Dysregulation of the dopaminergic system contributes to both pathophysiology and impaired executive function in BD (Bercik et al., 2011; Kao et al., 2018). Various gut microbiota can produce dopamine, leading to dopaminergic aberrations when gut microbial dysbiosis occurs in BD (Tsavkelova et al., 2000). Mice treated with the dopamine transporter protein inhibitor GBR12909 (15 mg/kg) constitute a validated animal model for BD, whereas germ-free mice are less susceptible to GBR12909 with less mania-like behavior, suggesting that the gut microbiota contributes to the disease progression of BD *via* the dopaminergic system (de Miranda et al., 2020). Additionally, para-Cresol-treated mice with social-behavioral deficits have a similar gut microbial profile to that of BD patients (e.g., depleted *Clostridiales*), with a reduction in excitability and number of evoked action potentials of dopamine neurons in the ventral tegmental area (Bermudez-Martin et al., 2021). The increase in *Bacteroides* and *Prevotella*, which is also observed in BD patients, is negatively associated with dopamine transporter expression in the brain (Hartstra et al., 2020). The application of probiotics has been demonstrated to prevent cognitive impairment by regulating the gut microbiota, thereby increasing serotonin, dopamine, and GABA levels and restoring neuronal impairment (Song et al., 2021). The evidence presented above strongly supports the interaction between the dopaminergic system and gut microbiota, underlying the cognitive impairment in BD.

Serotonin, also known as 5-HT, is significantly correlated with cognitive function in BD patients (Chou et al., 2012). In clinical studies, tryptophan levels and the kynurenine-dependent tryptophan index were reduced in patients with bipolar mania and were positively correlated with the Young Mania Rating Scale and Brief Psychiatric Rating Scale scores (Myint et al., 2007). Reduced 5-HT levels in the brain lead to impaired cognitive flexibility, which is the characteristic type of cognitive impairment in BD (Evers et al., 2007). Notably, the gut microbiota can divert tryptophan metabolism to the production of kynurenine instead of 5-HT by activating indoleamine 2, 3-dioxygenase and tryptophan 2,3-dioxygenase (Badawy, 2017). A lack of aryl hydrocarbon receptor ligands in the intestinal contents of germ-free mice has been consistently observed (Lamas et al., 2016). In germ-free mice, there are also relatively low plasma kynurenine levels accompanied by increased indoleamine 2,3-dioxygenase activity, and kynurenine levels increase in these animals after recolonization of the gut microbiota (Clarke et al., 2013; Van der Leek et al., 2017). The altered dynamic of the tryptophan metabolic pathway has also been implicated in a study of postmortem anterior cingulate gyrus in BD patients, showing increased tryptophan 2,3-dioxygenase activity and kynurenine levels (Miller et al., 2006). Thus, the activation of the kynurenine pathway by gut microbiota dysbiosis consequently increases tryptophan consumption, which potentially contributes to the reduced 5-HT neurotransmission in BD patients, a statement being supported by studies focusing on comorbid irritable bowel syndrome (IBS) in BD patients (Tseng et al., 2016) (**Figure 3**). IBS, characterized by abdominal pain, bowel movement disorders, and gut microbial dysbiosis, is considered to be a valid contributor to BD (Castellini et al., 2016; Rosenblat et al., 2020). A follow-up study showed that IBS patients in clinical remission still experience persistent attention impairment, with a continuous increase in plasma IL-6 levels, the kynurenine-to-tryptophan ratio, and inactivation of the cortisol awakening response (Clarke et al.,

2020). Acute tryptophan depletion significantly reduces plasma tryptophan and 5-hydroxyindoleacetic acid in IBS patients, inducing a negative shift in affective memory but without significant changes in mood (Kilkens et al., 2004). The evidence above suggests that 5-HT regulation by the gut microbiota may be involved in cognitive impairment in BD patients.

The gut microbiota profoundly affects peripheral GABA levels. Multiple organisms of gut microbiota are also involved in GABA synthesis, including a range of *Bifidobacterium*, *Lactobacillus*, and 16 intestinal *Bacteroides* strains (Barrett et al., 2012; Luck et al., 2021; Otaru et al., 2021). In germ-free animals, there is a significant reduction in GABA levels in the gut, serum, and brain (Matsumoto et al., 2013). Intestinal and peripheral GABA levels may influence the central nervous system through the gastrointestinal vagal nervous system. Bravo et al. (2011) found that treatment with *Lactobacillus rhamnosus* increases GABA(B1b) messenger RNA expression in cortical (cingulate and prefrontal) regions and decreases expression in the hippocampus and amygdala, consequently reducing corticosterone levels and manic-like behaviors. Moreover, the neurochemical and behavioral effects disappeared in vagotomized mice. *Lactobacillus casei* also stimulates gastrointestinal afferent vagal activity and inhibits stress-induced activation of cells producing pro-adrenocorticotropic hormone in the hypothalamic paraventricular nucleus in a dose-dependent manner, ameliorating somatic symptoms induced by learning stress (Takada et al., 2016). As reviewed by Wagner-Skacel et al. (2020), *Lactobacillus* has been significantly associated with the circadian rhythm in BD patients, linking a GABA disorder caused by the gut microbiota to cognitive function in BD. Thus, the gut microbiota may affect the GABA system mostly through the intestinal vagus nerve and modulate stress-related behavioral and cognitive functions in BD patients.

Multiple studies have demonstrated the versatility of glutamate, the agonist of N-methyl-d-aspartate receptor (NMDAR), as the foremost excitatory neurotransmitter in the central nervous system and modulator of gastrointestinal metabolism (Baj et al., 2019). A systematic review conducted by Reddy-Thootkur et al. (2020) shows that hippocampal glutamate levels are increased in BD patients, but no associations between glutamate metabolite levels and memory performance are detected. Considering that many of those included studies suffered from small sample sizes, the relationship between glutamate and cognitive impairment in BD require further exploration (Reddy-Thootkur et al., 2020). A postmortem study in 10 BD patients revealed significant lower protein and mRNA levels of NMDAR, indicating the presence of excitotoxicity induced by abnormal glutamatergic signaling in BD frontal cortex (Rao et al., 2010). Although there is evidence in support of the impact of the glutamatergic system in cognitive decline and disease progression in BD patients, information regarding the possible ways glutamate (either from dietary sources or microbial activities) may influence cognitive function in BD are still scarce. A Pilot study noted that the plasma and fecal glutamate levels, influenced by relative abundance of certain bacterial families, are negatively

associated with cognitive function including processing speed, mental flexibility and executive function (Palomo-Buitrago et al., 2019). Dietary or luminal glutamate may also activate vagal afferents which directly or indirectly influence brain areas including the cerebral cortex, limbic system, hypothalamus and basal ganglia (Kondoh et al., 2009). From the foregoing, further study is needed to investigate the impact of a dietary glutamate and gut-endogenous glutamate in cognitive function in BD.

As an important part of monoamine metabolism, the role of D-amino acids in the gut-brain axis is gaining wider attention. Gut microbiota contributes to the host pool of D-amino acids *via* intrinsic amino acid racemases within certain gram-negative microbiome (Radkov and Moe, 2014). Kawase et al. (2017) found that gut microbiota could modulate the metabolism of D-amino acids in the brain. They noted that D-aspartic acid, D-serine were higher in some brain regions of GF mice than in those of SPF mice, indicating that gut microbiota may regulate the activity of aspartic acid racemase and serine racemase in the host brain (Kawase et al., 2017). D-serine is an endogenous ligand for NMDAR and thus play a key role in synaptic plasticity (Schell, 2004). Long-term potentiation, which underlies learning and memory, depends on calcium dependent release of D-serine from astrocytes in adult rat CA1 pyramidal hippocampus cells (Henneberger et al., 2010). Genetic association studies convince the role of D-serine in the pathology of BD, showing an association between BD with the gene *G72*, whose product activates the D-serine degrading enzyme (Chen et al., 2004; Müller et al., 2011). Furthermore, ketamine metabolites (rac)-dehydronorketamine and (2S,6S)-hydroxynorketamine decrease intracellular D-serine concentrations in a concentration dependent manner in PC-12 cells (Singh et al., 2016). Previous study has found potential pro-cognitive effects with intravenous subanesthetic ketamine in BD patients (Zhou et al., 2021). Based on these findings, we suggest that gut microbiota may influence synaptic D-serine availability and thus modulate cognitive function in BD patients.

BDNF can also promote the growth and development of neurons as a neurotrophic factor. Alterations in a 5-HT receptor, BDNF, and NMDA receptor subunit expression in the hippocampal region have been demonstrated in germ-free mice (Bercik et al., 2011). However, studies of BDNF levels and specific functional and behavioral alterations in germ-free or antibiotic-treated animal models remain controversial (Holzer, 2016).

4 INTERACTIONS BETWEEN THE GUT MICROBIOTA AND PSYCHIATRIC MEDICATION

Significantly more attention is being given to the interactions between psychiatric drugs commonly used in BD and the gut microbiota. Cognitive side effects induced using antipsychotics in the treatment of BD include impairments in verbal learning, memory, cognitive control, and spatial working memory (Arts et al., 2013; Flowers et al., 2016). Cognitive side effects have also

been previously implicated in chronic inflammation and metabolic syndrome (Fang et al., 2019). Meanwhile, recent studies have considered the interaction between psychiatric medication and gut microbiota as a potential way by which the gut microbiota affects cognitive function (Flowers et al., 2019).

On the one hand, psychiatric medication has an important influence on the gut microbial profile, which, in turn, induces potential adverse events. Valproate significantly reduces fecal microbial richness and induces a gut microbial profile similar to that of patients with autism spectrum disorders (Liu et al., 2018). Flowers et al. (2017) showed that treatment with an atypical antipsychotic (AAP) in BD patients results in reduced gut microbial diversity, especially in women. A subsequent study focusing on patients with BD or schizophrenia also revealed significantly lower gut microbial diversity in AAP users compared to non-AAP users (Flowers et al., 2019). In contrast, Hu et al. (2019) found no significant changes in gut microbial α-diversity but noted an altered gut microbial composition in BD patients treated with AAP monotherapy (quetiapine). After such treatment, organisms of the *Klebsiella* and *Veillonella* genera are significantly increased in BD patients. Moreover, almost all AAPs can lead to weight gain, which may be closely related to gut microbial dysbiosis (McEvoy et al., 2005). Bahr et al. (2015b) found that the administration of risperidone inhibited non-aerobic resting metabolism in the gut microbiota, leading to a reduced total resting metabolism rate and increased body weight. The decreased Bacteroidetes-to-Firmicutes ratio induced by risperidone was linked to secondary weight gain in adolescent children (Bahr et al., 2015a). Zeng et al. (2021) also reviewed the potential role of the gut microbiota in cognitive impairment due to the metabolic side effects of AAPs used as a treatment in schizophrenic patients, which included disruption of inflammatory cytokine signaling and neurotransmitter disorders. Therefore, AAPs are likely to increase the risk of metabolic disorders by affecting the gut microbiota, consequently resulting in cognitive side effects.

On the other hand, the gut microbiota may also help to mediate the cognitive improvement effects of psychiatric medications for BD. Oral selective serotonin reuptake inhibitors (SSRIs) increase the excitability of the intestinal vagal nerve system through an intestinal epithelium-dependent mechanism. Critically, blocking the intestinal vagal signal by subdiaphragmatic vagotomy abolishes the antidepressant effect of oral SSRI treatment (McVey Neufeld et al., 2019). Both behavioral and neuroimaging studies have confirmed the positive effects of SSRI treatment on attention, appraisal, and memory before symptomatic remission. Additionally, Harmer and Cowen (2013) suggested that the antidepressant effects of SSRIs are cumulative results of improvements in cognitive functions related to emotion processing. Accumulated evidence highlights the potential role of vagal-dependent gut-brain signaling in cognitive improvement by SSRI treatment. Aripiprazole treatment also significantly increases the richness and diversity of the gut microbiota, especially the relative abundance of organisms of the minor genera *Clostridium*, *Peptoclostridium*, *Intestinibacter*, and *Christenellaceae*, also

accompanied by a rise in acetate, butyrate and isovalerate levels (Cussotto et al., 2019). Increased numbers of butyrate-producing bacteria may underlie the cognitive improvement of aripiprazole (Ning et al., 2021; Peitl et al., 2021). Studies targeting the *in vivo* interactions of various BD medications with both the gut microbiota and cognitive function will provide new insights into the mechanisms and side effects of these drugs. Microbiota-centered treatment will also be important for optimizing the management of BD patients.

5 POTENTIAL MICROBIOTA-CENTERED TREATMENTS FOR COGNITIVE IMPROVEMENT

Accumulated understanding of the brain-gut axis has led to the development of microbiota-centered treatment in mental illness acting through the gut flora. Both changes in diet habits and psychobiotic supplements can easily modify the gut microbiota. Fecal microbiota transplantation (FMT) facilitates a more stable evolution of gut microbial transplantation.

5.1 Diet

Changing one's dietary habits is the easiest method to modulate gut microbiota. Gut microbial dysbiosis is a key factor of cognitive impairment in diet-induced obesity (Desbonnet et al., 2014). A dietary survey of 97 BD patients showed that BD patients had greater intake levels of processed meat and sugar, fat, and salt (Davison and Kaplan, 2012). Long-term consumption of a high-fat diet increases the Firmicutes-to-Bacteroides ratio, which is associated with obesity (Shi et al., 2015; Ussar et al., 2015). A high-fat diet also led to insulin resistance and hyperglycemia in diet-induced obese mice who exhibited neurotransmitter disorders, including increased GABA and decreased tryptophan levels (Scott et al., 2020). Further, a high-fat diet can cause a significant decrease in tyrosine phosphorylation of insulin receptors, accompanied by an increase in inflammatory response signals (e.g., nuclear factor kappa-light-chain-enhancer of activated B-cells, c-Jun N-terminal kinase) in whole-brain lysate and a decrease in synaptic plasticity, leading to learning and memory impairment (Kothari et al., 2017).

In contrast, healthy diets have shown therapeutic potential for neurocognitive disorders. The Mediterranean diet, characterized by a high intake of fruits, vegetables, nuts, whole grains, and high-protein foods (i.e., fish), can help to reduce intestinal inflammation, cognitive impairment, and the risk of dementia (Pistollato et al., 2018). People at high risk of cardiovascular diseases also showed higher scores on the mean Mini-Mental State Examination and Cognitive Dysfunction Test after long-term consumption of the Mediterranean diet (Martínez-Lapiscina et al., 2013). In terms of dietary composition, a diet high in protein and saturated fat contributed to a greater abundance of *Bacteroides* (Wu et al., 2011). Fruits and vegetables are rich in dietary fiber. Microbiota-accessible carbohydrates found in dietary fiber increase the richness and α-diversity of the gut microbiota and inhibit the hippocampal glial activation and neuroinflammation induced by a high-fat

diet. In turn, these carbohydrates improved the performance of mice in nest-building and temporal order memory tests (Shi et al., 2020). Perez (2018) found that BD patients have lower compliance with the Mediterranean diet and higher biomarkers of insulin resistance compared to the healthy population. Therefore, dietary management of BD patients is crucial for reducing the risk of metabolic disorders and cognitive impairment in clinical practice.

The ketogenic diet is another dietary pattern that has attracted increasing attention in the treatment of neuropsychiatric diseases. Several studies have confirmed the role of the ketogenic diet in improving comorbidities and cognitive function in BD patients. The ketogenic diet reduces hyperinsulinemia in BD patients by alleviating mitochondrial dysfunction mediated by impairment of the phosphatidylinositol-3 kinase/protein kinase B/hypoxia-induced factor-1α signaling pathway (Campbell and Campbell, 2020). The ketogenic diet may affect the function of vesicular glutamate transporters and EAAT, Na+, K + -ATPase, Kir4.1, aquaporin-4, Cx34, and KATP channels by affecting the glutamate-glutamine cycle and glutamate synthase activity in astrocytes, thus reducing mild cognitive impairment in BD patients (Morris et al., 2020). Several studies have confirmed the role of the gut microbiota in the ketogenic diet, although only a few studies have discussed whether the gut microbiota is involved in the cognitive improvement effect of the ketogenic diet in BD patients. The ketogenic diet protects against seizures by regulating gut microbiota colonization, which increases GABA and glutamate levels in the hippocampus (Olson et al., 2018). In AD patients with mild cognitive impairment, a 6-week ketogenic diet increased the abundance of *Enterobacteriaceae*, *Akkermansia*, *Slackia*, *Christensenellaceae*, and *Erysipelotriaceae* and the synthesis of butyrate, accompanied by an improvement in cognitive function (Nagpal et al., 2020).

5.2 Psychobiotics

Probiotics are live microorganisms that offer non-specific benefits to the health of the host. Some of them may also modulate functions of the central nervous system, reducing psychiatric symptoms and improving cognitive function in patients, and are therefore known as psychobiotics (Evrensel et al., 2019). Clinical studies have demonstrated the role of probiotics in promoting gut microbial diversity and improving cognitive function in BD. Probiotics, including *Lactobacillus rhamnosus* strain GG and *Bifidobacterium animalis* subsp. lactis strain Bb12, reduce the risk of re-hospitalization among BD patients (Dickerson et al., 2018). A cohort study conducted by Reininghaus et al. (2018) showed a significant improvement in attention and psychomotor processing speed on the Digit Symbol Test and executive function on the Trail Making Test B in BD patients after long-term administration of a probiotic supplement, mainly containing organisms of the *Lactobacillus* genera and *Bifidobacterium* genera.

Because some prebiotics support the growth of specific gut microbiota with psychophysiological effects, some have been designated as psychobiotics, including fructooligosaccharides, inulin, and galactooligosaccharides (Sarkar et al., 2016). Currently, there are limited studies addressing the effect of

prebiotics in BD patients, but studies in animal models confirm their potential for cognitive improvement. Prebiotics might suppress inflammation that affects cognitive function. Chitosan oligosaccharides effectively reduced learning and memory impairment in an AD model by inhibiting oxidative stress and reducing the release of pro-inflammatory factors, such as IL-1 and TNF-α (Jia et al., 2016). Prebiotics also regulate the synthesis of gastrointestinal hormones. Four weeks of supplementation with prebiotics increased the expression of anorexigenic gastrointestinal hormones, such as peptide tyrosine-tyrosine, GLP-1, and leptin, while decreasing levels of ghrelin and other anorexigenic hormones and helping to improve learning and memory function in schizophrenia patients (Kao et al., 2018). Weight gain induced by antipsychotics, including olanzapine and risperidone, can also be reduced by prebiotics. Intake of the prebiotic galactooligosaccharide mixture significantly reduced olanzapine-induced weight gain, possibly in association with an increased and decreased number of organisms belonging to the *Bifidobacterium* and *Firmicutes* genera, respectively, together with increased cortical phospho-NMDA receptor 1 levels and decreased plasma TNF-α levels. Therefore, these effects suggest that prebiotics may prevent the metabolic and cognitive side effects of olanzapine.

5.3 Gastrointestinal Hormone Analogs

The possible mechanisms by which the gut microbiota modulates energy metabolism and cognitive function by influencing gastrointestinal hormones have been reviewed above. Gastrointestinal hormone analogs may also improve cognitive function in a mode similar to that of gastrointestinal hormones. Liraglutide, the GLP-1 receptor agonist, improved D-amphetamine-induced mania-like symptoms and working and recognition memory impairment in a BD mouse model (Chaves Filho et al., 2020). In non-diabetic BD patients, liraglutide also showed beneficial effects in several cognitive domains, including auditory verbal learning, working memory, and attention (Mansur et al., 2017). Therefore, the neuroprotective effect of liraglutide illustrates the potential for gastrointestinal hormone analogs to serve as promising adjunctive tools for BD treatment.

5.4 Fecal Microbiota Transplantation

FMT treatment involves the injection of filtrate feces from a healthy donor into a patient. FMT not only increases the microbial diversity but also provides long-term implantation of donor strains compared to the short-term impact on the gut microbiota achieved by changes in the diet or the addition of psychobiotics (Weingarden and Vaughn, 2017). In animal research, FMT treatment reversed cognitive impairment in AD model mice by altering the gut microbial composition and SCFA profile and increasing synaptic plasticity. Currently, few clinical studies have focused on the feasibility and efficacy of FMT in BD patients, although Hinton (2020) claimed that depressive symptoms disappeared with weight loss in a BD patient who had experienced 9 sessions of FMT treatment. Therefore, FMT may have a potential role in improving the cognitive function of BD patients, but further clinical experiments are warranted.

5.5 Vagus Nerve Stimulation

The vagus nerve establishes one of the important connections between emotional and cognitive areas of the brain and gut functions. Vagal afferent fibers express receptors for multiple gastrointestinal hormones (GLP-1, CCK, peptide YY, ghrelin, etc.), neurotransmitters (dopamine, GABA, NE, 5-HT, etc.), and gut microbial metabolites (SCFAs, LPS, etc.), to transfer microbiota signals to the central nervous system (Bonaz et al., 2018; Breit et al., 2018). Brain pathway activated by oral administration of Campylobacter jejuni, which has been proved to influence behavior and brain functions at subclinical doses, has been mapped using c-fos expression as a marker of neuronal activation (Gaykema et al., 2004). In this study, brain activation was observed in the nucleus tractus solitarius, the vagal afferent ending, and the projections of the nucleus tractus solitarius including parabrachial nucleus, paraventricular nucleus of the hypothalamus, amygdala and thalamus, indicating vagally-mediated microbiota effect on mood and cognitive function (Gaykema et al., 2004). Consistently, Bravo et al. (2011) found that treatment with *Lactobacillus rhamnosus* decreases GABA(B1b) messenger RNA expression in the hippocampus and amygdala, consequently reducing corticosterone levels and manic-like behaviors. Moreover, the neurochemical and behavioral effects disappeared in vagotomized mice, indicating therapeutic potential for treatment targeting vagal tone.

Vagus nerve stimulation, a medical treatment that is routinely used in the treatment of epilepsy and other neurological conditions, works by applying electrical impulses to the vagus nerve (Wheless et al., 2018; González et al., 2019). A 5-year prospective research in patients with treatment-resistant bipolar depression showed that treatment with vagus nerve stimulation was associated with better medication response and significantly greater mean reduction in suicidality compared to treatment-as-usual (McAllister-Williams et al., 2020). Chronic vagus nerve stimulation also produces sustained clinical and cognitive improvements in BD patients in a treatment-resistant depressive episode (McAllister-Williams et al., 2020). Therefore, vagus nerve stimulation seems to be a promising adjunctive therapy for cognitive impairment in BD patients.

6 CONCLUSION

There is an exciting future potential for research on the connection between gut microbiota and neurocognitive elements in BD patients. Accumulated studies have offered convincing evidence of the participation of microbial-derived metabolites, neurotransmitters, and gastrointestinal hormones in cognitive function. Complex interactions with the gut microbiota may also explain some of the cognitive side effects of certain psychiatric medications. Understanding the potential mechanisms underlying the gut microbiota and cognitive impairment in BD can unlock the door for the application of microbiota-centered treatments in BD management, which may help to prevent adverse events and improve the quality of life in BD patients. However, further investigation is needed before applying these findings in clinical practice despite applauding the recent rise in these strategies.

AUTHOR CONTRIBUTIONS

HW provided a significant contribution to the conceptualization. WD drafted the manuscript and constructed the figures and tables. JL and HY were responsible for data collection. YQ, SL and ZT reviewed and edited the manuscript. JH, HX, HT, and BW contributed to clinical content and proofreading. All authors contributed to the article and approved the submitted version.

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Gastrodin From *Gastrodia elata* Enhances Cognitive Function and Neuroprotection of AD Mice *via* the Regulation of Gut Microbiota Composition and Inhibition of Neuron Inflammation

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Gastrodin (Gas) is known to exhibit neuroprotective effects in Alzheimer's disease (AD). However, the detailed mechanism of action is still unclear. In the present study, we focused on the microbiome–gut–brain axis to investigate the mechanism of action of Gas using a D-galactose (Dgal)–induced AD model. Gas reversed the memory dysfunction of Dgal-administered mice. Neurons in the cerebral cortex and hippocampus were reduced in the Dgal-administered group, and the decrease of neurons was suppressed in 90 and 210 mg/kg Gas treatment groups. 16S rRNA sequence analysis was carried out to explore the composition of gut microbiota in fecal samples of mice. Gas treatment had a positive correlation with Firmicutes and had a negative correlation with Cyanobacteria, Proteobacteria, and Deferribaceters. Importantly, the LPS and proinflammatory cytokines in the brain increased in Dgal-administered mice, but these parameters recovered to normal levels after oral administration of Gas. To determine whether the microbiota–gut–brain axis is involved in the neuroprotective effect of Gas, the mice were given antibiotic cocktail before and during the trial period to decrease the gut microbiota of mice. The antibiotic cocktail partially eliminated the neuroprotective effect of Gas by changing the gut microbiome composition. These results indicated that Gas improves the memory of the AD mouse model *via* partly targeting the microbiota–gut–brain axis and mitigating neuron inflammation.

Keywords: AD mice, gut microbiota, memory, neuroinflammation, microglial cell

Abbreviations: AD, Alzheimer's disease; ABX, antibiotics; ANOVA, analysis of variance; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; Dgal, D-galactose; Done, donepezil; DAPI, 4′, 6-diamidino-2-phenylindole; GADPH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; Gas, gastrodin; IL-1β, interleukin-1 beta; IL-6, interleukin 6; Iba-1 ionized calcium-binding adapter molecule; LEFSE, linear discriminant analysis effect size; LDA, linear discriminant analysis; LPS, lipopolysaccharide; MWM, Morris water maze; NEUN, neuronal nuclear protein; NOR, novel object recognition; PCoA, principal coordinate analysis; PFA, paraformaldehyde; RT-PCR, real-time polymerase chain reaction; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha; and ZO-1, zonulin.

INTRODUCTION

The elderly population is growing at an unprecedented rate. Currently, there are about 703 million people above 65 years of age worldwide, which is estimated to progressively increase to more than two billion by 2050 (United Nations, 2019). Aging is a complicated progressive debilitation of physiological functions, which induces the degeneration of organ functions and exhibits several phenotypes that promote diseases such as cancer, type 2 diabetes, and hypertension (Xiao et al., 2018; Zhang et al., 2018). Alzheimer's disease (AD) is an important manifestation of aging, especially pathological brain aging. It is a pathological syndrome that is primarily characterized by major symptoms such as impaired cognitive function and perturbed daily life performance with behavioral abnormalities (Akhtar and Sah, 2020). The current drugs for AD, including four cholinesterase inhibitors and one N-methyl-D-aspartate (NMDA) receptor antagonist, have been able to mitigate AD symptoms but cannot cure the disease (Iraji et al., 2020). Thus, new therapeutic targets and drugs for the treatment and prevention of AD should be identified.

Animal models play an important role in studying AD. To date, transgenic mice such as APP/PS1, 3xTg-AD mice (Virgili et al., 2018), scopolamine- and D-galactose (Dgal)–induced mice (Li et al., 2016; Ali et al., 2020) are used to perform efficacy evaluation and determine the mechanism of action of certain compounds. Dgal, a normal reducing sugar, is widely used to induce age-related diseases, including AD (Oskouei et al., 2021). Rodents administered with Dgal show cognitive dysfunction, increased free radical production, mitochondrial dysfunction, and impaired calcium homeostasis (Jeong et al., 2021). Recently, some studies reported that Dgal promotes inflammation, neurodegeneration, and gut microbiota dysbiosis (Xiao et al., 2018; Zhang et al., 2018; Ali et al., 2020). In the present study, we used Dgal-induced AD mice for our experiments.

With the understanding of gut microbiota, more and more pieces of evidence indicate that the gut–brain axis plays a vital role in neuronal development and neurodegenerative diseases (Kennedy et al., 2017; Zhang et al., 2018). The status of gut function and the composition of gut microbiota can influence brain function and diseases. Several studies have reported the impact of dysbiosis on several neurodegenerative diseases such as AD and Parkinson's disease (Kennedy et al., 2017; Liu et al., 2020). Gut microbiota dysbiosis has been hypothesized to promote proinflammatory cytokines, induce neurotoxins, and cause metabolic disturbances (Tan et al., 2021). On the contrary, gut microbes, such as Bifidobacteria, Firmicutes, Actinobacteria, and Verrucomicrobia, have been implicated in promoting cognitive function (Sharma et al., 2021). Furthermore, several studies have shown the marked difference in gut microbiota composition and diversity in AD patients compared with those in their respective control (Sharma et al., 2021). The variance in the gut microbiota between diseased and healthy individuals in several diseases makes it a promising target for prevention and treatment of diseases. In addition, the promotion of dysbiosis leads to increased pathogenic bacteria that induce

brain damage, while eubiosis promotes the good functionality of the brain (Kennedy et al., 2017; Sharma et al., 2021).

Gastrodia elata, commonly known as Tian ma in Chinese, is an orchid used in traditional Chinese medicine to treat spasms, paralysis, dizziness, stroke, and dementia (Liu et al., 2018). *G. elata* contains gastrodin (Gas), parishin, p-hydroxybenzyl alcohol, vanillin, and vanillyl alcohol compounds, but the principal bioactive compound is Gas. Since it was first isolated in 1978, extensive investigations on the biological activity of Gas have been performed (Liu et al., 2018). Gas possesses a broad range of beneficial effects on neurodegenerative diseases, and its mechanisms of actions include modulating neurotransmitters, exerting antioxidative and anti-inflammatory effects, suppressing microglial activation, regulating mitochondrial cascades, and upregulating neurotrophins (Liu et al., 2018). Although the brain–gut–microbiota axis is an emerging pharmacological target in AD, the effect of Gas on the gut–brain axis remains unknown. In this study, we show that Gas treatment can promote cognition and neuroprotection and mitigates neuron inflammation by regulating the gut microbiota composition of AD model mice. To our knowledge, this study is the first to assess the influence of Gas on cognition by regulating gut microbiota.
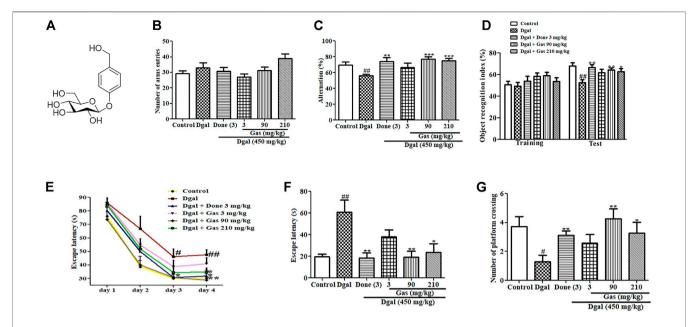
MATERIALS AND METHODS

Drugs and Reagents

Dgal and donepezil (Done) were purchased from Sangon Biotech Co., Ltd., Shanghai, China and Sigma-Aldrich Co., St. Louis, MO, United States, respectively. Antibiotics (vancomycin, neomycin, ampicillin, and metronidazole) and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Macklin Shanghai, China. Tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, lipopolysaccharide (LPS), and ELISA kits were provided by 4A Biotech, Beijing, China and Hefei Laier Biotech, Anhui, China. The antibodies of TLR4 (4A Biotech, Beijing China), occludin, pIKBα, zonulin (Santa Cruz Biotechnology, Dallas, Texas, United States), neuronal nuclei (NeuN), IKBα, Iba-1 (Cell Signaling Technology, Boston, United States), GADPH, secondary antibody (horseradish peroxidase [HRP]–linked anti-rabbit and anti-mouse IgGs), and ECL Western blot chemiluminescence detection kit (Beijing CoWin Biotech Company, Beijing, China) were used in the present study. All primers in this work were synthesized by Sangon Biotech Co., Ltd., Shanghai, China.

Extraction and Isolation of Gastrodin

The dried rhizome of *Gastrodia elata* was purchased from Chengdu, Sichuan Province, China, identified by associate professor Jianxia Mo and assigned a voucher specimen number (no. 20191114) of the plant deposited at the Institute of Materia Medica, Zhejiang University. Dried *G. elata* (1.5 kg dry weight) was crushed and extracted with 100% methanol for 48 h of shaking, filtration, and concentration with a vacuum pump to obtain 135 g crude extract. Under the guidance of the bioassay system (lifespan assay of K6001 yeast) (Lin et al., 2019), the crude extract (135 g) was separated by a silica gel (200–300 mesh,

FIGURE 1 | Chemical structure of Gas and changes in learning memory and spatial memory of AD mice after administering Gas. **(A)** Chemical structure of Gas. **(B)** Changes in the number of arm entries in the Y-maze test. **(C)** Alternation in the Y-maze test. **(D)** Object recognition index in the NOR experiment. **(E)** Escape latency in the training phase in the water maze test. **(F,G)** Escape latency and number of platform crossings in the water maze test. The number of animals in each group is eight, and the data are presented as means ± SEM. [#] and [##] indicate significant differences at $p < 0.05$ and $p < 0.01$ compared with those of the normal control, respectively; [*], [**], and [***] indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.01$ compared with those of the Dgal group, respectively.

Yantai Research Institute of Chemical Industry, Yantai, China) open column and eluted with $CH_2Cl_2/MeOH$ (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, and 0:100). The active fraction (12 g) eluted with $CH_2Cl_2/MeOH$ (75:25) was purified by HPLC (H&E SP ODS-A ($\Phi = 20 \times 250$ mm)) with 5% of aqueous methanol at a flow rate of 8 ml/min to obtain an active compound ($t_R = 41$ min) in batches. The chemical structure of the active compound was identified to be Gas by analyzing HR ESI-MS and ^{1}H NMR spectra and compared with that of the reported one (Lee et al., 2015). The chemical structure is given in **Figure 1A**.

Animal and Experimental Design

Gas and Done were dissolved in water prior to oral administration, while Dgal was dissolved in normal saline prior to intraperitoneal administration. Sixty Institute of Cancer Research (ICR) mice aged 8 weeks' old were purchased from Zhejiang Academy of Medical Sciences, Hangzhou, China. They were acclimatized under standard condition of 23°C temperature, 50% humidity, and 12 h light/dark cycle and provided chow diet and water *ad libitum*. After 1 week of acclimatization, the mice were randomly divided into the following groups: normal control, Dgal group, Dgal + Done 3 mg/kg group, Dgal + Gas 3 mg/kg, Dgal + Gas 90 mg/kg, and Dgal + Gas 210 mg/kg groups. The control and Dgal groups were given water, while others were given the respective drug dosage *via* oral gavage daily for 9 weeks. In addition, the control group was given normal saline, and all other groups were given Dgal dissolved in normal saline at 450 mg/kg *via* intraperitoneal injection daily for 9 weeks. The Y-maze and novel object recognition (NOR) tests started at the

eighth week, while the Morris water maze (MWM) test was started at the ninth week. The mice were killed at the end of the ninth week. Blood was taken *via* the orbital sinus, and brains and intestines of some mice were removed and preserved in a −30°C refrigerator for further analysis. At the same time, three mice in each group were perfused with cold PBS followed by cold 4% paraformaldehyde (PFA). Thereafter, the brains were embedded and sectioned for immunohistochemical staining.

In addition, the second study was carried out using 8-week-old male ICR mice. Forty mice were divided into the following groups: normal control, Dgal group, Dgal + Gas 90 mg/kg group, and Dgal + Gas 90 mg/kg + ABX group. The ABX group was administered with 100 μl of antibiotic (ABX) cocktail, which includes 0.5 g/L of vancomycin, 1 g/L of neomycin, 1 g/L of ampicillin, and 1 g/L of metronidazole, for 2 weeks in order to deplete the gut microbiota. The other groups were administered with 100 μl of Milli-Q water for 2 weeks. After 2 weeks, the normal control and Dgal group were given Milli-Q water *via* oral gavage, while other groups were administered with 90 mg/kg of Gas daily for 9 weeks. However, the Dgal + Gas 90 mg/kg + ABX group was treated with 100 μl of ABX cocktail daily for 9 weeks. The normal control group was treated with normal saline, and all other groups were given 450 mg/kg of Dgal dissolved in normal saline *via* intraperitoneal injection daily for 9 weeks. The Y-maze and NOR tests were started at the tenth week, while the MWM test was started at the twelfth week. At the end of the animal experiment, the samples and brain were taken as mentioned previously. All animal experiments were conducted with strict accordance to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. In

addition, the Committee on the Ethics of Animal Experiments of Zhejiang University Permit approved the experiment with permit number (ZJU20210155).

Y-Maze Test

The Y-maze test is used to measure working memory, and the method of assessment has been reported in a previous study (Li et al., 2016). The Y-maze comprises three arms (A, B, and C) with 30 cm length, 10 cm width, and 15 cm height with 120° relative to adjacent arms. The mouse activity in the Y-maze was recorded with a video recorder, and data were stored in ANY-MAZE software 6.35 (Stoelting, Illinois, United States). Each mouse was placed facing the wall of arm A and allowed to move freely in the maze for 5 min. The mice whose whole or half body entered an arm was recorded as an arm entry. Furthermore, an alternation was considered when a mouse moved using the three arms without revisiting the first arm. The total number of arm entries and alternations was calculated with ANY-MAZE software. The alternation was calculated by dividing the total number of alternations by the total number of arm entries −2 × 100.

Novel Object Recognition Test

The NOR test was carried out as previously described (Li et al., 2016). Briefly, the NOR test was performed in an open-field environment with an arena of a black plastic box (50 cm × 50 cm × 50 cm) with two identical objects and a novel object. The activity of mice in the open arena was recorded with a video recorder and stored in ANY-MAZE software. During the habituation phase, each mouse was placed in the open arena without objects for 5 min. In the middle of training, the open arena was cleaned with 75% ethanol and dried. After 24 h, the NOR test was performed, which included the training and test phases. In the training phase, each mouse was placed in the same arena with two identical objects (5 cm × 5 cm × 5 cm cubic steel placed on each other) set apart at equal distance for 5 min. After 1 h, one of the identical objects was substituted with a novel object (5 cm × 5 cm cylindrical steel placed on each other), and the exploration time of each mouse was recorded. The exploration was determined by the time spent by the mice either touching or sniffing the objects. The exploration time during the training and test phases was recorded with a video recorder, and data were stored in ANY-MAZE software. The alternation was calculated by dividing time spent on novel object by total time spent on both objects × 100 was calculated by ANY-MAZE software.

Morris Water Maze Test

The MWM test is used to measure spatial and long-term memory. It comprises a circular tank (1.25 m diameter and 50 cm depth) containing 25 cm deep warm water (22°C), a platform (10 cm diameter and submerged 1 cm below the water), a video camera recorder, and a computer. The experiment was carried out in 5 days. The mice were subjected to a 4-day acquisition experiment, where each mouse was placed at three different desired points, and the time taken to find the platform was recorded at each time. If the mouse did not find the platform in 120 s, it was directed toward the platform and allowed to stay on it for 10 s. On the fifth day, the platform was removed, and a desired point was selected. Each mouse was placed at the desired point and allowed to swim for 90 s. The number of times each mouse crossed the platform was recorded, and the escape latency was recorded.

Real-Time Polymerase Chain Reaction Analysis

Total RNA from the cerebral cortex was extracted and cDNA synthesized (Xiang et al., 2020). About 50 mg of the cerebral cortex was homogenized with TRIzol reagent (Invitrogen, California, United States) for RNA extraction. In addition, 2.5 µg of total RNA of each sample was used for cDNA synthesis using the HiFi-MMLV cDNA Kit (CoWin Biotech, Beijing, China). The transcript level was quantified by real-time PCR analysis using CFX96-Touch (Bio-Rad, California, United States) and SYBR Premix EX Taq (Takara, Otsu, Japan) under appropriate conditions. Relative gene expressions were analyzed by the $2^{-\Delta\Delta Ct}$ method. The 18S primer was used as internal control, and the primer sequences used are listed in **Supplementary Table S1**.

Western Blot Analysis

Western blot analysis was performed on the basis of a previous report (Li et al., 2016). Briefly, about 50 mg of cerebral cortex, one hippocampus, or 50 mg of small intestine was homogenized in 1% protease and 1% phosphatase inhibitor–containing buffer, and the protein concentration was determined. In addition, about 30 µg of protein of samples was transferred to a new tube for denaturation at 100°C for 20 min. Furthermore, 30 µg of protein of each sample was loaded in each well of sodium dodecyl sulfate–polyacrylamide gel. Gel electrophoresis was run at 80 V for 15 min and 120 V for 60 min. The protein on the gel was transferred to a polyvinylidene difluoride membrane and then blocked with 5% non-fat dry milk buffer for 60 min at room temperature. The membrane was incubated with primary antibodies to TLR4 (Beijing 4A Biotech Co., Ltd., Beijing China), IKBα (Cell Signaling Technology, Boston, United States), pIKBα, occludin, ZO-1 (Santa Cruz Biotechnology, Texas, United States), and GADPH (Beijing CoWin Biotech Company, Beijing, China) at 4°C overnight. After washing the membrane three times with Tris-buffered saline, the membrane was incubated with secondary antibody (HRP-linked anti-mouse or anti-rabbit IgGs, Beijing CoWin Biotech Company, Beijing, China) for 45 min. Then, the protein bands were developed with the ECL Western blot chemiluminescence detection kit, and the blot density was measured using ImageJ software (National Institutes of Health, Bethesda, MD, United States).

NeuN and Iba-1 Immunostaining

At the end of the animal experiment, three mice from each group were anesthetized through intraperitoneal injection of 10% chloral hydrate (0.35 ml/100 g) and perfused transcardially with cold PBS followed by cold 4% PFA dissolved in PBS. The brain was excised and fixed in 4% PFA solution overnight, then

dehydrated in 15% sucrose in PBS for 24 h, and finally transferred into 30% sucrose solution for 48 h. During brain sectioning, each brain was embedded in an optimal cutting temperature compound. A 20-μm-thick brain section was cut by a cryostat (Thermo Fisher, Massachusetts, United States) and placed in a well containing antifreeze (glycerin, ethylene, and PBS in a 3:3:4 ratio). The brain sections were then stored in a refrigerator at −30°C. During immunohistochemical staining, the brain sections were fixed on a slide hydrated with PBS for 5 min. Then, they were exposed to blocking buffer for 1 h followed by 1:1000 diluted primary antibodies (NeuN or Iba-1, Abcam, Cambridge, United Kingdom) and incubated overnight at 4°C. After incubation, the sections were washed three times with PBS and incubated with 1:1000 diluted secondary antibody (Alexa Fluor 488-linked goat antirabbit IgG, Abcam, Cambridge, United Kingdom) for 2 h at 37°C. The sections were covered with DAPI and covered carefully with a coverslip. The slides of the brain section were checked with an upright two-photon confocal microscope (Olympus BX61, Shinjuku, Japan), while those with Iba-1 antibody were checked with a fluorescent microscope (Leica DMI 3000 B, Wetzlar, Germany). The neurons in the hippocampus and cerebral cortex and microglia in the cerebral cortex were counted with ImageJ software.

Gut Microbiota Analysis

At the ninth week of experiment, fresh fecal samples of each mouse were collected and stored in liquid nitrogen until use for gut microbiota analysis. Gut microbiota analysis was performed according to a previous study (Xiang et al., 2020). Briefly, the total genomic DNA was extracted using the TIANamp Bacterial DNA Kit (DP302-02, Tiangen, Beijing, China), and V4 regions of the 16S rRNA gene were amplified with composite sense and antisense primers. The appropriate PCR condition was followed, and the broken sticky end of the target amplicon fragment was repaired by Klenow DNA polymerase, T4 DNA polymerase, and T4 PNK. Magnetic beads were used to purify amplicons, and replicate PCRs were pooled. Furthermore, sequence analysis was performed with UPARSE software. The sequence with ≥97% similarity was clustered for all samples, and OTUs were assigned for each representative sequence. This work was finished by LC-Bio Technology Co., Ltd., Hangzhou, China.

Enzyme-Linked Immunosorbent Assay

At the end of experiment, the blood samples of each mouse were collected from the orbital sinus by using a capillary tube. The samples were allowed to stand at room temperature for 2 h, centrifuged at 12,000 × g for 15 min at 4°C to obtain the plasma, and stored in a refrigerator at −30°C until use for biochemical analysis. In addition, cerebral cortex samples were homogenized, and the protein concentrations were determined as previously explained (Xiang et al., 2020). However, to measure the fecal LPS concentration, about 30 mg of the fecal sample of each group was suspended in 1 ml sterile PBS in pyrogen-free tubes. It was homogenized mildly to prevent disruption of bacterial cells and centrifuged at 12,000 × g for 15 min at 4°C to obtain the supernatant. The supernatant was filtered through a 0.22-μm to

0.45-μm filter consecutively followed by inactivation for 15 min at 90°C. The supernatants of control groups and treatment groups were diluted with PBS at ratio of 1:2.5 and 1:5, respectively. The fecal LPS, LPS, TNF-α, IL-1β, and IL-6 concentrations in the serum and cerebral cortex were measured using commercially available mouse ELISA kits (LPS, Hefei Laier Biotechnology Co., Ltd., Hefei, China; TNF-α, IL-1β, and IL-6, Beijing 4A Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Briefly, the sample and standard were added into the respective wells, followed by the addition of biotin conjugate, which was mixed properly and incubated for 2 h. After incubation, the wells were washed with wash buffer four times, and the HRP-streptavidin conjugate was added for 30 min and then washed with washing buffer four times. The substrate solution was added and incubated at 37°C for 15 min. The stop solution was added, and the optical density was checked with a microplate reader (BioTek, San Diego, California, United States) at 450 nm. The concentration of LPS, TNF-α, IL-1β, or IL-6 was calculated on the basis of the respective standard curve.
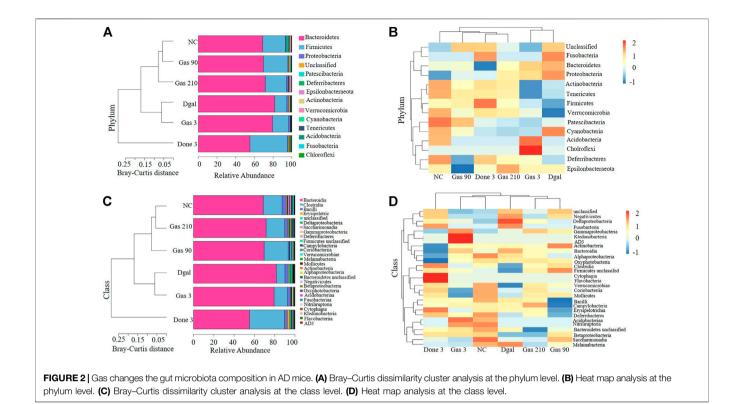
Biostatistical Analysis

Data were evaluated with two-tailed multiple t-tests using GraphPad Prism 6.0 (GraphPad Prism, San Diego, United States). Gut microbiota was statistically analyzed with a Kruskal test or a Wilcoxon test of R software (version 3.1.1). The results were presented as mean ± SEM with $p < 0.05$ considered to be statistically significant.

RESULTS

Gas Ameliorates Cognitive Dysfunction in Dgal-Induced AD Mice

To examine the cognitive function of mice, we designed various behavioral experiments including Y-maze and NOR tests to assess working memory and the MWM test to measure spatial long-term memory. The results of the Y-maze test are shown in **Figures 1B,C**. No statistical significant differences were observed in the numbers of arm entries in all groups (**Figure 1B**). However, the alternation of Dgal-only group was significantly reduced compared with that of the control (**Figure 1C**). Meanwhile, a significant increase in alternations was observed in Done 3 mg/kg (**Figure 1C**) and Gas treatment groups at the doses of 90 and 210 mg/kg, respectively (**Figure 1C**). The results of the NOR test, which also tests the working memory of mice, are displayed in **Figure 1D**. No statistical significant differences were observed in the object recognition index in all groups during the training phase. However, a marked reduction was observed in the object recognition index in the Dgal group compared with that in the normal control. At the same time, a significant increase was observed in the object recognition index in the Dgal + Done 3 mg/kg and Dgal + Gas treatment groups at doses of 90 and 210 mg/kg, respectively, compared with that in the Dgal group (**Figure 1D**). Furthermore, the MWM test was used to examine the spatial and long-term memory of mice, and the results are shown in **Figures 1E–G**. A significant increase in escape latency on training days 3 and 4 was

FIGURE 2 | Gas changes the gut microbiota composition in AD mice. **(A)** Bray–Curtis dissimilarity cluster analysis at the phylum level. **(B)** Heat map analysis at the phylum level. **(C)** Bray–Curtis dissimilarity cluster analysis at the class level. **(D)** Heat map analysis at the class level.
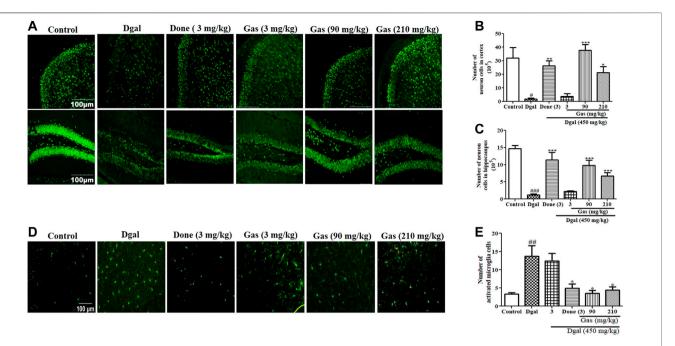
observed in the Dgal + Gas group compared with that in the normal control (**Figure 1E**). Meanwhile, the escape latency of the Dgal + Gas treatment group at a dose of 90 mg/kg was significantly reduced on trainings day 3 and 4 (**Figure 1E**). The significant reduction of escape latency in the Done 3 mg/kg and Gas 210 mg/kg groups was observed only on training day 4 (**Figure 1E**). In the test phase after training for 4 days, the same changes on escape latency of each group on test day as those on training day 4 were observed (**Figure 1F**). By contrast, the numbers of platform crossing in the Dgal-only group was obviously decreased compared with those in the normal control (**Figure 1G**). However, a significant increase in platform crossing numbers was observed in the Dgal + Done and Dgal + Gas treatment groups at doses of 90 and 210 mg/kg, respectively (**Figure 1G**). These results suggest that we successfully constructed the aging animal model with Dgal, while Gas and Done can improve working and long-term memory of AD mice.

Gas Changes the Gut Microbiota Composition in Dgal-Induced AD Mice

To understand how Dgal and Gas affect gut microbiota, Illumina high-throughput sequencing was conducted to read the 16S rRNA sequences of the V4 region of 36 fecal microbiota samples of the normal control; Dgal, Dgal +3 mg/kg Done, and Dgal + Gas treatment groups at doses of 3, 90, and 210 mg/kg, respectively. We used Bray–Curtis dissimilarity distance to assess overall diversity in gut microbiome composition. The gut microbiome composition of the normal control and Dgal group was vastly different from each other. However, the gut microbiome composition of the Dgal +3 mg/kg

Gas group was not different from that of the Dgal-only group, while the gut microbiome composition of the Dgal +90 and 210 mg/kg Gas groups was close to that of the normal control group (**Figures 2A,C**). Interestingly, the gut microbiota of the Dgal + Done group was significantly different from that of other groups. Thus, these results suggested that gut dysbiosis was reversed by Gas at doses of 90 and 210 mg/kg. To explore the effect of Gas on the relative abundance of microbes in the gut, we analyzed the relative abundance at the phylum and class levels. The results of phylum analysis are displayed in **Figures 2A,B**. A significant decrease in Tenericutes and Verrucomicrobia and a marked increase in Bacteroidetes and unclassified were observed in the Dgal group compared with that in the normal control group. Furthermore, a marked increase in the relative abundance of Firmicutes and Verrucomicrobia and significant reduction in Bacteroidetes were observed in the Dgal + Done 3 mg/kg group compared with those in the Dgal group. However, treatment with Dgal +3 mg/kg of Gas induced a significant decrease in the unclassified group compared with the Dgal group. After administration of Gas at doses of 90 and 210 mg/kg, a significant increase in Firmicutes and Verrucomicrobia was observed in these groups compared with that in the Dgal group. In addition, the class analysis displayed in **Figures 2C,D** showed a significant increase in abundance of Bacteroidia, unclassified class, and Negativicutes and a significant decrease in class Clostridia, Verrucomicrobiae, Coriobacteriia, and Mollicutes in the Dgal group compared with that in the normal control. However, treatment with 3 mg/kg of Done significantly increased the abundance of Clostridia, Verrucomicrobiae, and Firmicutes unclassified and

FIGURE 3 | Gas promotes neuroprotection and mitigates microglial activation in Dgal-induced AD mice. **(A)** Fluorescent image of neurons in the hippocampus and cerebral cortex of all groups. **(B)** and **(C)** represent digital number of neurons in the cerebral cortex and hippocampus, respectively. **(D)** Fluorescent image of microglia in the cerebral cortex. **(E)** Digital number of microglia in the cerebral cortex. The brains of three mice in each group were cut, and six sections of the cerebral cortex and hippocampus of each mouse were used to calculate neurons or microglia. The data were presented as means ± SEM. ## and ### indicate significant differences at $p < 0.01$ and $p < 0.001$ compared with those of the normal control, respectively; *, **, and *** indicate significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with those of the Dgal group, respectively.

decreased that of Bacteroidia and Negativicuts compared with that in the Dgal group. A significant decrease in the abundance of Negativicutes in all Gas groups, unclassified class in the 3 and 210 mg/kg Gas-treated group, and Erysipelotrichia in the 90 mg/kg Gas group was observed compared with that in the Dgal group. Meanwhile, a significant increase in Clostridia in the 90 mg/kg Gas group and Gammaproteobacteria, Coriobacteriia, and Verrucomicrobiae in the 210 mg/kg Gas group was observed compared with that in the Dgal group. These results indicated that Gas significantly increased the relative abundance of Firmicutes and Verrucomicrobiae at the phylum and class levels.
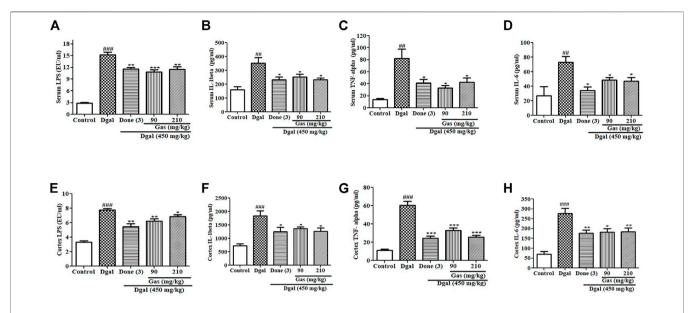
Gas Promotes Neuroprotection and Mitigates Microglial Activation in Dgal-Induced AD Mice

To determine whether Gas exerts neuroprotective effects and affects the activity of microglia in the brain, we used immunohistochemical staining to detect the changes of neurons in the cerebral cortex and hippocampus of the brain. The photographs and digitally calculated number of neurons in the cerebral cortex and hippocampus are displayed in **Figures 3A–C**. The number of neurons in the cerebral cortex and hippocampus was significantly decreased in the Dgal group compared with that in the normal control group. No clear change in the number of neurons was observed in the cerebral cortex and hippocampus of the D-gal + 3 mg/kg Gas treatment group compared with that in the Dgal group. However, the

Dgal + Done group and Dgal + Gas 90 and Dgal +210 mg/kg groups had significantly more neurons in the cerebral cortex and hippocampus than the Dgal group (**Figure 3B**). Gas at a dose of 90 mg/kg showed the best neuroprotective effect. Subsequently, we detected the changes of microglia in the cerebral cortex of the brain, and the results are shown in **Figures 3D,E**. The numbers of microglia in the cerebral cortex were significantly higher in the Dgal group ($p < 0.01$). Nevertheless, the numbers of microglia in the cerebral cortex of the Dgal + Done group and all Dgal + Gas groups were obviously lower than those of the Dgal alone group. Furthermore, we assessed BDNF gene expression in the cerebral cortex of mice. A marked decrease of BDNF expression in the Dgal group and evident increase of BDNF expression in the Dgal + Done and Dgal + Gas treatment groups at doses of 90 and 210 mg/kg were observed compared with those in the normal control and Dgal group (**Supplementary Figure S1A**). The results indicated that Gas improved the memory of AD mice *via* modifying neuroprotection and decreasing microglia, and the dose of 90 mg/kg was best among the tested concentrations.

Gas Mitigates Serum Inflammation and Neuroinflammation in Dgal-Induced AD Mice

Gut dysbiosis has been implicated in promoting increased LPS levels in the blood, which induces serum inflammation and promotes neuroinflammation (He et al., 2021). In this study,

FIGURE 4 | Effects of Gas on proinflammatory cytokines in the serum and cerebral cortex. **(A–D)** Changes in the serum LPS, IL-1β, TNF-α, and IL-6 of AD mice after treatment with Gas, respectively. **(E–H)** Concentration of cerebral cortex LPS, IL-1β, TNF-α, and IL6. Sample numbers are five, and the data are presented as means ± SEM. [#], [##], and [###] indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with those in the normal control, respectively; [*], [**], and [***] indicate significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with those of the Dgal group, respectively.

we investigated the signaling pathway of inflammation. The fecal LPS and plasma LPS in the Dgal group were significantly higher than those in the normal control (**Supplementary Figure S5D**, **Figure 4A**). After drug treatment, the fecal LPS and plasma LPS levels in the Dgal + Done and Dgal + Gas 90 and 210 mg/kg groups were lower than those in the Dgal group (**Supplementary Figure S5D**, **Figure 4A**). Furthermore, we measured the proinflammatory cytokines in the plasma, and the results are displayed in **Figures 4B–D**. Similar increase and reduction of serum IL-1β, TNF-α, and IL-6 were observed in the Dgal group and all drug treatment groups (**Figures 4B–D**). We examined these parameters in the brain, and the results are shown in **Figures 4E–H**. Increased cerebral cortex LPS in the Dgal group and decreased cerebral cortex LPS in the Dgal + Done and Dgal + Gas groups at doses of 90 and 210 mg/kg were observed compared with those in the normal control and Dgal group (**Figure 4E**). The proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6 in the brain were significantly increased in the Dgal group and decreased in the drug treatment groups (**Figures 4F,H**). These results revealed that Gas can mitigate neuroinflammation of AD mice induced by Dgal *via* attenuating endotoxin and proinflammatory cytokines in the plasma and brain.
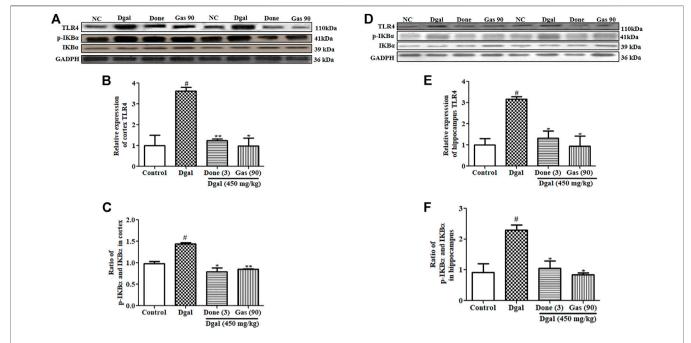
Gas Ameliorates Neuroinflammation Through the TLR4/NF-κB Pathway in Dgal-Induced AD Mice

An increase in LPS has been shown to activate the TLR4 signaling pathway, which increases the expression of proinflammatory markers (Jamar et al., 2021). We considered that LPS might activate the TLR4/NF-κB signaling pathway in the brain of mice.

Thus, we investigated the changes of this signaling pathway in the cerebral cortex and hippocampus. A marked increase in TLR4 and phosphorylated IκBα protein levels was observed in the cerebral cortex and hippocampus of the Dgal group compared with that in the normal control (**Figures 5A–F**; **Supplementary Figure S2A,B**). At the same time, these patterns in the cerebral cortex and hippocampus were significantly decreased in the Dgal + Done and Dgal + Gas treatment groups at a dose of 90 mg/kg (**Figure 5**, **Supplementary Figure S2A,B**). In addition, NF-κB and IKKβ gene expression was significantly increased in the Dgal group compared with that in the normal control (**Supplementary Figure S1B,C**) but was significantly reduced in the Dgal + Done and Dgal + Gas treatment groups at a dose of 90 and 210 mg/kg, respectively (**Supplementary Figure S1B,C**). These results revealed that Gas reduced neuroinflammation in the brain of AD mice by regulating the TLR4/NF-κB signaling pathway.

Gas Upholds the Integrity of the Intestinal Barrier and Blood–Brain Barrier (BBB)

The intestinal barrier and BBB are important in preventing microorganism invasion into blood circulation and the brain. Intestinal tight junction leakage increases LPS and bacteria in the blood (Jamar et al., 2021). Brain tight junction leakage has been implicated in the progression of neurodegenerative diseases (Varatharaj and Galea, 2017). Thus, we explored two important tight junction proteins, namely, zonulin and occludin, and the results are shown in **Figure 6**. The zonulin and occludin proteins of the intestine were significantly decreased in the Dgal-only group (**Figures 6A–C**, **Supplementary Figure S2C**) compared with those in the normal control. After administering Gas at 90 mg/kg, zonulin and occludin protein

FIGURE 5 | Effect of Gas on the TLR4/NF-κB signaling pathway in the cerebral cortex and hippocampus. **(A)** Western blot analysis of the cerebral cortex TLR4, pIKBα, and IKBα. **(B)** and **(C)** represent digitalized results of Western blot analysis. **(D)** Western blot analysis of TLR4, pIKBα, and IKBα in the hippocampus. **(E)** and **(F)** represent digitalized Western blot analysis of TLR4, pIKBα, and IKBα in the hippocampus, respectively. Sample number of each group is three, and the data are presented as means ± SEM. [#] indicates significant differences at $p < 0.05$ compared with that in the normal control; [*] and [**] indicate significant difference at $p < 0.05$ and $p < 0.01$, respectively, compared with those in the Dgal group.
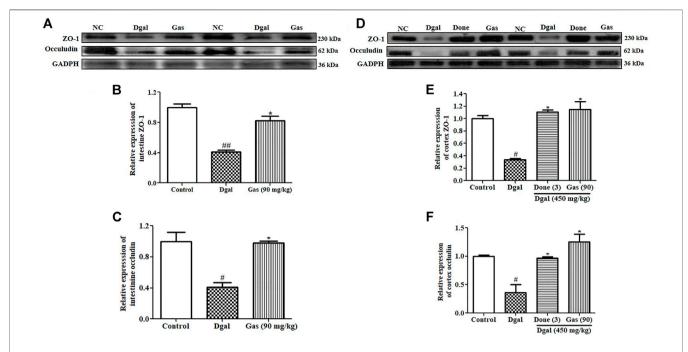
expression levels in the intestine were significantly increased compared with those in the Dgal group (**Figures 6A–C**; **Supplementary Figure S2C**). As we expected, the changes of zonulin and occludin in the brain were similar to what is observed in the intestine (**Figures 6D–F**, **Supplementary Figure S2D**). These findings suggest that Dgal damaged the intestinal barrier and BBB of Dgal-induced AD mice, and Gas ameliorated these damages and promoted neuroprotection.

Gas Promotes Cognition and Neuroprotection Partly by Regulating Gut Microbiota Composition
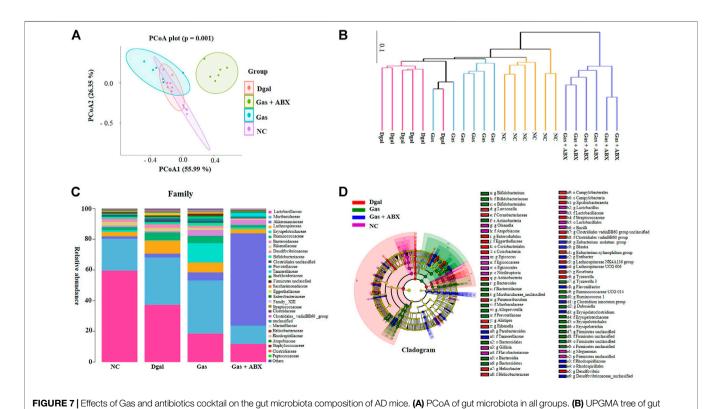
To determine whether gut microbiota is involved in the neuroprotection of Gas, we administered an antibiotic cocktail to mice for 2 weeks to deplete the gut microbiota of mice before administering Dgal and Gas. The gut microbiota of AD mice at the end of the animal experiment was analyzed again with 16S rRNA sequence analysis. The results of principal coordinate analysis (PCoA) based on the OTU abundance are shown in **Figure 7A**. The Dgal + Gas + ABX group and other groups could be distinguished clearly on the basis of the gut microbiota composition. Meanwhile, 90 mg/kg of Gas significantly affected the gut microbiota composition of AD mice induced by Dgal (**Figure 7A**). The results of multivariate ANOVA of the PCoA matrix scores indicated a statistically significant difference between the microbiota of the Dgal + Gas + ABX group and other groups. Significant differences were also observed in the

normal control, Dgal, and 90 mg/kg Gas groups (**Figure 7B**). To understand how Gas and ABX affected the gut microbiota of AD mice, we further focused on family-level analysis, and the results are shown in **Figure 7C**. Dgal significantly increased the abundance of the Clostridiales_vadinBB60_ group, Staphylococcaceae, and Marinifilaceae and reduced that of Erysipelotrichaceae, Atopobiaceae of gut microbiota in mice. A significant increase in the abundance of Erysipelotrichaceae, Bacteroidaceae, Rhodospirillaceae, Tannerellaceae, and Atopobiaceae and significant decrease in the abundance of the Clostridiales_vadinBB60_ group and Helicobacteraceae were observed in the Gas treatment groups compared with those in the Dgal group. Meanwhile, ABX cocktail significantly reduced the abundance of Bifidobacteriaceae, Eggerthellaceae, Atopobiaceae, Muribaculaceae, Staphylococcaceae, Peptococcaceae, Marinifilaceae, Firmicutes_ unclassified, Streptococcaceae, and Erysipelotrichaceae and significantly increased the abundance of Akkermansiaceae, Tannerellaceae, Enterobacteriaceae, and Rhodospirillaceae compared with those in the Gas treatment group. These results demonstrated that Gas may increase the relative abundance of cognition beneficial microbes, such as Bacteroidaceae, Muribaculaceae, and Erysipelotrichaceae. Meanwhile, ABX significantly reduced these beneficial microbes for memory and significantly increased Akkermansiaceae to diminish the neuroprotective function of Gas.
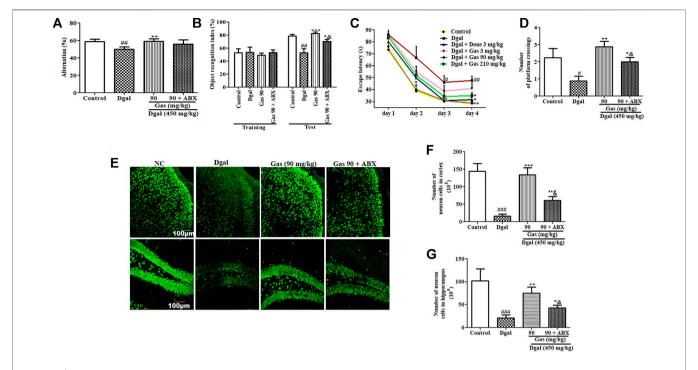
To compare the gut microbial community composition, a logarithmic LDA score cutoff of 3 and profiling of LDA effect size

FIGURE 6 | Gas upholds the integrity of the intestinal barrier and BBB. **(A)** Western blot analysis of the small intestine ZO-1 and occludin. **(B)** and **(C)** represent digitalized Western blot results of ZO-1 and occludin in the small intestine. **(D)** Western blot analysis of the cerebral cortex ZO-1 and occludin. **(E)** and **(F)** represent digitalized Western blot results of the cerebral cortex ZO-1 and occludin. Sample number of each group is three, and the data are presented as means ± SEM. [#] and [##] indicate significant differences at $p < 0.05$ and $p < 0.01$ compared with those of the normal control, respectively; * indicates significant difference at $p < 0.05$ compared with that in the Dgal group.

FIGURE 7 | Effects of Gas and antibiotics cocktail on the gut microbiota composition of AD mice. **(A)** PCoA of gut microbiota in all groups. **(B)** UPGMA tree of gut microbiota in all experimental groups. **(C)** Stacked bar chart of the relative abundance of gut microbiota at the family level. **(D)** Cladogram of gut microbiota in all groups.

FIGURE 8 | Antibiotic cocktail partially diminishes the neuroprotective effect of Gas *via* the interference of gut microbiota. **(A)** Changes of alternation in the Y-maze test. **(B)** Object recognition index in the NOR test. **(C)** Escape latency in the training phase in the MWM test. **(D)** Number of platform crossings in the test phase in the MWM experiment. **(E)** Fluorescent image of the cerebral cortex and hippocampus neurons of AD mice after treatment with Gas. **(F,G)** Digitalized results of hippocampus and cerebral cortex neurons. The brains of three mice in each group were cut, and six sections of the cerebral cortex and hippocampus of each mouse were used to calculate the neuron. The data are presented as means ± SEM. The data were presented as means ± SEM. [#], [##], and [###] indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with those of the normal control, respectively; [*], [**], and [***] indicate significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with those in the Dgal group, respectively. [&] represents significant difference at $p < 0.05$ compared with that of the Gas group.

analysis were used to identify important taxonomic differences between groups. In the normal control group, the significantly different microbiota was Olsenella, Atopobiaceae, Egicoccus, Egicoccales, Nitriliruptoria, *Lactobacillus*, Lactobacillaceae, Lactobacillales, Bacilli, Firmicutes, Flavobacteriaceae, Megamonas, Gillisia, and Xanthomonadaceae unclassified. Meanwhile, the abundance of Actinobacteria, Desulfovibrio, Coriobacteriia, Epsilonbacteraeota, Roseburia, Rikenella, Helicobacter, Anaerotignum, and Clostridiales vadinBB60 group showed a significant difference in the Dgal group. After administering Gas, a significant difference was observed in the abundance of Bacteroidetes, Muribaculaceae, Erysipelotrichales, *Lactobacillus*, Ruminococcaceae UCG 014, Actinobacteria, Bifidobacterium, Firmicutes, and Ruminococcus in the Gas treatment groups. Interestingly, significant differences were observed in Akkermansiaceae, Verrucomicrobia, Parabacteroides, *Bacteroides caecimuris*, Blautia, Enterobacteriaceae, *Clostridium innocuum*, Desulfovibrionaceae, Eubacterium nodatum group, Insolitispirillum, Lachnospiraceae UCG 006, Erysipelatoclostridium, Parasutterella, Extibacter, Flavonifractor, and Enterobacteriaceae in the ABX-treated group (**Figure 7D**, **Supplementary Figure S3**). These results demonstrated that Gas induces the proliferation of cognition beneficial microbes, such as Muribaculaceae, Erysipelotrichales,

and Bifidobacterium, while ABX significantly reduced beneficial microbes for memory and significantly increased Akkermansiaceae to offset the effects of Gas.

ABX Partially Diminishes the Neuroprotective Effect of Gas *via* the Interference of Gut Microbiota

Furthermore, we performed animal behavioral experiments to assess changes in cognition of AD mice after administering the antibiotic cocktail. Significant reduction of alternation in the Y-maze test and object recognition index in the NOR test was observed in the Dgal group. Meanwhile, these parameters were recovered in the 90-mg/kg Gas treatment group (**Figures 8A,B**, **Supplementary Figure S4A**). Furthermore, the increase of training day 4 escape latency and reduction of platform crossing numbers in the MWM test were observed in the Dgal group compared with those in the normal control (**Figures 8C,D**, **Supplementary Figure S4B**). Meanwhile, these parameters were regained in the 90-mg/kg Gas treatment group (**Figures 8C,D**, **Supplementary Figure S4B**). As we expected, the increase of these parameters in the 90-mg/kg Gas treatment group were offset or partially offset by antibiotic cocktail (**Figures 8A–D**, **Supplementary Figure S4A–B**). Moreover, we performed tissue biopsies and immunostaining of the brain to detect changes in the

cerebral cortex and hippocampus of AD mice after administering ABX. The significant reduction of neurons in the cerebral cortex and hippocampus was observed in the Dgal group (**Figures 8E–G**) and 90-mg/kg Gas treatment suppressed the reduction (**Figures 8E–G**). However, treatment with ABX significantly reduced neurons in the cerebral cortex and hippocampus compared with the Gas treatment groups (**Figures 8F,G**). Interestingly, ABX did not completely eliminate the effects of Gas. These results suggested that gut microbiota partially contribute in neuroprotection.

DISCUSSION

Gastrodia elata is a Chinese herbal medicine used for treating neurodegenerative diseases, such as AD and Parkinson's disease (Jang et al., 2015). Gas is the main constituent of *G. elata* (Liu et al., 2018) and has been reported to have antioxidative, anticancer, anticonvulsive, and neuroprotective properties (Liu et al., 2018). In spite of Gas being reported to improve the memory of AD mice such as APP/PS1 and Tg 2576 (Virgili et al., 2018; Liu et al., 2018; Hu et al., 2014), the detailed mechanism of action of Gas remains unclear. Growing evidence shows that the gut–brain axis is a pharmacological target for cognitive impairments. Therefore, in this study, we used Dgal to induce AD in mice and investigated the mechanism of action of Gas *via* the gut–brain axis. The results of animal behavior experiments in **Figures 1B–G** suggested that we successfully constructed AD model mice with Dgal, and Done and Gas at doses of 90 and 210 mg/kg, respectively, could improve the memory of AD mice. Our results are consistent with those of other studies (Obrenovich et al., 2020; Zeng et al., 2021)

Recently, other studies have reported that the gut–brain axis plays a vital role in brain functions, making the gut microbiota an emerging pharmacological target for brain aging (Kowalski and Mulak, 2019; Sharma et al., 2021). To elucidate the effect of Gas on gut microbiota, we used the 16S rRNA gene sequencing technology. The increase of Bacteroidetes and reduction of Tenericutes and Verrucomicrobia in the Dgal group and significant increase of the relative abundance of Firmicutes and Verrucomicrobiae after administering Gas (**Figures 2A–D**) indicated that Dgal induced gut microbiota dysbiosis, and Gas administration reversed it.

In the central nervous system, the hippocampus and cerebral cortex have important roles for memory. The hippocampus is involved in memory formation and storage of short-term memory traces, meanwhile the cortex took part in long-term storage of memory (Gräff et al., 2012). Moreover, they produced the interaction *via* modification of histone posttranslation. The hippocampus transferred memory traces to the cortex for memory consolidation and long-term storage and had feedback interactions for memory retrieval (Gräff et al., 2012; Preston and Eichenbaum, 2013)**.** Therefore, we detected changes in neurons of the cerebral cortex and hippocampus with immunostaining. As we expected, the significant reduction of the cortex and hippocampus neurons in the Dgal group was observed and the neuron-decrease

was fixed by Done and Gas administration (**Figures 3A–C**). The observations shown in **Figures 1–3** suggested that the gut microbiome is involved in the neuroprotective effects of Gas and Done. In the present study, we found that Gas has neuron protection effects on both the cortex and hippocampus. The results of the animal behavior experiment (Figure 1and **Figure 8**) indicated that Gas can improve the short-term memory and long-term storage of memory. We will focus of Gas effect on modification of histone posttranslation in future studies.

Neuroinflammation was a design feature of central nervous system disease and was recognized as a potential mediator of cognitive impairments. Systemic inflammation levels are increased with advanced age and neurodegeneration. The impact of age on neuroinflammatory responses including glial activation, increase of proinflammatory cytokines, and aberrant neuronal signaling could lead to the worse of the central nervous system microenvironment in disease and acceleration of cognitive impairment (Kumar, 2018). Furthermore, pathological increase in proinflammatory cytokines in the brain promoted neuron inflammation that caused neuronal death and cognitive dysfunction (He et al., 2021). Dgal induced gut dysbiosis, which increases LPS levels in blood (He et al., 2021). Gut dysbiosis affects intestinal integrity by promoting a leaky gut, which causes LPS to enter the blood circulation (He et al., 2021). This process induces systemic inflammation, which reaches the brain and promotes neuroinflammation by activating the TLR4 signaling pathway (Liu et al., 2019). In the present study, we focused on the integrity of the intestinal barrier and BBB; LPS of the plasma, cortex, and hippocampus; and TLR4/NF-κB signaling pathway to investigate the mechanism of action of Gas. The changes in these parameters in **Figures 4–6** and **Supplementary Figure S5D** revealed that Gas improved the memory of Dgal-induced AD mice by preventing LPS produced in the intestine to enter the blood and brain, reducing proinflammatory cytokines and mitigating the TLR4/NF-κB signaling pathway. Furthermore, we investigated the effect of Gas on microglia in the cortex. The results in **Figures 3D,E** suggested that microglia are involved in the neuroprotective effect of Gas.

The reduction of tight junction proteins, such as zonulin and occludin in Gal-induced AD mice were observed in this study. However, the downregulation of tight junction proteins does not necessary imply loss of function of the barriers. It is merely an indirect pointer and does not imply the loss of function of the intestine barrier and blood–brain barrier. In the future study, we will select phenolphthalein excretion test and circulating D-lactic acid assay to measure the permeability of the intestinal barrier and use the time-domain optical near-infrared imaging to evaluate the permeability of the blood–brain barrier of AD mice to confirm whether the intestinal barrier and BBB take important roles during neuroprotection of Gas.

To obtain direct evidence to support our hypothesis that the gut–brain axis plays an important role in the neuroprotective effect of Gas, we used antibiotic cocktail to deplete gut microbiota of mice and examine the effect of Gas on the gut microbiota and memory of AD mice again. The significant changes on gut microbial composition of AD mice (**Figure 7**), reduction of alteration in Y-maze and NOR index in NOR tests (**Figures 8A,B**), reduction of neurons in the cerebral cortex and hippocampus (**Figures 8D–F**), and increase of escape latencies
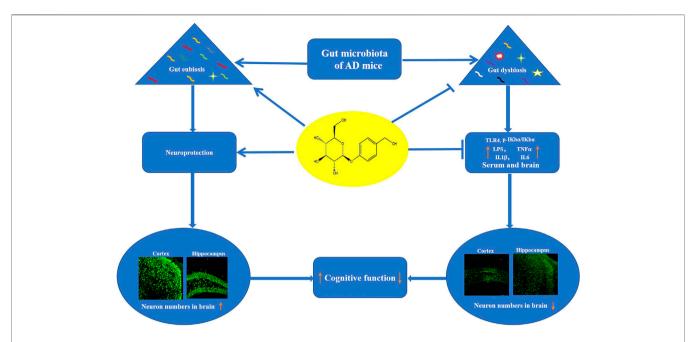
FIGURE 9 | Proposed mechanism of action of Gas. Gas improved the memory of Dgal-induced AD mice *via* regulating the composition of gut microbiota and inhibition of neuroinflammation.

and reduction of crossing numbers of the platform in the MWM experiment (**Figures 8C,D**) after administering antibiotics indicated that the gut–brain axis plays a vital role in the neuroprotective effect of Gas. However, the effect of Gas on the memory of AD mice was not completely diminished by antibiotics. This evidence illustrated that Gas not only targets the gut microbiota but also directly enters the blood circulation, traverses the BBB, and arrives in the cerebral cortex and hippocampus to exert neuroprotection.

In the previous studies, Rikenellaceae, Desulfovibrio, Cyanobacteria, Proteobacteria, Deferribacter, Helicobacter, and Escherichia have been indicated to be the harmful gut microbiota for cognitive function. Rikenellaceae was overrepresented in old mice, and the genus Alistipes of this family could modulate neuronal signals in anxiety (Murros et al., 2021; Wu et al., 2021). Desulfovibrio produce hydrogen sulfide, induce gut permeability, and promote toxin leakage that debilitates brain function (Murros et al., 2021). Cyanobacteria may produce the neurotoxin β-N-methylamino-ʟ-alanine (BMAA) to promote nervous system dysfunction and increase the formation of Aβ plaques (Silva et al., 2020). Proteobacteria can induce neuroinflammation and microglia activation and promote cognitive decline (Carabotti et al., 2015; Silva et al., 2020). Deferribacter as a pathobiont increased inflammation (Carabotti et al., 2015). Helicobacter can induce several cytokines such as IL-6 and TNF-α that can contribute to BBB disruption and promote neurodegeneration (Akhmedov et al., 2009). Escherichia produces extracellular amyloid that can bind to Toll-like receptor 2 and trigger downstream inflammatory responses, which affect cognitive functions (Tükel et al., 2010). On the contrary, Ruminococcus, *Akkermansia muciniphila*, Negativibacillus, Clostridium, Enbacterium, and Odoribacter are beneficial gut microbiota for cognitive function. Ruminococcus produces butyrate, inhibits histone deacetylase,

mitigates the secretion of proinflammatory cytokines, and promotes cognitive function (Jiang et al., 2021). Furthermore, the butyrate level in the plasma is negatively correlated with Aβ deposition in cognitively impaired individuals and butyrate decreases BBB permeability and IL-1β expression in LPS-induced AD mice (Marizzoni et al., 2020). *Akkermansia muciniphila* mitigates intestinal barrier dysfunction, reduces the deposition of Aβ plaques in the brain, promotes cognitive function, and delays pathological changes in the brain (Ou et al., 2020; Higarza et al., 2021). Negativibacillus has a positive correlation with cognition (Sadovnikova et al., 2021). *Clostridium* can synthesize 3-indolepropionic acid, a strong antioxidant that can scavenge free radicals produced by D-gal, thus protecting the brain from oxidative damage. It also reduces neuronal apoptosis, mitigates histopathological changes and BBB permeability, and promotes cognitive function (Tükel et al., 2010; Liu et al., 2015; Zhang et al., 2018; Obrenovich et al., 2020). Eubacteria and Odoribacter are positively correlated with cognitive function (Zhou et al., 2021). These data and the significant changes on these gut microbiotas after administering Gas in our study (**Figures 2A,B**, **Supplementary Figure S5A,B**) strongly supported our conclusion that Gas promotes beneficial gut microbes and mitigates non-beneficial ones to improve the memory of AD mice *via* the modification of the gut–brain axis.

Interestingly, the abundance of *Akkermansia muciniphila*, which produce beneficial metabolites for the brain, was significantly increased after antibiotic treatment in our study. However, the memory of AD mice was not increased by the excessive increase of *Akkermansia muciniphila*. This finding indicated that the ratio of gut microbiota is very important for optimal function. Our results were consistent with those of other studies, which reported that the overcolonization of *Akkermansia muciniphila* promotes inflammation and *Akkermansia*

muciniphila is overrepresented in Parkinson's disease patients (Seregin et al., 2017; Lubomski et al., 2021).

In the present study, we used intraperitoneal injection of Dgal to induce AD in mice. Although significant changes in gut microbiota were observed, the results of PCoA showed the overlap of gut microbiota in the normal control and Dgal groups (**Supplementary Figure 5C**). It is possible that the effect of intraperitoneal injection of Dgal on gut microbiota was weaker than that of oral administration. In a future study, we will compare the effect of these two methods on gut microbiota and select the best way to construct an AD model. Furthermore, we will use transgenic AD mice or natural aging mice to confirm whether Gas has the same efficacy and mechanism of action in different AD models.

On the other hand, we performed bacterial phenotypic prediction of gut microbiota in our study. The reduction of aerobic, gram-positive, and containing mobile elements and the increase of anerobic, gram-negative, and potentially pathogenic were observed in the Dgal group compared with those in the normal control (**Supplementary Figure S6A–F**). These parameters were reversed to the normal levels in the Done and 90 mg/kg Gas groups (**Supplementary Figure S6A–F**). In addition, Gas activates biosynthesis of tetrahydofolate, biotin, stearate, oleate, palmitate, palmitoleate, and lipid IVA (**Supplementary Figure S7**). These results confirmed that the microbiome–gut–brain axis is involved in the neuroprotective effect of Gas.

CONCLUSION

In conclusion, Dgal induced the dysbiosis of gut microbiota to produce LPS and impaired the integrity of the intestinal barrier and BBB. Furthermore, LPS enters the blood to induce neuroinflammation and cognitive decline. Gas improves the memory of AD mice *via* increasing abundance of *Lactobacillus* and Firmicutes, which produce GABA, acetylcholine, and histamine to promote cognitive function and reducing abundance of pathogenic bacteria. Moreover, the Gas maintained intestinal barrier and BBB function, which was damaged by Dgal treatment to protect the brain from LPS and proinflammatory cytokines (**Figure 9**). Our study provides a scientific basis for the development of drugs targeting gut microbes.

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/bioproject; PRJNA781105.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of Zhejiang University Permit which approved the experiment with permit number (ZJU20210155).

AUTHOR CONTRIBUTIONS

OF and JW performed animal experiments, mechanism of action analysis, and data analysis; JM identified *Gastrodia elata* and performed data analysis; OF wrote the draft; HOs, HOh, WP, LX, and JQ contributed to designing the overall research strategies. LX, HOs, HOh, and JQ supervised and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.814271/full#supplementary-material

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The Role of Gut Microbiota—Gut—Brain Axis in Perioperative Neurocognitive Dysfunction

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With the aging of the world population and advances in medical and health technology, more and more elderly patients are undergoing anesthesia and surgery, and perioperative neurocognitive dysfunction (PND) is receiving increasing attention. The latest definition of PND, published simultaneously in November 2018 in 6 leading journals in the field of anesthesiology, clarifies that PND includes preoperatively cognitive impairment, postoperative delirium, delayed neurocognitive recovery, and postoperative cognitive dysfunction and meets the diagnostic criteria for neurocognitive impairment in the Diagnostic and Statistical Manual of Mental Disorders -fifth edition (DSM-5). The time frame for PND includes preoperatively and within 12 months postoperatively. Recent studies have shown that gut microbiota regulates central nervous function and behavior through the gut microbiota - gut - brain axis, but the role of the axis in the pathogenesis of PND remains unclear. Therefore, this article reviews the mechanism of the role of gut microbiota-gut-brain axis in PND, so as to help explore reasonable early treatment strategies.

Keywords: gut microbiota, gut-brain axis, perioperative neurocognitive dysfunction, postoperative cognitive dysfunction, cognition

INTRODUCTION

PND refers to alterations in cognitive function before and/or after surgery, including preoperatively diagnosed cognitive decline, postoperative delirium (POD), delayed neurocognitive recovery, and postoperative cognitive dysfunction (POCD) (Evered et al., 2018a). (**Figure 1**). The incidence of PND rangs from 41 to 75% at 7 days postoperatively to 18–45% at 3 months postoperatively (Austin et al., 2019). PND tends to occur in elderly patients and is mainly characterized by diminished attention, memory, and verbal thinking skills. The risk factors for PND are multifaceted and may be related to age, infection, preexisting cognitive disorders, surgery duration, anesthetic management, tissue damage, psychological stress and genetic susceptibility (Subramaniyan and Terrando, 2019; Eckenhoff et al., 2020). It has been shown that PND is mainly associated with neuroinflammation, cholinergic dysfunction, oxidative stress, abnormal accumulation of β-amyloid and impaired neurosynaptic function (Pepeu and Giovannini, 2010; Lin et al., 2020). PND is very harmful as it can lead to prolonged hospital stays, increased hospital costs, high social burden and increased mortality. Currently, there are many prevention and treatment strategies for PND, including drug prevention (Su et al., 2011; Tian et al., 2015; Lee et al., 2017; Zhang et al., 2018), antibiotic anti-inflammatory (Liang et al., 2018), control of fasting time and carbohydrate load (Batchelor et al., 2019), physical activity and social participation (Lai et al., 2021), anesthesia management, such as

multi-mode analgesia including preemptive analgesia, dexmedetomidine, and epidural analgesia (Lei et al., 2020; Fan et al., 2021), and blood pressure management (Maheshwari et al., 2020) and so on. However, these strategies have not achieved ideal clinical effects. Therefore, it is particularly important to explore the pathogenesis and effective prevention and treatment strategies of PND.

In recent years, gut microbiota has become an important topic in biological research. Signals from the gut can regulate the function of specific brain regions, and patients with central nervous system (CNS) diseases are often accompanied by gastrointestinal symptoms such as constipation and diarrhea, so the concept of gut microbiota - gut - brain axis emerged. Germ-free mice can show impaired learning and memory (Diaz Heijtz et al., 2011; Clarke et al., 2013), and recent studies have shown that changes in gut microbiota are associated with abnormal cognitive behavior. The abnormal gut microbiota caused by surgical anesthesia was age-dependent, which manifested by a significant decrease in the abundance and diversity of the microbiota with increasing age (Liufu et al., 2020). The reduction of beneficial bacteria (*lactic acid bacteria, bifidobacterium*) increases the risk of postoperative cognitive impairment, while surgical anesthesia exacerbates gut microbiota dysbiosis and shifts gut microbiota to a more toxic phenotype (Guyton and Alverdy, 2017). Many perioperative factors can affect the gut microbiota, including the operation itself, antibiotics, opioids or acid-inducing drugs (Krezalek and Alverdy, 2016). Studies have found a strong link between abnormal gut microbiota composition and the onset of autism, depression, schizophrenia and Alzheimer's disease (Mangiola et al., 2016; Lv et al., 2017; Yang et al., 2017a; Yang et al., 2017b; Zhan et al., 2018). There is growing evidence that gut microbes communicate with the central nervous system and can influence brain function and behavior through neural, endocrine, and immune pathways (Lynch and Hsiao, 2019). Thus, it would be beneficial for the prevention and treatment of CNS diseases to regulate gut microbiota composition and improving its physiological functions.

Intestinal barrier dysfunction is considered to be an important cause of gut-brain axis dysfunction. Normal gut microbiota is equivalent to a protective biological barrier for the intestine, while gut microbiota dysbiosis can not only produce neurotoxic factors, but also affect intestinal mucosal permeability, resulting in "leaky gut", which can cause a large number of inflammatory and neurotoxic factors to enter the CNS (Osadchiy et al., 2019). The phenomenon of "leaky gut" with increased intestinal permeability and gut microbiota dysbiosis occurs after general anesthesia surgery and is closely related to postoperative cognitive impairment.

Gut Microbiota Dysbiosis and Neuroimmune Dysfunction

Central nervous inflammatory response is the main pathologic process of PND (Nathan, 2019; Yang et al., 2019; Yang et al., 2020a). Tissue damage and oxidative stress induced by surgery and anesthesia can induce the release of local or systemic pro-

inflammatory cytokines and the activation of corresponding inflammatory signaling pathways, resulting in systemic inflammation (Huber-Lang et al., 2018). Pro-inflammatory cytokines can utilize specific receptors and transporters on the surface of endothelial cells of the blood-brain barrier (BBB) and directly cross the BBB, triggering neuroinflammation and ultimately leading to POCD (Abrahamov et al., 2017; Yang S. et al., 2017; Yang et al., 2020a). Microglia are resident immune cells of the CNS, responsible for immune monitoring and detection of their designated brain regions (Kettenmann et al., 2011). As systemic inflammation progresses, microglia develop an activated morphological phenotype and release increased pro-inflammatory factors such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and reactive oxygen species (ROS) (Liu et al., 2020). Microglia release proinflammatory factors that contribute to subsequent astrocyte activation and further promote neuroinflammation (Liddelow et al., 2017; Liu et al., 2020). In addition, cytokines entering the brain can activate the hypothalamic-pituitary-adrenal (HPA) axis to release cortisol and act on downstream pathway of the glucocorticoid receptor in the hippocampus, triggering depressive-like behaviors (Luo et al., 2018). Activation of complement system is another necessary inflammatory response activated by the damage associated molecular patterns triggered by surgery. For example, deposition of c-reactive protein activates and modulates the classical complement pathway, leading to inflammatory dysregulation. Use of drugs that block the complement cascades, such as C3 receptor blockers, improves neuroinflammation and memory function in PND (Xiong et al., 2018), also suggesting the role of neuroinflammation in PND.

Gut microbiota dysbiosis aggravates postoperative systemic inflammatory response. Intestinal mucosal immune barrier is an important component of the intestinal barrier, mainly composed of abundant lymphocytes and macrophages, and is the largest immune cells reservoir of the body. The normal gut microbiota maintains a good balance with the host mucosal immune system. A systematic retrospective study shows that patients with or without digestive surgery experienced significant changes in their gut microbiota, characterized by an increased proportion of gram-negative bacteria (Lederer et al., 2017). Gram-negative bacterial cell wall lipopolysaccharide (LPS) activates Toll-like receptors 4 (TLR4) on the surface of intestinal epithelial cells and mediates enhanced intestinal permeability, the activation of TLR in plasma and brain cells by LPS can induce the release of pro-inflammatory cytokines and lead to memory deficits (Yang et al., 2020b). At the same time, dendritic cells directly capture the antigens of intestinal dysregulated bacteria, or Intestinal Microfold Cells phagocytose antigens and then deliver them to dendritic cells and other antigen-presenting cells, which affect the development and differentiation of CD4$^+$ and CD8$^+$ T cells, resulting in the imbalance of intestinal immune homeostasis (Ekmekciu et al., 2017). The damaged intestinal barrier can then promote intestinal bacteria and intestinal toxic metabolites to enter the blood circulation. Pro-inflammatory factors can reduce the expression of tight junction proteins

such as occludin and claudin-5 at mRNA and protein levels, destroy the integrity of BBB, and can enter the brain to activate adaptive immune cells, resulting in brain immune dyshomeostasis (Ni et al., 2018). In animal studies, oral *lactobacillus* reduced the BBB permeability, and consequently protected the postoperative cognitive functions of the aged and gut dysbiosis mice (Wen et al., 2020). And after oral prebiotic Bimuno® (galactooligosaccharide (B-GOS) mixture), the number of probiotics such as *Lactobacillus and Bifidobacterium* in intestine of PND mice increased, the level of inflammatory factors in hippocampus of brain decreased, and the cognitive function improved (Yang et al., 2018). A meta-analysis suggested that probiotics enhanced cognitive performance in patients with Alzheimer's disease (AD) or mild cognitive impairment (MCI) possibly by reducing levels of inflammatory and oxidative biomarkers (Den et al., 2020). It was found that probiotics VSL#3 treatment could upregulate the expression of microRNA-146a (miR-146a) and block the BTG2/Bax (B-cell translocation gene 2/Bcl-2-associated X protein) axis in POCD mice, thus inhibiting neuronal apoptosis and reducing oxidative stress (Mao et al., 2021). In addition, miR-146a overexpression alleviated hippocampal dependent learning and memory impairment and hippocampal inflammation in POCD mice (Chen et al., 2019). MicroRNA-146a protects neurons by altering microglial phenotypes, reducing pro-inflammatory cytokines and enhancing phagocytosis, thereby ameliorating cognitive deficits in AD mice (Liang et al., 2021). Therefore, postoperative gut microbiota dysbiosis may promote the development of PND by aggravating peripheral and central inflammatory responses. (**Figure 2**).

Gut Microbiota Dysbiosis and Neurotransmitter Products

The occurrence of PND is closely related to the concentration of neurotransmitters in brain. Neurotransmitters such as acetylcholine (Ach), 5-hydroxytryptamine (5-HT), dopamine (DA), gamma amino butyric acid (GABA), etc. affect the function of CNS through central cholinergic and dopaminergic nerves, and the decline of learning and memory ability is often accompanied by the changes in neurotransmitter levels in related brain regions. Application of cardiopulmonary bypass (CPB) can reduce the mortality caused by myocardial infarction, heart failure, and fatal arrhythmias, but CPB-related complications are prevalent, such as POCD and gastrointestinal injury. α7nAchR activation markedly reduced intestinal injury, POCD, neuronal apoptosis, proinflammatory factor expression, and number of CD4$^+$ IL-17$^+$ cells. In contrast, the lack of α7nAChR significantly aggravated the pro-inflammatory response and POCD caused by CPB (Kong et al., 2015; Chen et al., 2018). There is increasing evidence that the pathophysiology of delirium is caused by multiple neurotransmitter system dysfunctions. The concentrations of DA and 5-HT metabolites in cerebrospinal fluid (CSF), hippocampus and basolateral amygdala of POD rats were significantly increased. After treatment with the selective

5-HT1A antagonist WAY-100635, POD symptoms were reversed at some extent in rats (Qiu et al., 2016).

Surgical anesthesia disturbs the gut microbiota, destroys the 5-HT synthesis and metabolic homeostasis involved in intestinal endocrine cells and gut microbiota, and can lead to the fluctuation of the level of 5-HT in the body, thus affecting the mood, behavior and postoperative gastrointestinal peristalsis. In addition, *lactobacillus, Bifidobacterium, Streptococcus* and other bacteria in the intestinal tract are involved in the process of GABA synthesis by glutamic acid metabolism, and the GABA synthesized by gut microbiota directly stimulates enterochromaffin cells to secrete 5-HT, affecting the levels of brain-derived nutritional factors and dopamine. Supplementing probiotics, regulating gut microbiota and promoting the stability of neurotransmitter level will be beneficial to postoperative recovery (Yang et al., 2020b; Lv et al., 2021). Fructooligosaccharides have been found to have positive neural effects in AD rats, such as increasing Ach, serotonin, and adrenaline, and reducing damage to the CA1 region of the hippocampus (Chen et al., 2017). Therefore, postoperative gut microbiota dysbiosis may promote the development of PND by affecting the balance of neurotransmitters. (**Figure 2**).

Gut Microbiota Dysbiosis and Abnormal Gut Microbiota-Host Co-Metabolites

Gut microbiota can provide the host with biological enzymes and biotransformation pathways that the human body does not possess, and co-metabolize with the host, producing metabolites such as vitamins, fatty acids, bile acids and trimethylamine N-oxide (TMAO) and other metabolites through various metabolic pathways and transferring them to the circulatory system, thus regulating the microenvironment and function of the brain.

Vitamin D (VD) plays a vital role in gut homeostasis (Malaguarnera, 2020). VD administration improved cognitive dysfunction by modulating gut microbiota and increased *Bacteroidetes/Firmicutes* ratio (Hussein et al., 2022). VD supplementation increased the relative abundance of *Bacteroides* (Charoenngam et al., 2020). Vitamin A deficiency exacerbated learning and memory deficits, decreased abundance of *Lactobacillus* and α- and β-diversity. Gut microbiota α diversity decreased, β diversity increased in depressed bipolar disorder patients, mainly for the abundance levels of *Streptococcaceae* and *Bacteroidaceae* increased, while the abundance levels of *Akkermansia Muciniphila* and *Faecalibacterium prausnitzii* decreased. Meanwhile, further analysis showed that the level of serum metabolites changed significantly in depressed bipolar disorder patients, including B-vitamins, kynurenic acid, gamma-aminobutyric acid and short-chain fatty acids. These metabolites may be associated with the abundance of gut microbiota, including *Akkermansia muciniphila, Citrobacter spp, Phascolarctobacterium spp, Yersinia spp, Enterobacter spp* and *Flavobacterium spp* (Li et al., 2022).

Short-chain fatty acids (SCFAs) are essential metabolites of intestinal microbial activity and are trophic factors for the intestinal mucosa and epithelium. They are produced by

microbial fermentation of dietary fiber in colon, and mainly include acetate (C_2), propionate (C_3) and butyrate (C_4) (Martin-Gallausiaux et al., 2021). SCFAs induce inflammation and immune responses by activating G protein-coupled receptors (GPR41 and GPR43) on the surface of intestinal epithelial cells and immune cells (Xiong et al., 2004; Kimura et al., 2013). SCFAs activate the sympathetic nervous system by binding to the GPR41 receptor in sympathetic ganglion neurons (Kimura et al., 2011). SCFAs can cross BBB, affect neurotransmission and neurotransmitter production, and induce abnormal behavior (DeCastro et al., 2005; Mitchell et al., 2011). Microbiota can also regulate the metabolism of tryptophan, which plays a key role in the normal operation of the immune system and gut-brain axis (Agus et al., 2018), the three major metabolic pathways for the production of kynurenine, indole and 5-HT from tryptophan are all under the indirect or direct control of microbiota. DA, Ach and GABA can also be produced by gut microbiota (Fung et al., 2017; Sherwin et al., 2018). In addition, LPS, a metabolite of bacteria, can directly affect the CNS by activating TLR4 in microglia, resulting in massive production of inflammatory cytokines in the CNS (Leitner et al., 2019). In mice with AD, fecal microbiota transplantation (FMT) treatment reversed the changes of gut microbiota and the SCFA butyrate, and the symptoms were improved (Sun et al., 2019).

TMAO, a gut microbe-dependent metabolite is implicated in the development of age-related cognitive decline. Increased levels of circulating TMAO may induce the susceptibility to surgery, contributing to aggravating neuroinflammation and decreasing cognitive ability in elderly rats after surgery (Meng et al., 2019). Reduced circulating TMAO levels by 3,3-Dimethyl-1-butanol ameliorated the cognitive decline in APP/PS1 mice, accompanying a decrease in neuroinflammation and the Amyloid β (Aβ)1–42 levels in the hippocampus (Gao et al., 2019). The study found that a decrease in the number of *lactobacillus plantarum* in the gut was associated with cognitive impairment. *Lactobacillus plantarum* enhanced the therapeutic effects of memantine treatment in APP/PS1 mice by remodeling the intestinal microbiota and inhibiting the synthesis of TMAO (Wang et al., 2020).

Gut microbiota metabolites have been proved to have neuroactive or toxic effects. Recent studies have shown significant metabolic abnormalities in patients with postoperative cognitive impairment. Therefore, postoperative gut microbiota dysbiosis may promote the development of PND by causing abnormal gut microbiota-host co-metabolites. (**Figure 2**).

Gut Microbiota Dysbiosis and Amyloid-Beta and Tau Protein

Advanced age is an independent risk factor for PND, and neuronal degeneration may occur in elderly patients before surgery, which is manifested by Aβ protein accumulation and protein hyperphosphorylation in the brain. The accumulation of Aβ protein and hyperphosphorylation of Tau protein were increased in neurons of aged mice after surgery and anesthesia (Terrando et al., 2011). In animal studies, isoflurane exposure led

to spatial memory impairment with increases of levels of Aβ and phosphorylated Tau protein in the brain (Zuo et al., 2018). In addition, it has been reported that elevated Aβ and Tau protein in CSF after surgery are risk factors for PND (Evered et al., 2018b). The accumulation of Aβ and Tau protein induces neuroinflammation, leading to glial cell activation, pro-inflammatory cytokines release and neuronal damage (Calsolaro and Edison, 2016).

Aβ proteins from the gut microbiota themselves (produced by *E. coli, Bacillus subtilis, Salmonella*, etc.) can enter bloodstream through the damaged intestinal wall. Although the primary structure of enteric-derived Aβ is different from that of cerebral Aβ, its tertiary structure is very similar, suggesting that enteric-derived Aβ may trigger cross-immune responses and trigger overactivation of pro-inflammatory signaling pathways in the brain. The increased proportion of *Escherichia coli* in the intestinal tract after surgery and anesthesia can promote Aβ protein deposition inside and outside the neuronal cells, and can promote the synaptic dysfunction and even lead to cell death by activating the reactive changes of glial cells surrounding the neuronal cells. In addition, deposition of Aβ protein interferes with NMDA receptor mRNA expression in hippocampal neurons and cortex, reducing synaptic plasticity and leading to cognitive impairment (Newcombe et al., 2018). The increase in Aβ protein caused by gut microbiota dysbiosis can be ameliorated by transplantation of normal fecal microbiota. Germ-free mice showed cognitive dysfunction after receiving FMT from human amyloid precursor protein knock-in mice, which indirectly indicated that gut microbiota dysbiosis could promote Aβ protein deposition to cause cognitive impairment (Kundu et al., 2022). Therefore, postoperative gut microbiota dysbiosis may promote the development of PND by causing abnormal accumulation of Aβ and Tau protein. (**Figure 2**).

Gut Microbiota Dysbiosis and Anxiety and Depression

Anxiety and depression are the most common psychiatric disorders with increasing numbers of people worldwide suffering from them (Kessler and Bromet, 2013). Anxiety, depression and functional bowel disorders are highly comorbid, suggesting that the gut-brain axis may be involved in the pathological mechanism of these psychosis (Mussell et al., 2008). Patients with anxiety and depression often present with elevated inflammation levels, HPA axis dysfunction, neurotransmitter signaling dysfunction, etc. As previously described, gut microbiota regulates these events, and thus they may have the potential to regulate depression and anxiety disorders (Dinan and Cryan, 2013).

Studies have shown that gut microbiota can regulate anxiety in mice. For example, compared with the SPF control, GF Swiss Webster, National Institutes of Health Swiss, and Naval Medical Research Institute mice showed decreased anxiety-like behavior. Conversely, GF BALB/c and C57BL/6 mice showed increased anxiety-like behavior (Sampson and Mazmanian, 2015). These results suggested that anxiety-like behavior in mice was highly correlated with gut microbiota.

FIGURE 1 | Nomenclature of PND. PND is divided into five categories by time period, including preoperative neurocognitive dysfunction (preoperative, measurable, objective impairment of cognitive function), POD (occurred within 1 week after surgery or before discharge and met the DSM-5 diagnostic criteria for delirium), delayed neurocognitive recovery (cognitive decline within 0–30 days after surgery), postoperative neurocognitive disfunction (pNCD or POCD, mild and severe cognitive decline existed from 30 days to 12 months after surgery) and first diagnosed cognitive decline more than 12 months after surgery. Gut microbiota dysbiosis and intestinal barrier dysfunction.
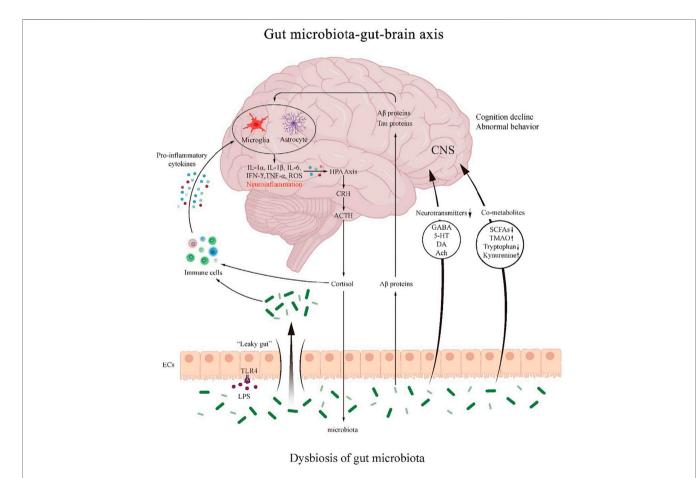
FIGURE 2 | The mechanism of the role of gut microbiota-gut-brain axis in PND. The gut microbiota regulates the interactions between the gut and brain through neural, endocrine and immune pathways. Dysbiosis of the gut microbiota regulates immune activity and produces pro-inflammatory cytokines that can either promote the formation of neuroinflammation and further activate the HPA axis.

A growing number of studies have shown that people with major depressive disorder (MDD) have altered gut microbiota structure in comparison with healthy controls. Gut microbiota dysbiosis can lead to depression-like behaviors in GF mice. For example, GF mice exhibited depressive-like behaviors and

metabolic disturbances after receiving FMT from MDD patients (Kelly et al., 2016; Zheng et al., 2016). Thus gut microbiota dysbiosis is an important factor causing MDD.

Recently, a large amount of evidence has shown the potential anti-anxiety and anti-depression activity of probiotics. Treating

mice with *Lactobacillus rhamnosus* JB-1 reduced stress-induced HPA response and anxiety-like behavior (Bravo et al., 2011). Administration of *Lactobacillus helveticus* improved anxiety and depression caused by restraint stress in adult SPF rats (Liang et al., 2015). Moreover, the combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 showed potential anti-anxiety like activity in rats and promoted psychological properties in healthy people (Messaoudi et al., 2011). *Bifidobacterium longum* 1714 or *Bifidobacterium breve* 1205 reduced stress-related behavior (anxiety or depression) in innately anxious BALB/c mice (Savignac et al., 2014). Prebiotics have also been shown to improve anxiety and depression. Galacto-oligosaccharides (GOS) or the combination of GOS and fructo-oligosaccharide improved anxious and depressant behavior in rodents (Burokas et al., 2017).

Patients with PND often present with a variety of neuropsychiatric symptoms like anxiety and depression, and anxiety and depression will accelerate the decline of their cognitive function (Ismail et al., 2018). We already know that gut microbiota is linked to anxiety and depression, so gut microbiota may promote the development of PND by inducing neuropsychiatric symptoms such as anxiety and depression.

Gut microbiota dysbiosis leads to altered levels of neurotransmitters, such as GABA, 5-HT, DA and Ach. It also causes abnormal gut microbiota-host co-metabolites modulating SCFAs, TMAO and tryptophan/kynurenine pathway metabolites. Gut microbiota and LPS affect intestinal mucosal permeability, resulting in "leaky gut". Aβ proteins from the gut microbiota themselves can enter bloodstream through the damaged intestinal wall and play a role in neuroinflammation.

CRH: corticotropin releasing hormone; ACTH: adrenocorticotropic hormone; HPA: hypothalamic-pituitary-adrenal; EC: enteroendocrine cell; CNS: central nervous system; GABA: gamma amino butyric acid; 5-HT: 5-hydroxytryptamine; DA: dopamine; Ach: acetylcholine; SCFA: short-chain fatty acids; TMAO: trimethylamino oxide; LPS: lipopolysaccharide; TLR4: toll-like receptor 4.

CONCLUSION

In summary, there is a strong link between gut microbiota and PND that depends on the gut-brain axis. Gut microbiota modulates neurological function in the brain through various pathways such as participation in immune regulation and neuroendocrine regulation (Xu et al., 2020), affecting patients' cognitive function. Mechanisms by which gut microbiota regulate the CNS have been proposed, including the biotransformation of neurotoxicants mediated by gut microbiota, changes in neuroactive products in response to environmental stressors, bidirectional communication within the gut brain axis to alter the integrity of the intestinal barrier and regulation of mucosal immune function (Dempsey et al., 2019). Surgery and anesthesia may disrupt gut microbiota homeostasis in a direct or indirect manner, causing cognitive dysfunction. The research on PND and gut microbiota dysbiosis is getting more and more advanced, but there is a lack of research on gut microbiota alterations and postoperative cognitive changes after surgery and anesthesia, and more basic and clinical research is needed in the future to focus on the role of specific gut microbiome and specific targets of gut microbiota and provide new approaches for the treatment of PND.

AUTHOR CONTRIBUTIONS

JL and WH did literature retrieval and prepared the draft, SG and YeZ made the first revision of the manuscript, YoZ finalized the manuscript.

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Zi Shen Wan Fang Attenuates Neuroinflammation and Cognitive Function *Via* Remodeling the Gut Microbiota in Diabetes-Induced Cognitive Impairment Mice

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Background: Cognitive dysfunction is a critical complication of diabetes mellitus, and there are still no clinically approved drugs. Zi Shen Wan Fang (ZSWF) is an optimized prescription composed of Anemarrhenae Rhizoma, Phellodendri Chinensis Cortex, and Cistanches Herba. The purpose of this study is to investigate the effect of ZSWF on DCI and explore its mechanism from the perspective of maintaining intestinal microbial homeostasis in order to find an effective prescription for treating DCI.

Methods: The diabetes model was established by a high-fat diet combined with intraperitoneal injections of streptozotocin (STZ, 120 mg/kg) and the DCI model was screened by Morris water maze (MWM) after 8 weeks of continuous hyperglycemic stimulation. The DCI mice were randomly divided into the model group (DCI), the low- and high-ZSWF–dose groups (9.63 g/kg, 18.72 g/kg), the mixed antibiotic group (ABs), and the ZSWF combined with mixed antibiotic group (ZSWF + ABs). ZSWF was administered orally once a day for 8 weeks. Then, cognitive function was assessed using MWM, neuroinflammation and systemic inflammation were analyzed by enzyme-linked immunosorbent assay kits, intestinal barrier integrity was assessed by hematoxylin-eosin (HE) staining and Western blot and high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Furthermore, the alteration to intestinal flora was monitored by 16S rDNA sequencing.

Results: ZSWF restored cognitive function in DCI mice and reduced levels of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. Moreover, ZSWF protected

Abbreviations: ZSWF, Zi Shen Wan Fang; DCI, diabetic cognitive impairment; STZ, streptozotocin; ABs, antibiotic; MWM, morris water maze; HE, hematoxylin-eosin; UPLC-MS/MS, high performance liquid chromatography tandem mass spectrometry; DM, diabetes mellitus; SCFAs, short-chain fatty acids; HFD, high-fat diet; Muc2, Mucin-2; SIgA, secretory immunoglobulin A; PCoA, weighted unifrac principal coordinate analysis; FBG, fasting bood glucose.

the integrity of the intestinal barrier by increasing intestinal ZO-1 and occludin protein expression and decreasing urinary lactulose to mannitol ratio. In addition, ZSWF reshaped the imbalanced gut microbiota in DCI mice by reversing the abundance changes of a wide range of intestinal bacteria at the phyla and genus levels. In contrast, removing gut microbiota with antibiotics partially eliminated the effects of ZSWF on improving cognitive function and reducing inflammation, confirming the essential role of gut microbiota in the improvement of DCI by ZSWF.

Conclusion: ZSWF can reverse cognitive impairment in DCI mice by remolding the structure of destructed gut microbiota community, which is a potential Chinese medicine prescription for DCI treatment.

Keywords: ZSWF, DCI, inflammation, intestinal barrier, gut microbiome

INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disease with increasing incidence. Cumulative studies confirm that DM is a key risk factor for cognitive impairment (Zilliox et al., 2016; Biessels and Despa, 2018; Biessels and Whitmer, 2020). Although many excellent studies of DCI exist, its potential mechanisms remain elusive and effective drugs are lacking. Several studies suggested that DCI might be attributed to cerebrovascular dysfunction (Prakash et al., 2012), neuroinflammation (Rom et al., 2019), and metabolic disorders (Zhang et al., 2020a). In recent years, emerging studies noticed that intestinal microbiota structure and function disorders are closely related to brain function. Gut-brain crosstalk is a complex network system, which maintains the stability of gastrointestinal tract on the one hand and affects the brain homeostasis on the other hand (Rhee et al., 2009). Intestinal microbes regulate brain homeostasis through multiple pathways, among which intestinal barrier-inflammation is one of the main pathways (Rogers et al., 2016). Next, the intestinal barrier plays a crucial role in maintaining of intestinal microbiome and peripheral homeostasis. In contrast, intestinal microbiome dysbiosis can provoke disruption of the intestinal barrier. Increased intestinal permeability can cause excessive translocation of bacterial lipopolysaccharide into the bloodstream, which can trigger systemic inflammation (Zhang et al., 2019). Microbiota-derived inflammatory response ultimately leads to neuroinflammation and neuronal damage (Bairamian et al., 2022).

Increasing evidence indicates that the structural changes of intestinal microbiota are associated with the development of DCI (Xu et al., 2017). Clinical studies found significant differences in gut microbiome composition between diabetic patients with and without cognitive impairment (Zhang et al., 2021a). Also, preclinical studies suggested that DCI was associated with alterations of the gut microbiome (Gao et al., 2019). Furthermore, Yu et al. (2019) transplanted fecal bacteria from DCI and non-DCI mice into the gut of pseudo-germ-free mice, respectively, and they found that the escape latency was significantly longer in pseudo-germ-free mice receiving DCI mouse fecal bacteria than those receiving non-DCI mouse

fecal bacteria. These studies suggest that maintaining intestinal microbial homeostasis is an effective strategy for the prevention and treatment of DCI.

Traditional Chinese medicine (TCM) believes that "poison damage brain collateral" and "deficiency of kidney essence" are the main pathogenesis of DCI, and "clearing heat-fire and detoxifying" and "invigorating kidney for protecting semen" are the key therapeutic principles. Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex are the most commonly used heat-clearing drug pair in clinical practice, and they were originally derived from "Tong Guan Wan." Previous modern pharmacological evidence suggests that Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex drug pair exhibit an effect on improving diabetes-related complications (Zhang et al., 2014), and their active ingredients demonstrate a clear protective effect on cognitive function (Liu et al., 2012; Xian et al., 2013; Liang et al., 2017; Piwowar et al., 2020). However, studies also found that the bioavailability of saponins and alkaloids (the main components of Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex, respectively) is low (Singh and Chaudhuri, 2018; Baldim et al., 2020), suggesting that regulation of intestinal microbial homeostasis may be the key pathway for them to improve diabetes-related complications. Furthermore, considering that the treatment of diabetes-related complications is a long process and that Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex are severe cold medicines, the compatibility of warm drugs to neutralize cold is the basic principle of the compatibility of TCM prescriptions. Cistanches Herba (*Cistanche deserticola* Y. C. Ma), a classic Chinese medicine with the effect of tonifying kidney and nourishing essence, is warm in property. Also, modern pharmacological studies suggest that Cistanches Herba demonstrates the function of maintaining intestinal flora homeostasis and neuroprotection (Li et al., 2017; Wang et al., 2017). Therefore, under the guidance of basic theories of TCM, we formed ZSWF by the compatibility of the Cistanches Herba (*C. deserticola* Y. C. Ma) and Anemarrhenae Rhizoma (*Anemarrhena asphodeloides* Bge)-Phellodendri Chinensis Cortex (*Phellodendron chinense* Schneid) herb pair, hoping to find a new prescription to treat DCI.

In this study, we investigated the effects of ZSWF on the cognitive function of DCI mice, and we explored the possible mechanism by regulating intestinal flora. Cognitive function, neuroinflammation and systemic inflammation, intestinal barrier integrity, and gut microbiome diversity were examined. Furthermore, an antibiotic intervention was used to assess the possibility of a causal relation between ZSWF improving cognitive and gut microbiota.

METHODS

Preparation of Zi Shen Wan Fang Extract

ZSWF is composed of Anemarrhenae Rhizoma (dried rhizome of *A. asphodeloides* Bge.), Phellodendri Chinensis Cortex (dried bark of *P. chinese* Schneid.), and Cistanches Herba (dried fleshy stem of *C. deserticola* Y. C.Ma) with a ratio of 1:1:1 in weight. The drug materials of Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex were purchased from Hebei anguo Chinese Herbal Medicine Co., Ltd., and Cistanches Herba was purchased from Inner Mongolia Mandera Biological Technology Co., Ltd. These herbs were identified by professor Tianxiang Li (TianJin University of Traditional Chinese Medicine) and were extracted according to our previous method (Zheng et al., 2018). In simple terms, Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex were mixed at the ratio of 1: 1 by weight, then extracted by reflux for 3 times with 80% ethanol of 8 times (2 h each time), and filtrate was collected. The same weight Cistanches Herba was first extracted by reflux for 3 times with 8 times the amount of 80% ethanol (2 h each time), and filtrate was collected and then extracted with 10 times the amount of distilled water for 3 times (2 h each time). Next, the volume of liquid required for ingastric administration of mice during the treatment cycle was calculated, and all filtrate was mixed and concentrated to 0.94 g/ml ZSWF crude extract by rotary evaporator, which was separated and stored in a refrigerator at −80°C for use. Moreover, our previous study systematically investigated the main chemical components of ZSWF extract (Zheng et al., 2018), including *in vitro* components (e.g., Neomangiferin, Berberine, TimosaponinBII, and Cistansinenside A), absorbed components (e.g., Mangiferin, Timosaponin C, and 3/5-O-Feruloylquinic acid), as well as prototypical (e.g., Phellodenrine, Tetrahydropalmatine, and Oxoberberine) and metabolic components [e.g., 3,4-dihydroxybenzenepropionic acid and (20R, 25S)-timosaponin AI] in feces.

Establish the Diabetic Cognitive Impairment Model and Administration Method

Two hundred male C57BL/6J mice (8 weeks-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., [SCXK (Jing) 2016-006]. The animals were housed in the Laboratory Animal Center of Tianjin University of Traditional Chinese Medicine under a standard laboratory condition (temperature 22 ± 2°C, humidity at 50 ± 15%, 12-h-light/12-h-dark cycle) and were given *ad libitum* access to water and food.

All experimental procedures were approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (TCM-LAEC2019083, Tianjin, China).

After 1 week of adaptive feeding, the diabetic mouse model was replicated as previously reported (Kusakabe et al., 2009). In simple terms, mice were intraperitoneally injected with 120 mg/kg streptozotocin (STZ, Sigma, United States) after being fed a high-fat diet with 60% energy from fat for 3 weeks (Beijing Vital River Laboratory Animal Technology Co., Ltd.). One week after injection, fasting blood glucose (FBG) of mice after 12 h of fasting was detected, and mice with FBG > 11.1 mmol/L were selected to continue high-fat diet feeding. After 8 weeks of continuous hyperglycemic stimulation, mice with cognitive impairment were screened with MWM (detailed in 2.3) and used in follow-up studies.

To investigate the effect of ZSWF on the cognitive function of DCI mice and its potential mechanism, the mice with cognitive impairment were randomly divided into five groups: vehicle treated group (DCI), ZSWF low dose (9.36 g/kg, clinical equivalent dose) treated group (ZSWFL), ZSWF high dose (18.72 g/kg, 2 times the clinical equivalent dose) treated group (ZSWFH), mixed antibiotic (natamycin 3 mg/ml, neomycin 2 mg/ml and bacitracin 2 mg/ml) treated group (ABs), and ZSWF combined with mixed antibiotic treated group (ZSWF + ABs). Mice in the treatment group were orally gavaged with crude extract of ZSWF for 8 weeks, and mice in Con group and DCI group were gavaged with equal volume of distilled water. The control group was fed the normal diet, and the other groups were kept on HFD during drug administration. Weight was measured every 2 weeks, and FBG was measured weekly during continuous treatment.

Morris Water Maze Experimental Evaluation of Cognitive Function

To screen mice with abnormal cognitive function after 8 weeks of hyperglycemia and investigate the effect of ZSWF treatment on cognitive function, the MWM experiment was performed as previously described (Morris., 1984). In short, to acclimate to the maze environment, all the mice were allowed to swim freely in the maze without a platform for 1 min the day before the experiment began. Next, the positioning navigation experiment was conducted for five consecutive days, during which the mice were allowed to swim freely for 60 s. If they did not reach the platform within 60 s, the mice were slowly guided to the platform and held there for 10 s. In the space exploration experiment, the mice were allowed to swim freely for 60 s in a maze where the platform was removed. During this process, the time to first reach the original platform, the duration in the platform quadrant, and the frequency of crossing the platform were recorded. During the whole test, data acquisition is completed by the automatic image surveillance and processing system.

Cresyl Violet Staining

To investigate the effect of ZSWF on hippocampal neurons of DCI mice, cresyl violet staining was performed. At the end of the treatment, the mice were deeply anesthetized by inhalation with

4% isoflurane, and blood samples were collected. Apical perfusion with PBS was then performed to remove residual blood from the brain tissue, and whole brain tissue was collected. After fixation with 4% paraformaldehyde, the brain tissue was dehydrated with gradient sucrose solution (10%, 20%, and 30%, respectively), and the coronal sections with thickness of 10 μm were obtained using a frozen slicer. Sections were stained with 0.1% cresol violet solution, dehydrated with ascending grades of alcohol, cleared with xylene, mounted with neutral resin, and images were taken using Leica DM4000B biological microscope (Beijing, China).

Enzyme-Linked Immunosorbent Assay

To investigate the effects of ZSWF on neuroinflammation in DCI mice, the mice were sacrificed by cervical dislocation after deep anesthesia. Then, the skull was cut along the midline of the skull to expose the whole brain tissue, the cortex was separated to expose the hippocampus, and the hippocampus was collected with bamboo sticks and stored at −80°C for detection. Also, commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure the hippocampus IL-6 (JYM0012Mo, Colorful-Gene, 2–150 pg/ml), IL-1β (JYM0531Mo, Colorful-Gene, 1.5–100 pg/ml), TNF-α (JYM0218Mo, Colorful-Gene, 8–500 pg/ml), and monocyte chemotaxis protein-1 (MCP-1, JYM0099Mo, Colorful-Gene, 6–450 pg/m) according to manufacturer's instructions. To investigate the effects of ZSWF on proinflammatory cytokines in peripheral circulation in DCI mice, blood samples from each group were collected and centrifuged at 3,000 g for 15 min. The levels of IL-6, IL-1β, TNF-α, and INF-γ (abs552811, Absin Bioscience Inc., 7.81–500 pg/ml) in the serum were also detected using ELISA kits. Furthermore, to investigate the effect of ZSWF on the integrity of intestinal mucosal barrier in DCI mice, colon tissue approximately 5 cm connected to the cecum of mice in each group was taken, colon contents were scraped, and then, the colon and colon contents were stored in EP tubes, respectively. Next, colon tissue was lysed with lysate, and the content of mucin-2 (MUC2) in colon was determined by the ELISA kit (SEA705Mu, Cloud Clone Biotechnology Co., LTD., 0.78–50 ng/ml). The colon contents were diluted with 0.9% Nacl, homogenized and centrifuged (3,000 g, 15 min), and the content of secretory immunoglobulin A (SlgA) in the colon contents was determined by the ELISA kit (SEA641Mu, Cloud Clone Biotechnology Co., LTD., 0.156–10 ng/ml). All experimental procedures were performed according to the manufacturer's instructions. The optical density at 450 nm was obtained with an ELISA microplate reader (Infinite® 200 PRO, Tecan, Swit). There were six biological repetitions in each group for proinflammatory cytokines and five biological repetitions in each group for Muc2 and SlgA.

Hematoxylin-Eosin Staining of Colon

To evaluate the effect of ZSWF on intestinal barrier integrity in DCI mice, the colon was fixed in 4% paraformaldehyde for 24 h, and then, the paraffin section was performed. Sections with a thickness of 0.5 μm were stained with hematoxylin

(5 min) and 0.5% eosin (3 min), and the histopathological images of the colon were taken with a Leica DM4000B biologic microscope.

Western Blot Analysis

To investigate the effect of ZSWF on intestinal permeability of DCI mice, Western blot analysis was used to detect the protein expressions of occludin and ZO-1. Proteins from colon tissues were extracted and quantified using the BCA protein quantification kit. Total proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane by wet transfer apparatus. Tight junction protein associated antibodies ZO-1 (ab216880, Abcam, United Kingdom) and occludin (ab216327, Abcam, United Kingdom) were used to incubate the bands overnight at 4°C, where β-actin (13E5, CST, United States) was used as a reference protein, and then, goat anti-rabbit secondary antibodies (ab205718, Abcam, United Kingdom) were used to incubate the bands at room temperature for 2 h. In conclusion, the immunoreactive bands were visualized using enhanced chemiluminescence reagents, and the luminescence intensity was quantitatively analyzed using Image-pro Plus6.0.

HPLC-MS/MS Analysis Lactulose/Mannitol Ratio in Urine

To observe the effect of ZSWF on intestinal barrier integrity in DCI mice, the ratio of lactulose to mannitol in urine was determined by HPLC-MS/MS according to the previous method (Kubica et al., 2012). At the end of treatment, mice were given a 2:1 lactulose-mannitol solution, and urine was collected in a metabolic cage for 6 h. The supernatant was obtained by centrifugation and stored at −80°C. A Waters ACQUITY UPLC BEH Amide (2.1 mm × 50 mm, 1.7 μm, VK) was used for separation. The mobile phase consisted of Methanol (A) and acetonitrile-water (B), and the flow rate was set at 0.25 ml/min. The gradient profile was: 0–1 min (A: 90%; B: 10%), 1–8 min (A: 60%; B: 40%), 8–15 min (A: 90%; B: 10%). The sample injection volume was 2 μL. The ESI source operates in negative mode, with a capillary voltage of 2.0 kV and a desolvation temperature of 550°C. The source of the gas was set as follows: desolvation at 200 L/h and cone at 0 L/h. The collision cell pressure was 4.5×10^{-3} mbar. Dates were processed using MassLynx™4.1 software (Waters Corp, Milford, MA, United States). Lactulose and mannitol contents were calculated as well as the standard curve and corresponding peak area.

Gut Microbiota Composition Analysis

To investigate the effect of ZSWF on intestinal microflora of DCI mice, feces of each group were collected by individual metabolic chamber for approximately 24 h after treatment, and 16S rDNA sequencing was performed. The metagenomic DNA from each feces sample was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hamburg, Germany). Thereafter, the DNA concentration was determined, and the V3-V4 regions of bacterial 16S rDNA was amplified by PCR (95°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for

10 min, 10°C until halted by user) using 10 ng DNA as a template. The 16S primers 338F-ACTCCTACGGGAGGCAGCAG and 806R-GGACTACHVGGGTWTCTAAT were used as fusion primers containing Ion Torrent sequencing adapters. PCR reactions were performed in triplicate 20 μl reaction mixtures containing 4 μl of 5 × FastPfu Buffer (1 μM), 2 μl of 2.5 mM dNTPs, 0.8 μl of Forward Primer (5 μM), and 0.8 μl of Reverse Primer (5 μM), 0.4 μl of FastPfu Polymerase, 2 μl of Microbial DNA (5 ng/μl), and add ddH2O to 20 μl. Afterwards PCR products were gel-purified, and the amplicon DNA concentration was determined. Sequencing of pooled amplicons was performed with an Illumina MiSeq platform (Illumina Inc., San Diego, United States), and the resulting analysis using the MiSeq Reporter software (MSR) and the classification is based on the Greengenes database. Subsequent cord diversity analysis applied to determine α (within sample) and β (between samples) diversity. To demonstrate the clustering of different groups, the Nonmetric multidimensional scaling and weighted unifrac principal coordinate analysis (PCoA) were conducted.

Statistical Analysis

Data were processed and analyzed using the statistical package SPSS (version 17.0), and the results were expressed as mean ± standard deviation (SD). Data were tested for normality before difference analysis, escape latency data were analyzed by repeated measures ANOVA, the remaining data were analyzed by one-way ANOVA, followed by a post hoc Tukey's Honest Significant Difference test for multiple comparisons among the groups. A p value of less than 0.05 was considered to indicate statistical significance. For 16S rRNA gene sequence analysis, all reads were deposited and grouped into operational taxonomic units (OTU) at a sequence identity of 97%, and the taxonomic affiliation of the OTUs was determined with quantitative insights into microbial ecology (version 1.8.0) against the Greengenes database (version 13.5). Principal component analysis was performed using SIMCA 14.0, and metastats was used to analyze differences between groups.

RESULTS

Zi Shen Wan Fang Ameliorated Cognitive Impairment and Prevented Neuron Damage in Diabetic Cognitive Impairment Mice

To investigate the effect of ZSWF on the body weight of DCI mice, we analyzed the changes of body weight of each group of mice every 2 weeks during the treatment of ZSWF, and we found that ZSWF tended to increase the body weight of DCI mice, but no statistical difference ($p > 0.05$) was found, indicating that ZSWF demonstrated no effect on the body weight of DCI mice. Considering that hyperglycemia is the primary cause of cognitive dysfunction, we first investigated the effect of ZSWF on FBG in DCI mice. The results showed that no significant difference in FBG was found between the ZSWF group and the DCI group during continuous treatment ($p > 0.05$), whether the ZSWFL or ZSWFH group (**Supplementary Figure S1**). Next, to investigate the effect of ZSWF on cognitive impairment in DCI mice, the

MWM experiment was performed. The results of the positioning navigation experiment showed that the escape latency of DCI group was significantly increased compared with the Con group ($p < 0.05$), while the escape latency of mice in the ZSWF group was significantly decreased compared with the DCI group ($p < 0.05$) (**Figure 1A**). Next, the results of the space exploration experiment showed that ZSWF significantly increased the frequency of crossing the platform ($p < 0.01$, $F = 6.590$) (**Figure 1B**) and the duration of swimming in the platform quadrant (**Figure 1C**), and it reduced the time for DCI mice to reach the platform for the first time ($p < 0.05$, $F = 6.434$) (**Figure 1D**). Moreover, the swimming trajectory of the space exploration experiment showed that the swimming trajectory of the ZSWF group is oriented, while that of the DCI group is disordered (**Figure 1E**).
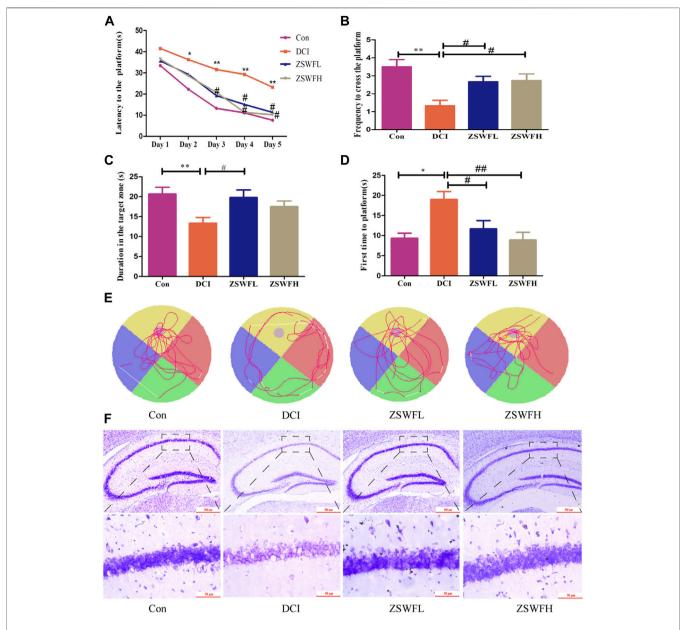
The hippocampus is the main brain region regulating learning and memory, and the damage of hippocampus neurons is the final pathological change of cognitive dysfunction. Therefore, we investigated the protective effect of ZSWF on DCI hippocampal neurons by cresol violet staining the neuronal Nissl bodies. As described in **Figure 1F**, the DCI animals neuron layer is thin, irregularly and loosely arranged, intercellular spaces are widened, and the cellular structure is incomplete, even with the loss of large amounts of cells. Meanwhile, this phenomenon can be reversed by the ZSWF administration, whether ZSWFL or ZSWFH.

Zi Shen Wan Fang Suppressed Hippocampal and Peripheral Inflammation in Diabetic Cognitive Impairment Mice

Cumulative studies reported that neuroinflammation is an important pathological mechanism of DCI (Rom et al., 2019), and chronic systemic inflammation is one of the important characteristics of DM (Esser et al., 2014). Thus, we measured the levels of classic proinflammatory cytokines IL-1β, IL-6, TNFα, MCP-1, and INF-γ in the hippocampus and serum. Our results indicated that the hippocampus levels of IL-1β ($F = 8.909$, $p < 0.01$) (**Figure 2A**), IL-6 ($F = 20.64$, $p < 0.01$) (**Figure 2B**), TNF-α ($F = 6.935$, $p > 0.05$) (**Figure 2C**), and MCP-1 ($F = 7.033$, $p < 0.05$) (**Figure 2D**) in DCI mice were significantly increased, and the levels of IL-1β ($F = 10.12$, $p < 0.01$) (**Figure 2E**), IL-6 ($F = 4.719$, $p < 0.05$) (**Figure 2F**), TNF-α ($F = 6.767$, $p < 0.01$) (**Figure 2G**), and INF-γ ($F = 4.727$, $p < 0.05$) (**Figure 2H**) in serum were also significantly increased. In contrast, these proinflammatory cytokines were significantly reduced in the ZSWF group compared with the DCI group ($p < 0.05$), although no significant dose dependence occurred.

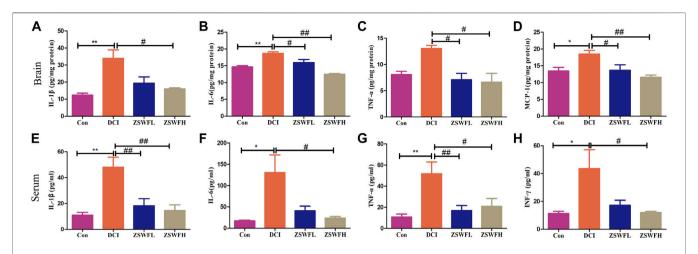## Zi Shen Wan Fang Maintains Intestinal Integrity in Diabetic Cognitive Impairment Mice

Given that intestinal dysbiosis in diabetes animals may affect gut integrity and subsequently lead to release of bacterial proinflammatory cytokines into the circulation, we examined the effect of ZSWF on intestinal barrier integrity in DCI mice. HE staining results showed that compared with the Con group, the

FIGURE 1 | ZSWF ameliorated cognitive impairment and prevented neuron damage in DCI mice. **(A)** escape latency, **(B)** the frequency of crossing the platform, **(C)** duration in the platform quadrant, **(D)** time of first arrival at platform. These data are expressed as the mean ± SD, (n = 15). Escape latency data were analyzed by repeated measurement ANOVA, other data were analyzed by one-way ANOVA. $**p < 0.01$, $*p < 0.05$ vs. Con group, $^{##}p < 0.01$, $^{#}p < 0.05$ vs. DCI group. **(E)** representative swimming tracks of each group of mice in the space exploration experiment. **(F)** representative images of cresol violet staining in hippocampus of each group of mice.

colonic mucosa of mice in the DCI group was damaged, the number of goblet cells was reduced, and mucosal muscle layer was thinned, while ZSWF protected the colonic injury of DCI mice (**Figure 3A**). Furthermore, intestinal SIgA plays a key role in the intestinal immune system. Our data showed that compared with the Con group, the SIgA level of colon contents was significantly reduced in the DCI group ($F = 8.092$, $p < 0.01$), while ZSWF treatment significantly reversed the decreased SIgA level in DCI mice ($p < 0.05$) (**Figure 3B**). Next, we examined the level of Muc2 secreted by goblet cells and found that ZSWF treatment significantly increased the content of

Muc2 in the DCI mice colon ($F = 33.39$, $p < 0.01$) (**Figure 3C**). Moreover, the urinary lactulose/mannitol (L/M) ratio was used to determine intestinal permeability and found that the urinary L/M ratio of the ZSWF group was significantly lower than that of the DCI group ($F = 12.06$, $p < 0.01$) (**Figure 3D**). In addition, we also investigated the effect of ZSWF on tight junction protein expression in the colon of DCI mice and found that compared with the Con group, the expression of tight junction proteins ZO-1 and occludin in the colon of DCI mice was significantly decreased, while ZSWF treatment significantly increased the expression of ZO-

FIGURE 2 | ZSWF suppressed hippocampal and peripheral inflammation in DCI mice. **(A)** IL-1β contents in the hippocampus, **(B)** IL-6 contents in the hippocampus, **(C)** TNF-α contents in the hippocampus, **(D)** MCP-1 contents in the hippocampus, **(E)** IL-1β levels in the serum, **(F)** IL-6 levels in the serum, **(G)** TNF-α levels in the serum, **(H)** INF-γ levels in the serum. All data were expressed as mean ± SD ($n = 6$). One-way ANOVA was used to compare statistical differences between groups. $**p < 0.01$, $*p < 0.05$ vs. Con group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. DCI group.

1($F = 326.8$, $p < 0.01$) and occludin ($F = 181.2$, $p < 0.01$) in the colon of DCI mice (**Figures 3E,F**).
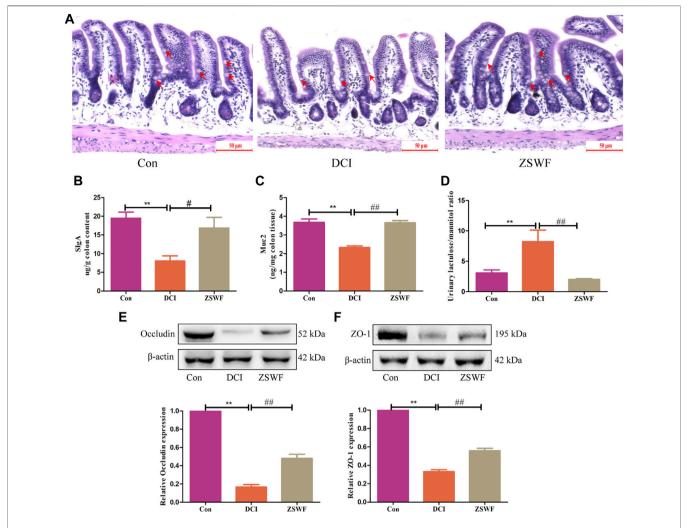
Zi Shen Wan Fang Reversed Gut Microbiota Dysbiosis and Increased Short-Chain Fatty Acids in Diabetic Cognitive Impairment Mice

Gut microbiota may be an ideal target for understanding and treating DCI. To reveal the effect of ZSWF in regulating gut microbiota in DCI mice, the feces were sampled and sequenced by performing a pyrosequencing-based analysis of bacterial 16S rDNA (V3-V4 region). Weighted UniFrac distance-based PCoA revealed a distinct clustering of microbiota composition for each group (**Figure 4A**). At the phylum level, the DCI mice displayed an increased the relative abundance of *Firmicutes* (54.27% vs. 27.53%) and *Proteoobacteria* (30.08% vs. 3.24%) and decreased abundance of *Bacteroidetes* (15.21% vs. 64.25%) compared with Con mice. In contrast, the microbiota imbalance was ameliorated by ZSWF administration as it decreased the abundance of *Firmicutes* (32.95% vs. 54.27%) and *Proteoobacteria* (14.71% vs. 30.18%) and increased *Bacteroidetes* (50.34% vs. 15.2%) (**Figure 4B**). Furthermore, treatment with ZSWF reduced the ratio of *Firmicutes* to *Bacteroidetes* in DCI mice (**Figure 4C**). At the genus level, the relative abundance accounted for the top 30 was analyzed, and after ZSWF treatment, several important modifications of the gut microbiota composition were found. The relative abundance of *Bacteroides* ($F = 14.48$, $p < 0.01$) and *Alistipes* ($F = 4.559$, $p < 0.05$) were markedly increased, and *Dorea* ($F = 5.396$, $p < 0.01$), *Intestinimonas* ($F = 11.79$, $p > 0.05$), *Desuifouibrio* ($F = 12.45$, $p < 0.05$), and *Allobaculum* ($F = 10.32$, $p < 0.05$) were significantly decreased in the ZSWF administration group compared to the DCI group (**Figures 4D–J**). These results confirmed that the homeostasis of the gut microbiota in DCI individuals was destroyed, and ZSWF could play an important role in modulating the composition of gut microbiota on DCI mice.

Furthermore, considering that short-chain fatty acids (SCFAs) are major metabolites of intestinal microbiota and play an important role in gut-brain axis communication, we examined the effect of ZSWF on fecal SCFAs concentration and found that ZSWF could increase the concentration of SCFAs in feces of DCI mice (**Supplementary Figure S3**).

Microbiota Ablation With Antibiotics Eliminated Zi Shen Wan Fang in Improving Cognitive Function and Reducing Neuroinflammation as Well as Systemic Inflammation of Diabetic Cognitive Impairment Mice

The above results suggest that the gut microbiota-brain axis plays an important role in ZSWF in improving cognition impairment of DCI mice. To further investigate the relationship between the improvement of cognitive function and the regulation of intestinal microbiota by ZSWF, a cocktail of oral antibiotics was used to eliminate the gut microbiota. The mice were administrated with antibiotics in the drinking water starting 7 days before ZSWF treatment and throughout the experiment. First, we found that antibiotics demonstrated no effect on FBG in DCI mice (**Supplementary Figure S4**). Furthermore, antibiotic treatment significantly reduced the amount of OTU in the feces of DCI mice (**Figure 5A**), suggesting that most intestinal microbiota were eliminated. Antibiotics alone did not significantly alter cognitive function and inflammation in the DCI group, suggesting antibiotics did not further impair cognitive function and increase inflammation induced diabetes (**Figures 5B–E**). Of note, the antibiotic treatment abrogated the beneficial effects of ZSWF treatment in DCI mice. We found that the cognition improvement with ZSWF was abolished by antibiotics treatment, with longer escape times (**Figure 5B**), longer times of first to arrival platform (**Figure 5C**), lower times in the target quadrant (**Figure 5D**), and lower frequency to cross platform (**Figure 5E**).

FIGURE 3 | ZSWF maintains intestinal integrity in DCI mice. **(A)** representative images of colon HE staining, the goblet cells marked by the red arrow, scale bar 50 μm (n = 3). **(B,C)** SIgA and Muc2 contents in the colon, **(D)** lactulose to mannitol ratio, these data were expressed as mean ± SD (n = 5). **(E,F)** occludin and ZO-1 represented Western blotting bands and relative protein expression levels in the colon, data were expressed as mean ± SD (n = 3). All data were analyzed using one-way ANOVA, **p < 0.01 vs. Con group, ##p < 0.01, #p < 0.05 vs. DCI group.
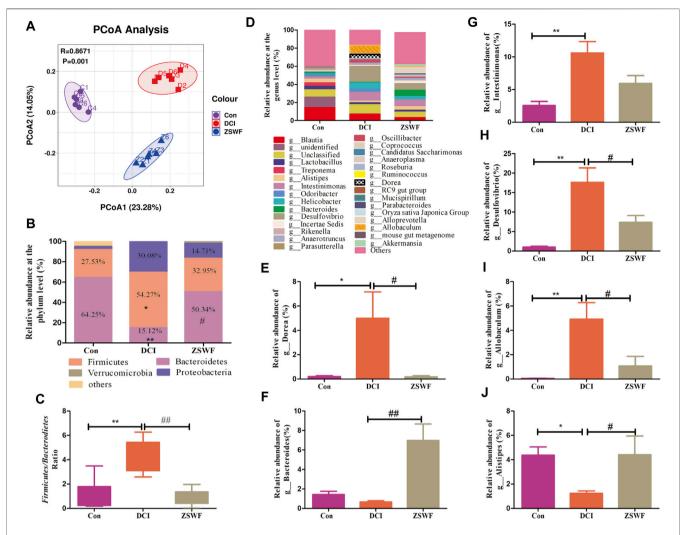
Moreover, we also observed that the effect of ZSWF on reducing hippocampus and serum proinflammatory cytokines was eliminated by antibiotic intervention (**Figures 5F–M**). Meanwhile, the effect of ZSWF intervention on increasing fecal SCFAs in DCI mice was eliminated (**Supplementary Figure S5**). These results suggest that maintaining intestinal microflora homeostasis is a critical pathway for ZSWF to reverse cognitive function and reduce inflammation in DCI mice.

DISCUSSION

Cognitive dysfunction is one of the complications of diabetes, and no clinically approved treatment drug exists. Previous cumulative studies showed that DCI is closely associated with intestinal microbiome dysregulation (Xu et al., 2017; Zhang et al., 2021a), suggesting that maintaining intestinal microbiome

homeostasis may be an effective strategy to prevent and treat DCI. In this study, we investigated the effects of ZSWF on the cognitive function of DCI mice, and we explored the mechanism from the perspective of gut-microbial-brain axis. Next, we present evidence that suggests that ZSWF supplementation ameliorated the diabetes-induced cognitive dysfunction and reduced neuroinflammation and systemic inflammation. Furthermore, ZSWF treatment protected intestinal barrier integrity and maintained intestinal microbiota homeostasis in DCI mice. In particular, the effects of ZSWF treatment on improving diabetes-induced cognitive impairment and inflammation were eliminated after antibiotic treatment deleted intestinal bacteria, highlighting the essential role of gut microbiota in improving cognitive function of ZSWF.

In this study, we established a diabetic mouse model according to the method previously reported (Kusakabe et al., 2009). Given that our previous studies confirmed a certain incidence of

FIGURE 4 | ZSWF reversed gut microbiota dysbiosis in DCI mice. **(A)** principal coordinates analysis (PcoA), **(B)** relative abundances at phylum levels, **(C)** ratio of *Firmicutes* to *Bacteroidetes*, **(D)** relative abundance of bacteria at the genus level, **(E–J)** relative abundance of *Dorea, Bacteroides, Intestinimonas, Desulfourbrio, Allobaculum,* and *Alistipes*. All data were expressed as mean ± SD ($n = 6$), and one-way ANOVA was used to compare statistical differences between groups, $**p < 0.01$, $*p < 0.05$ vs. Con group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. DCI group.

diabetes-induced cognitive impairment (Song et al., 2017), mice with cognitive impairment were screened by MWM after 8 weeks of hyperglycemic stimulation. Our screening results revealed that diabetic mice demonstrated an approximately 80% chance of developing cognitive impairment (Unpublished). Then, we treated DCI mice with ZSWF extract for 8 weeks. FBG was measured weekly during treatment, and cognitive function was assessed after treatment. Likewise, we found that ZSWF significantly improved cognitive function and protected hippocampal neuron damage in DCI mice. However, this study found that ZSWF could not improve FBG, the initial pathological factor of DCI mice, suggesting that improving the subsequent complex pathological links mediated by hyperglycemia may be the mechanism by which ZSWF improves DCI.

Accumulated evidence suggests that neuroinflammation is an important pathological mechanism of DCI (Muriach et al., 2014;

Jeong et al., 2021). IL-6, IL-1β, and TNF-α are common proinflammatory cytokines, which are significantly altered in a variety of acute and chronic inflammatory diseases. IFN-γ is secreted mainly by natural killer cells and natural killer T cells and plays a role in innate immunity. A large number of excellent previous studies confirmed that the diabetic hyperglycemic environment mediates changes in innate immune system function and significantly increases IFN-γ levels. MCP-1 is a major chemokine that recruits monocyte/macrophage to the site of tissue injury and plays a critical role in microvascular complications of diabetes. Therefore, in order to investigate the effects of ZSWF on the system and neuroinflammation of DCI mice, we used the ELISA kit to detect the changes of the above cytokines, and we found that the levels of these proinflammatory factors in the hippocampus of DCI mice were significantly increased, which was consistent with previous reports (Wang et al., 2019; Zeinivand et al., 2020).
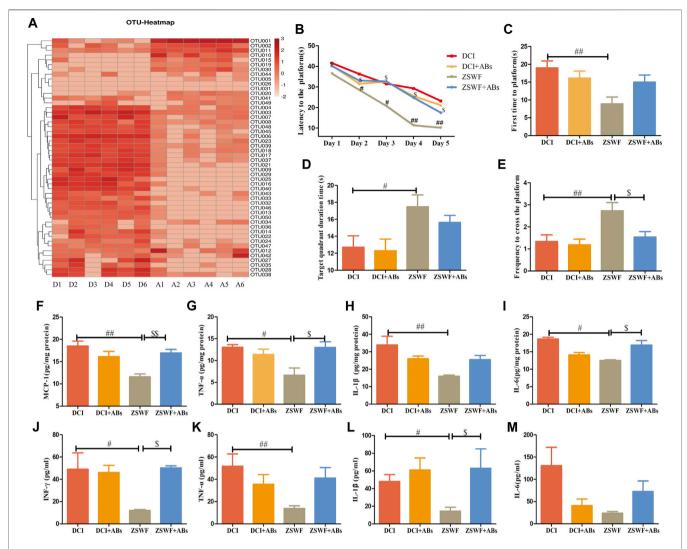
FIGURE 5 | Microbiota ablation with antibiotics eliminated the effects of ZSWF on improving cognitive function and reducing inflammation in DCI mice. **(A)** effects of antibiotic treatment on relative abundance of intestinal bacteria at genus level in DCI mice. The heat map represents the relative sequence abundance of Top50 OTU of genus level bacteria. The redder color represents more bacteria composition or the higher relative abundance of genus level bacteria. The horizontal axis represents groups, D1–D6 are six samples from the DCI group, and A1–A6 are six samples from the antibiotic group. **(B)** escape latency, **(C)** time of first arrival at platform, **(D)** duration in platform quadrant, **(E)** the frequency of crossing the platform. These data were expressed as mean ± SD ($n = 15$). Escape latency data were analyzed by repeated measurement ANOVA, and other data were analyzed by one-way, $^{##}p < 0.01$, $^{#}p < 0.05$ vs. DCI group, $^{$}p < 0.05$ vs. ZSWF group. **(F)** MCP-1 contents in the hippocampus, **(G)** TNF-α contents in the hippocampus, **(H)** IL-1β contents in the hippocampus, **(I)** IL-6 contents in the hippocampus, **(J)** INF-γ levels in the serum, **(K)** TNF-α levels in the serum, **(L)** IL-1β levels in the serum, **(M)** IL-6 levels in the serum. These data were expressed as mean ± SD ($n = 6$), one-way ANOVA was used to compare statistical differences between groups, $^{##}p < 0.01$, $^{#}p < 0.05$ vs. DCI group, $^{$$}p < 0.01$, $^{$}p < 0.05$ vs. ZSWF group.

Likewise, our current study also revealed that ZSWF reduced hippocampal proinflammatory cytokines levels in DCI mice. Also, we found that ZSWF inhibited the activation of brain-resident immune cells (microglia and astrocytes) in DCI mice (unpublished). In addition to brain-resident immune cells releasing proinflammatory cytokines, peripheral circulating proinflammatory cytokines crossing the blood-brain barrier is also a key pathologic pathway of neuroinflammation. In a similar manner, our results showed that ZSWF significantly reduced peripheral circulating proinflammatory cytokines in DCI mice. These results suggest that the reduction of peripheral circulation and neuroinflammation is the mechanism of ZSWF to improve

DCI. However, studies showed that the bioavailability of main chemical components of Anemarrhenae Rhizoma and Phellodendri Chinensis Cortex is low (Singh and Chaudhuri, 2018; Baldim et al., 2020), and our previous *in vivo* chemical analysis of ZSWF also showed that most components in Cistanches Herba were concentrated in feces (Zheng et al., 2018), suggesting that regulation of gastrointestinal function or intestinal flora may play a crucial role in the reversal of DCI inflammation by ZSWF.

Increasing evidence also indicates that diabetes-induced systemic inflammation is mainly caused by impaired intestinal barrier integrity and dysbiosis of intestinal flora (Cani et al., 2008;

Cani et al., 2009; Li et al., 2019). Our detection results of intestinal barrier integrity related parameters showed that the intestinal barrier integrity was damaged in DCI mice, which was consistent with previous reports (Visser et al., 2010; Li et al., 2022; Xiao et al., 2022). Likewise, our results show that ZSWF exhibits a protective effect on intestinal barrier integrity in DCI mice by increasing colon tight junction protein expression (ZO-1 and occludin) and mucin (Muc2) content, as well as colon content immunoglobulin A (SIgA) levels. These results systematically confirmed that ZSWF maintained intestinal barrier integrity in DCI mice.

Intestinal microbiome is a key component of the intestinal barrier, and its homeostasis can lead to an increase in the permeability of the intestinal barrier. The results of our 16S rDNA sequencing revealed that the intestinal microbiome composition of DCI mice was significantly altered, and ZSWF demonstrated a significant reversal effect on the microbiome disorder of DCI mice. The relative abundance results of phylum level bacteria showed that DCI mice increased the relative abundance of *Firmicutes* and *Proteobacteria*, and they significantly decreased the abundance of *Bacteroidetes*, which was consistent with previous reports (Zhu et al., 2016). More notably, ZSWF improved the imbalance of intestinal bacteria, as demonstrated by a decreased ratio of *Firmicutes* to *Bacteroides*. At the genus levels, our results showed that ZSWF treatment significantly increased the abundance of *Bacteroides* and *Alistipes*, and it decreased the abundance of *Desulfouibrio*, *Dorea*, and *Allobaculum*. Clinical studies linked changes in *Bacteroides* to cognitive and neurodegenerative diseases (Cattaneo et al., 2017; Saji et al., 2019). Also, previous studies found that *Bacteroides* can reduce inflammation (Tan et al., 2019) and protect intestinal mucosal permeability (Hooper et al., 2001). Studies found that the abundance of *Alistipes* is significantly reduced in patients with mild cognitive impairment, suggesting that *Alistipes* is negatively correlated with cognitive function (Zhang et al., 2021b). *Desulfovibrio* is a proinflammatory bacterium (Simpson et al., 2021) and can produce hydrogen sulfide, which demonstrates a cytotoxic effect (Gibson, 1990). The above evidence suggested that ZSWF could maintain intestinal microbiome homeostasis in DCI mice, which was reflected in that ZSWF increased the relative abundance of beneficial bacteria in DCI mice and decreased the abundance of bacteria that produced cytotoxic substances. Antibiotic cocktail treatment is an appropriate method to explore the effects of intestinal bacteria on physiology and disease in animal (Kennedy et al., 2018). Data from ZSWF combined with antibiotic therapy revealed that oral antibiotics did not improve or worsen FBG, cognitive function, as well as inflammation in DCI mice. However, oral antibiotics treatment eliminated the effect of ZSWF on cognitive function and inflammation in DCI mice, suggesting that gut microbes are necessary to ZSWF to improve cognitive function and inflammation in DCI mice.

The regulation of intestinal flora by ZSWF in DCI mice may be bidirectional. On the one hand, the chemical composition of ZSWF maintained the composition of intestinal microbes in DCI mice, and on the other hand, intestinal microbes in DCI mice metabolized the components with low bioavailability of ZSWF. The potential active components of ZSWF regulating intestinal flora of DCI mice may be polysaccharides in Cistanches Herba and alkaloids in Phellodendri Chinensis Cortex. Previous studies confirmed that Cistanche polysaccharides demonstrate a clear regulatory effect on intestinal flora (Fu et al., 2020; Fan et al., 2021; Gao et al., 2021). Although accumulative studies confirmed that berberine, the main alkaloid component of Phellodendri Chinensis Cortex, can protect cognitive function (Aski et al., 2018; Shinjyo et al., 2020; Yi et al., 2021), subsequent studies gradually recognized that regulating intestinal flora may be the initial link of berberine improving cognitive function (Zhang et al., 2020b; Habtemariam, 2020). The ZSWF components metabolized by intestinal flora of DCI mice may be saponins in Anemarrhenae Rhizoma (Tian et al., 2016; Dong et al., 2021). Furthermore, besides the immune signaling pathway investigated in this study, the mechanism of ZSWF regulating intestinal flora to improve DCI may also include the following aspects: first, regulating the tryptophan-kynurenine metabolic pathway. Previous cumulative studies confirmed that gut microbiota plays an important role in regulating tryptophan-kynurenine metabolism (Kennedy et al., 2017; Agus et al., 2018; Deng et al., 2021), and several studies also found that tryptophan-kynurenine metabolism is involved in the regulation of glutamate neurotransmitters and synaptic excitability, which is crucial for the protection of cognitive function (Oxenkrug, 2007; Forrest et al., 2015; Tanaka et al., 2020; Bakker et al., 2021). More notably, our previous study found abnormal kynurenine metabolism in DCI mice, and ZSWF treatment could improve kynurenine metabolism in DCI mice (Yin et al., 2022). These studies suggest that regulation of tryptophan-kynurenine metabolic pathway may be the mechanism by which ZSWF improves cognitive function by maintaining intestinal microbiome homeostasis in DCI mice. Second, the increase the level of SCFAs derived from gut microbiota. As the main metabolite of gut microbiota, SCFAs have been reported to ameliorate cognitive impairment mediated by a variety of etiologies, including DCI (Lee et al., 2020; Enry et al., 2021; Qian et al., 2021; Zheng et al., 2021). Our previous study also found that ZSWF increased fecal SCFAs levels in DCI mice (unpublished). These results suggest that increasing the level of SCFAs derived from gut microbiota may also be the mechanism by which ZSWF regulates intestinal microflora to improve cognitive function in DCI mice. However, this study still demonstrates some limitations. For example, although our previous studies identified the main chemical components of ZSWF extract *in vivo* and *in vitro*, including the main archetypes and metabolic components in feces (Zheng et al., 2018), which components are involved in maintaining intestinal microbiota homeostasis and their mechanisms remain unclear. Moreover, since no drug with a definite therapeutic effect for DCI exists in clinical practice at present, this study did not select a suitable positive control.

CONCLUSION

ZSWF can improve cognitive dysfunction and neuroinflammation in DCI mice, while oral antibiotics can partially eliminate these effects, suggesting that maintaining

intestinal microbial homeostasis may be the underlying mechanism of ZSWF to improve cognitive function in DCI mice.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

YZ and PZ designed research; JS, QY, LZ, YW, PY, MG, LY, and ZW performed research; JS, QY, LZ, YW, PY, MG, LY, and ZW analyzed data; JS, QY, LZ, and HL wrote the paper. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.898360/full#supplementary-material

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Probiotics (*Bacillus clausii* and *Lactobacillus fermentum* NMCC-14) Ameliorate Stress Behavior in Mice by Increasing Monoamine Levels and mRNA Expression of Dopamine Receptors (D$_1$ and D$_2$) and Synaptophysin

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Stress is a physiological consequence of the body to adversity. The gut–brain axis and probiotics are gaining interest to provide better treatment for stress and other neurological disorders. Probiotic (*Lactobacillus fermentum* NMCC-14 and *Bacillus clausii*, 10^{10} colony-forming unit/day/animal, per oral) effects were investigated in acute (up to day 7) and subacute (days 8–14) restraint-stressed and normal mice through behavioral paradigms (elevated plus maze: EPM, light dark box/dark light box: LDB, and open field test: OFT). Time spent in the open arms of the EPM, time spent in the light compartment of the LDB, and movable time and time spent in the center of the OFT were significantly ($p \leq 0.05$, $n = 5$) increased in probiotic-treated restraint-stressed mice. Enzyme-linked immunoassay determined blood cortisol and adrenocorticotropic hormone (ACTH) levels, which were reduced significantly ($p < 0.05$, $n = 5$) in probiotic-treated restraint-stressed mice. Hematoxylin and eosin-stained hippocampal slides also showed less or no neurodegeneration in the probiotic-treated animals. High-performance liquid chromatography and quantitative polymerase chain reaction were performed to determine the monoamine levels and mRNA expression of dopamine receptor

Abbreviations: ACTH, Adrenocorticotropic hormone CA, Cornu ammonis; CFU, Colony-forming unit; corticotrophin-releasing hormone, Corticotropin-releasing hormone; cDNA, Complementary DNA; DNA, Deoxyribonucleic acid; D$_1$, Dopamine Receptor 1; D$_2$, Dopamine Receptor 2; DG, Dentate gyrus; ELISA, Enzyme-linked immunoassay; EPM, Elevated plus maze; GADPH, Glyceraldehyde 3-phosphate dehydrogenase; HPLC, High-performance liquid chromatography; H&E, Hematoxylin and eosin; i.p, Intraperitoneal; LDB, light dark box/dark light box; mRNA, Messenger ribonucleic acid; NE, Norepinephrine; NS, Normal saline; OFT, Open field test; p.o, Per oral; qPCR, Quantitative polymerase chain reaction; vs., versus.

TABLE 1 | Animal groups, probiotic treatment schedule, and behavioral test details.

Experimental set	Group (n = 5/group/ experiment)	Drug/Probiotic treatment and behavioral studies schedule
Normal	I	No stress and probiotic treatment, Behavioral tests (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)
Negative	II	Stress induced for 120 min/day, Behavioral tests performed 24 h after last stress induction, Behavioral tests (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)
Positive	III	Fluoxetine 10 mg/kg in NS (i.p) + stress induced for 120 min/day, Behavioral tests performed 24 h after last stress induction, Behavioral tests (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)
Lactobacillus fermentum NMCC-14 + Stress (LF-S)	IV	10^{10} CFU/day (p.o) + stress induced for 120 min/day, Behavioral tests performed 24 h after last stress induction (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)
Bacillus clausii + Stress (BC-S)	V	10^{10} CFU/day (p.o) + stress induced for 120 min/day, Behavioral tests performed 24 h after last stress induction (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)
Lactobacillus fermentum NMCC-14 (LF)	VI	10^{10} CFU/day (p.o), Behavioral tests performed (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)
Bacillus clausii (BC)	VII	10^{10} CFU/day (p.o), Behavioral tests performed (EPM: acute, day 4; subacute, day 11; LDB: acute, day 5; subacute, day 12; OFT: acute, day 6; subacute, day 13)

Abbreviations: EPM, elevated plus maze; LDB, light dark box; OFT, open field test; i.p, intraperitoneal; p.o, per oral; NS, normal saline.

fermentum NMCC-14 (non-spore former) and commercially available spores of *Bacillus clausii* (Enterogermina®) were determined for their potential as curative/therapeutic agents in stress.

2 MATERIALS AND METHODS

2.1 Animals

Albino C57BL/6J mice (male) weighing 23 ± 3 g, age 6–8 weeks, were purchased from the National Institute of Health, Islamabad, Pakistan, and transferred for initial acclimatization of 5 days to the animal house facility in the Department of Pharmacy, Quaid-i-Azam University, Islamabad, Pakistan. The temperature was maintained at 25 ± 2°C under 12/12 hours dark–light cycle. All experiments were performed according to the "guidelines and principles of laboratory animals" provided by the Bioethical Committee of Quaid-i-Azam University, Islamabad, with approval number **#BEC-FBS-QAU2021-266**.

Animals were randomly divided into seven groups (**Table 1**), each having five mice/experiment. Group I served as the normal group, where animals were allowed free access to food and autoclaved water without any probiotic treatment or stress induction. Group II acted as the negative control and included animals with free access to food and autoclaved water, followed by the restraint-stress procedure for 120 min/day in the early morning (**Table 1**). Group III was a positive group, and animals were administered fluoxetine (10 mg/kg/day, intraperitoneally (i.p)). Freshly prepared *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* at a dose of 10^{10} colony-forming unit (CFU)/ml/day were administered orally to the animals in

Groups IV and V, respectively, followed by the restraint-stress procedure for 120 min. Animals in groups VI and VII were treated simply with freshly prepared *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/ml/day) and *Bacillus clausii* (10^{10} CFU/ml/day) without any induction of stress. All treatments were continued for 7 days to evaluate the acute effect and 14 days to evaluate the subacute effect.

2.2 Probiotics

Spores of *Bacillus clausii* by Sanofi, 11 Viale Europa, Origgio, were purchased from the local pharmacy, available with the trade name Enterogermina® (Batch Number: X1009, Reg. No. in Pakistan: 095289). *Lactobacillus fermentum* NMCC-14 (Accession number: MK611941.1, 586 bp, linear deoxyribonucleic acid (DNA)) (Ghazanfar et al., 2019), isolated from the milk of "Nilli Ravi Buffalo," was provided by the probiotics lab, National Institute for Genomics and Advanced Biotechnology, *National Agricultural Research Centre*, Islamabad, Pakistan.

2.3 Chemicals

Chloroform, ethanol, formalin, orthophosphoric acid, 2-propanol, phosphate buffer—tween (Lot. N0. 00352, Part Number: 00501, Agdia, United States), dopamine hydrochloride (Lot No. BCBS3110V, Sigma Aldrich, Germany), nor-adrenaline (Alfa Aesar, Lot No. 1017520), fluoxetine (gift from Ferozson Laboratories, Nowshera, Pakistan), ACTH enzyme-linked immunoassay (ELISA) kit (Catalog No E026Mo, Bioassay Technology Laboratory, Shanghai, China) and cortisol ELISA kit (Catalog No. MBS269130, MyBioSource, Inc., San Diego, United States), TRIzol™ reagent (Catalog Number 15596018, Invitrogen ™,

ThermoFisher Scientific, United States), Onescript cDNA synthesis kit (Catalog No. G236, Applied Biological Materials), and Maxima SYBER® Green/ROX qPCR Master Mix (Lot No. 00737730 and 00720013, Thermoscientific, Lithuania) were used.

2.4 Stress Induction

Restraint or immobilization is a widely used and accepted model to study stress and depression in animals (Paré and Glavin, 1986; Liang et al., 2015; Seewoo et al., 2022). Restrainers (polyvinyl chloride, 50 ml syringe ventilated from the front by producing small vents) were used to induce stress in mice through previously reported protocols (Paré and Glavin, 1986; Zafir and Banu, 2009). After the administration of the probiotics (10^{10} CFU/ml/day, p.o) and fluoxetine (10 mg/kg/day, i.p), animals were locked static inside the syringe barrel by pulling the plunger toward the animal except leaving space for cervical movement in the front of the restrainer and placed for 120 min on an opaque surface. Mice were then removed from restrainers and returned to their cages for 5 min. Each animal was then evaluated for behavioral changes by using elevated plus maze (EPM), open field (OF), and dark light box (LDB) tests. All behavioral trials conducted were video recorded using a top-fitted camera system (iPhone 7). Parameter details for results were extracted from the videos for evaluation. All tests were performed on a separate day (EPM: acute on day 4 and subacute on day11, light dark box/dark light box (LDB): acute on day 5 and subacute on day 12, and OFT: acute on day 6 subacute on day 13) to avoid apparatus-induced stress in the mice.

2.5 Behavioral Tests

2.5.1 Elevated Plus Maze

The EPM test has been extensively used to evaluate rodent behavior in stress, anxiety, and depression. EPM is a plus sign (+)-shaped wooden apparatus that consists of 2 open arms (length and width: 15 cm × 5 cm) and 2 closed arms (length, width, and height: 15 cm × 5 cm × 20 cm). Experiments were conducted through well-established protocols by Walf and Frye (2007), with slight modifications. EPM was adjusted 50 cm above the ground. After 120 min of restraining and 5 min of stay in the cage, on day 4 (for acute) and day 11 (for subacute), each animal of every group (**Table 1**) was put in the center of the EPM with its head facing one of the open arms. The animal was then allowed to explore the apparatus for 5 min, and time spent in the open and closed arms was used to assess stress behavior. All experiments were video recorded by the top-fitted camera.

2.5.2 Dark Light Box

A DLB was used, following established protocols to measure stress behavior in mice (Bourin and Hascoët, 2003). The apparatus was composed of a wooden box divided into two chambers, one light compartment and one dark compartment. Animals from all groups (**Table 1**) were assessed for stress behavior after 120 min of restraining and 5 min of intermediate stay in the cage, on day 5 for acute and day 12 for subacute effects. Each animal was placed in the light compartment with its head facing the wall of the chamber. Time spent in the light and dark compartments (parameters

for stress evaluation) was video recorded for 5 min using the top-fitted camera.

2.5.3 Open Field Test

The open field test (OFT) assesses the locomotor activity and exploratory drive in rodents (Gould et al., 2009). The apparatus consists of square chambers (length, width, and height: 72 cm × 72 cm × 15 cm) divided into small squares (4 × 4) having an area of 18 cm × 18 cm. Each restrained (120 min) animal (**Table 1**) was kept in the center of the field on day 6 (for acute) and day 13 (for subacute) and allowed to explore the field for 5 min. Movable time and time spent in the center of the paradigm were recorded to evaluate the stress behavior using the top-fitted camera.

2.6 Serum Cortisol and Adrenocorticotropic Hormone Level by Enzyme-Linked Immunoassay

Blood samples were collected by cardiac puncture and transferred to a collecting tube. Serum was then extracted/separated by centrifugation for 15–20 min at 4°C and 2,000–3,000 rpm. Serum cortisol and ACTH levels were determined by ELISA according to the manufacturers' instructions using an ACTH ELISA kit (Catalog No. E026Mo, Bioassay Technology Laboratory, Shanghai, China) and a cortisol ELISA kit (Catalog No. MBS269130, MyBioSource, Inc., San Diego, United States) respectively.

2.7 Histological Examination of Hippocampus

Animals were euthanized by cervical dislocation 24 h after the last restraining and activity in the behavioral paradigm. Brains removed were fixed in 4% paraformaldehyde and embedded in paraffin. Brains were then cut into a 5-μm thick section using a rotary microtome (Leica Biosystems). Hematoxylin and eosin (H&E) staining of each section was performed, and slides were developed and examined under an optical light microscope (Olympus. Model: CX41RF, Olympus Corporation, Tokyo, Japan).

2.8 Quantification of Monoamine Levels in Prefrontal Cortex and Hippocampus

The brain was dissected on an ice-cold Petri dish. Parts of the brains, HC, and PFC were removed and preserved in prechilled phosphate-buffered saline (PBS) (pH: 3.5, ingredients: 6.8 g potassium dihydrogen phosphate dissolved in 1000 ml of distilled water, and orthophosphoric acid was used to adjust the pH). Samples were then homogenized and centrifuged for 15–20 min at 4°C and 15,000 rpm. Supernatant collected was then filtered and used to detect the level of neurotransmitters in it. The mobile phase was composed of PBS and methanol (3:1, respectively). The column (material: 5 μm C18, dimensions: 4.6 × 250 mm, P/N 227-30017-08, S/N 17L05417) attached to high-performance liquid chromatography (HPLC, Shimadzu,

detector: SPD-20AV, degasser unit: DGU-20ASR, and pump: LC-20AT) was adjusted to a wavelength of 270 nm and a flow rate of 1.5 ml to detect the neurotransmitter levels in the PFC and HC of mice. Next, 20 μl of the tissue sample prepared in PBS buffer was loaded to detect dopamine, serotonin, and NE levels in the PFC and HC. Standard solutions of dopamine, serotonin, and NE serially diluted (1×10^9 ng/L, 5×10^8 ng/L, 2.5×10^8 ng/L, 1.25×10^8 ng/L, and 0 ng/L) were first analyzed to confirm the retention time of each neurotransmitter (serotonin: 9 min, dopamine: 3.4 min, and NE: 1.9 min). Various neurotransmitter levels were analyzed using HPLC (LC-20AT Shimadzu, Kyoto, Japan). The concentration in each sample was calculated by the operating software LabSolution Lite (2008) provided by Shimadzu Corporation, Japan.

2.9 Quantitative Real-Time Polymerase Chain Reaction to Determine mRNA Expression of Dopamine Receptors (D_1 and D_2) and Synaptophysin

Left and right HC and PFC were removed from the brain on an inverted precooled Petri dish kept on ice. Both isolated HC and PFC were used to extract total RNA using TRIzol™ reagent (Catalog Number: 15596018, Invitrogen™, ThermoFisher Scientific, United States) according to the manufacturer's protocols. After adjusting the concentration, RNA samples were converted to complementary DNA (cDNA) using a Onescript cDNA synthesis kit (catalog number: G236, Applied Biological Materials). cDNA of each sample was synthesized according to the provided protocols by the manufacturer. Samples were then used in qPCR for the measurement of messenger ribonucleic acid (mRNA) expression of glyceraldehyde 3-phosphate dehydrogenase (GADPH, housekeeping gene, primer used: forward 5′–3′: ACTCCA CTCACGGCAAATTCA; reverse 3′–5′: TCTCGCTCCTGG AAGATGGT) (Allais et al., 2007), dopamine receptor subtypes, i.e., dopamine receptor 1 (D_1, primer used: forward 5′–3′: AACTGTATGGTGCCCTTCTGTGG; reverse 3′–5′: CATTCGTAGTTGTTGTTGCCCCG) (Zhu et al., 2007) and dopamine receptor 2 (D_2, primer used: forward 5′–3′: CACTCCGCCACTTCTTGACATACA; reverse 3′–5′: TCTCCTCCGACACCTACCCCGA) (Gould et al., 2009), and synaptophysin (primer used: forward 5′–3′: AGACAGGCAGGTGAAGAGGA; reverse 3′–5′: TTGGCTCTTCCCAGGTTATG) (Allais et al., 2007). Maxima SYBER® Green/ROX qPCR Master Mix (Lot Number: 00737730 and 00720013, Thermoscientific, Lithuania) was used in Applied Biosystem-installed with StepOne™ software Real-Time PCR System (Version 2.3, Life Technologies Corporation) for the relative quantification of each sample; then, $2^{-\Delta\Delta CT}$ was calculated after analyzing for expression level. Fold expression of dopamine receptor subtypes (D_1 and D_2) and synaptophysin (normalized with GADPH) was then expressed as fold change related to the normal (**Table 1**).

2.10 Statistical Analysis

Experimental data obtained from all groups are presented as mean ± standard error to the mean (SEM). Graphs were designed using Origin Lab 6.0 (United States). Statistical assessment was performed using two-way analysis of variance (ANOVA) to determine the significance of the difference ($p \leq 0.05$) through IBM® SPSS® Statistics, version 25.

3 RESULTS

3.1 Probiotics Ameliorate Stress Behaviors in Mice
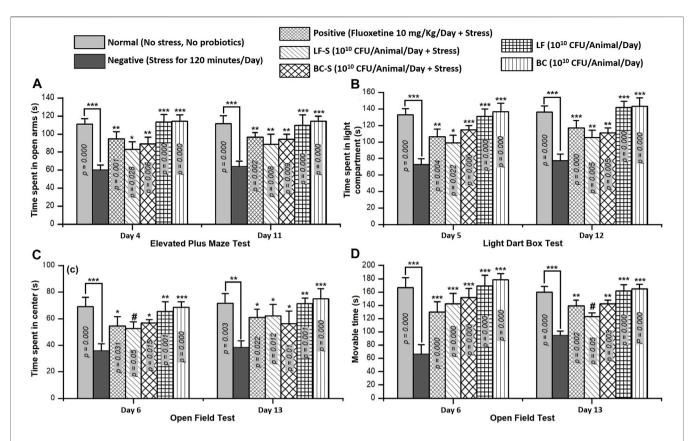
Behavioral changes are primary indicators and can be observed in humans and animals to evaluate stress and depression. In animals, stress induction techniques vary from species to species. The restraint stress technique in mice is a well-reported method to observe the therapeutic effects of different drugs through various behavioral paradigms and other molecular and hormonal changes. The EPM, DLB, and OF tests are known paradigms used to evaluate the stress behavior of mice.

3.1.1 Elevated Plus Maze

Time spent in the open arms of EPM is a parameter to evaluate the stress level in mice. Compared with the normal group ($n = 5$, **Table 1**), stress was induced in the negative group ($n = 5$) and time spent in the open arms was reduced significantly ($p < 0.001$, $n = 5$/group) both in acute and subacute restraint-stressed mice (**Figure 1A**). Fluoxetine (10 mg/kg, i.p), acting as a positive control (**Table 1**), suppressed stress behavior and significantly ($p < 0.05$, $n = 5$) increased the time spent in the open arms of the EPM in acute and subacute restraint-stressed mice (**Figure 1A**). Administration of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) in Group VI (LF-S) and V (BC-S) animals (**Table 1**) also significantly ($p < 0.05$, $n = 5$) increased the time spent in the open arms of EPM in acute and subacute restraint-stressed mice in comparison to the negative group. Compared to the normal group (Group I), *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/ day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) showed no significant difference ($p < 0.05$, $n = 5$) in LF (Group VI) and BC (Group VII) mice, both in acute and subacute probiotics treatments (**Figure 1A**). In comparison, *Bacillus clausii* showed more positive effects than *Lactobacillus fermentum* NMCC-14 in the EPM experiment.

3.1.2 Light Dark Box

Time spent in the light compartment indicates normal behavior, while time spent in the dark chamber represents stress behavior of the animal. Oral administration of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day) and *Bacillus clausii* (10^{10} CFU/day) suppressed stress behavior in the LF-S and BC-S groups (**Table 1**) and significantly ($p < 0.05$, $n = 5$) increased the time spent in the light compartment in acute and subacute restraint-stressed mice (**Figure 1B**). Fluoxetine (10 mg/kg, i.p) also significantly ($p < 0.01$, $n = 5$) increased the time spent in the light compartment both in acute and subacute restraint-stressed mice (**Figure 1B**).

FIGURE 1 | Effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) in restraint-stressed and normal mice in **(A)** elevated plus maze (EPM) test, **(B)** light dark box (LDB) test, and **(C,D)** open field test (OFT). Compared to the negative group, *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) significantly ($n = 5$/group) increased the time spent in the open arms of EPM and the time spent in the light compartment of LDB as well as the movable time and time spent in the center of OFT in acute and subacute restraint-stressed mice. Compared to the normal group, no significant ($p < 0.05$) change in the animals' behavior in groups BC and LF occurred. Values expressed are mean ± standard error to the mean (SEM). Note that $^{\#}p = 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the negative group by applying two-way ANOVA.

Compared to the normal group, no significant ($p < 0.05$, $n = 5$) changes were observed in the time spent in the light compartment by administration of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) in the LF and BC groups (**Table 1**) in acute and subacute probiotics-fed mice. Results from all groups showed more stress-suppressing effects of *Bacillus clausii* than *Lactobacillus fermentum* NMCC-14 in the LDB test.
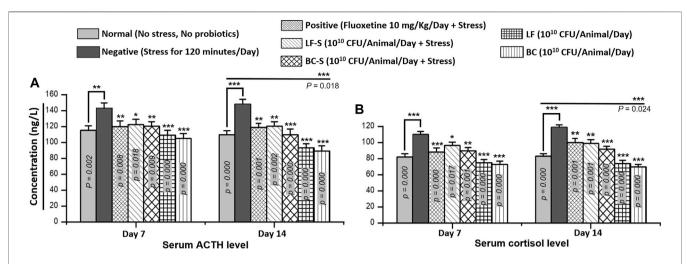
3.1.3 Open Field Test
Movable and immovable time and time spent in the central arena of the apparatus are parameters used to evaluate stress behavior in mice. Compared to the negative group ($n = 5$), *Bacillus clausii* (10^{10} CFU/day, p.o) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) significantly ($p \leq 0.05$, $n = 5$) increased the movable time in the LF-S and BC-S animal groups (**Table 1**) in acute and subacute restraint-stressed mice (**Figure 1C**). Time spent in the central arena was also increased significantly ($p \leq 0.05$, $n = 5$) by the oral administration of *Bacillus clausii* (10^{10} CFU/day) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day) in the LF-S and BC-S group animals, both in acute and subacute

restraint-stressed mice (**Figure 1D**). Fluoxetine (10 mg/kg, i.p, positive group) ($n = 5$, **Table 1**) also significantly ($p < 0.05$, $n = 5$) increased the movable time and time spent in the center of the OF both in acute and subacute restraint-stressed mice (**Figures 1C and D**). Compared to the normal group, no significant ($p < 0.05$, $n = 5$) changes were observed by acute and subacute administration of *Bacillus clausii* (10^{10} CFU/day, p.o) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) in animals in the LF and BC groups (**Table 1**) in movable time and time spent in the center of the open field. Results from OFT showed that treatment with *Bacillus clausii* produces more positive effects than treatment with *Lactobacillus fermentum* NMCC-14, except in movable time in subacute conditions, where the effects of *Lactobacillus fermentum* NMCC-14 were more prominent than those of *Bacillus clausii*.

3.2 Serum Cortisol and Adrenocorticotropic Hormone Level
Cortisol, the main glucocorticoid from the adrenal gland, plays a central role in glucose metabolism and in the response of the body to stress. The production of adrenal cortisol is regulated by the

FIGURE 2 | Effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) on serum **(A)** adrenocorticotropic hormone (ACTH) and **(B)** cortisol levels. Compared to the negative group, *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) significantly ($n = 5$/group) reduced the ACTH and cortisol levels in the LF-S and BC-S groups. Compared to the normal group, BC significantly decreased (day 14: ACTH, $p = 0.018$; cortisol, $p = 0.024$) the ACTH level in the BC group animals on day 14 and no significant ($p < 0.05$) change in the ACTH and cortisol levels was observed both in the LF and other BC group animals. Values expressed are mean ± standard error to the mean (SEM). Note that $^{\#}p = 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the negative group by applying two-way ANOVA.

pituitary hormone adrenocorticotropic hormone, synthesized in response to hypothalamic corticotrophin-releasing hormone. Compared to the negative group ($n = 5$), *Bacillus clausii* (10^{10} CFU/day, p.o) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) treatment significantly ($p < 0.05$, $n = 5$) reduced the adrenocorticotropic hormone and cortisol levels in Group IV (LF-S) and Group V (BC-S) animals (**Table 1**), both in acute and subacute restraint-stressed mice (**Figures 2A and B**). Fluoxetine (10 mg/kg, i.p) also significantly ($p < 0.05$, $n = 5$) reduced the adrenocorticotropic hormone and cortisol levels in acute and subacute restraint-stressed mice (**Figures 2A and B**). Compared to the normal group, except ACTH and cortisol levels in mice in group VII (BC) on day 14, no significant ($p < 0.05$, $n = 5$) change in serum ACTH and cortisol levels in Group VI (LF) and VII (BC) was observed, both with acute and subacute administration of *Bacillus clausii* (10^{10} CFU/day, p.o) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) (**Table 1**; **Figures 2A andB**). All results of ACTH and cortisol levels in the blood serum of mice showed that *Bacillus clausii* produced a greater decrease in ACTH and cortisol levels than *Lactobacillus fermentum* NMCC-14 and will be more effective to treat stress conditions.
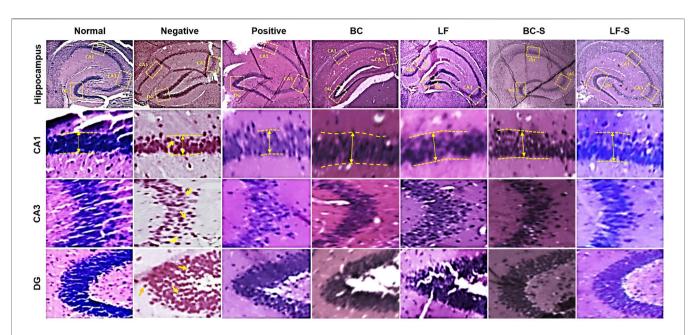
3.3 *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* Improve Neurodegeneration in the Hippocampus

Neurodegeneration in the cornu ammonis (CA1 and CA3) and dentate gyrus (DG) of the HC was investigated for the effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) in restraint-stressed and normal mice (**Table 1**; **Figures 3** and **4**). Histomorphological alterations showed neurodegeneration and pyknosis in acute and
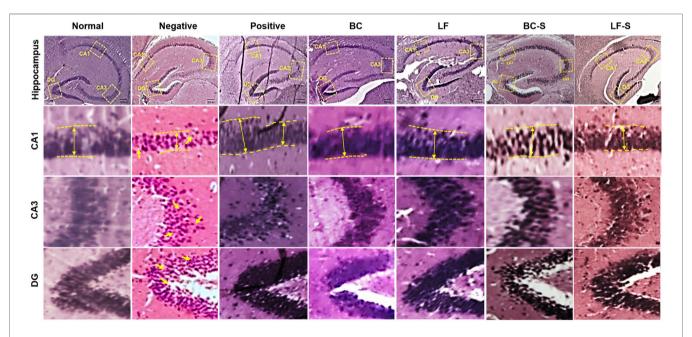
subacute restraint-stressed mice (negative group—Group II, **Table 1** and **Figures 3** and **4**). Slides of the brains of negative group mice, both in acute and subacute restraint-stressed conditions, showed retraction of neuronal processes, condensed arborization of neurons toward the nucleus (pyknosis), and reduced width of CA1 of the HC, as shown in **Figures 3** and **4**. Animals in the normal, LF (Group VI), and BC (Group VII) showed no obvious abnormalities or degeneration of neurons of the CA1, CA3, and dentate gyrus of the HC (**Figures 3** and **4**). Orally administered *Bacillus clausii* (10^{10} CFU/day) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day) also resulted in improvement in the neuronal arborization in DG, CA1, and CA3 of the HC in the brains of restraint-stressed mice of Group IV and V as compared to negative group (Group II).

3.4 Effects of *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* on Monoamine Level in the Prefrontal Cortex and Hippocampus

Stress and depression are associated with monoamine levels in the synaptic cleft. An increase in the monoamine level at synapses improves mood disorders, as proposed in the "monoamine hypothesis" (Hirschfeld, 2000). Stressors with short bursts and less duration increase the monoamine level, but a decrease is observed with persistent and recurrent induction of stress in animals (Dishman, 1997). Compared to the negative group (**Table 1**), *Bacillus clausii* (10^{10} CFU/day, p.o) and *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) significantly ($p < 0.05$, $n = 5$/group) increased the serotonin, dopamine, and NE levels in acute acute and subacute restraint-stressed mice (**Figures 5A–F**). Fluoxetine (10 mg/kg, i.p, positive group) also significantly ($n = 5$, $p <$

FIGURE 3 | Effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) on the hippocampus CA1, CA3, and DG of acute restraint-stressed and normal mice. *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) improved stress-induced neurodegenerative changes in the hippocampus CA1, CA3, and DG regions (H&E staining; magnification ×10, scale bar: 200 μm).

FIGURE 4 | Effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) on the hippocampus CA1, CA3, and DG of subacute restraint-stressed and normal mice. *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) improved stress-induced neurodegenerative changes in the hippocampus CA1, CA3, and DG regions (H&E staining; magnification ×10, scale bar: 200 μm).

0.05) increased only the level both in acute and subacute restraint-stressed mice. The fluoxetine group (10 mg/kg, i.p) did not show any significant ($n = 5$, $p < 0.05$) increase versus the negative group in the level of dopamine and NE in acute acute and subacute restraint-stressed mice. Compared to the normal group animals (**Table 1**), no significant ($p < 0.05$, $n = 5$, **Figures 5A–F**) changes in the serotonin, dopamine, and NE levels were observed in the LF (Group VI) and BC (VII), both in acute acute and subacute restraint-stressed mice. In comparison, *Bacillus clausii* showed a greater increase in

the monoamine level than *Lactobacillus fermentum* NMCC-14.

3.5 Effects of *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* on the mRNA Expression of Dopamine Receptors (D₁ and D₂) and Synaptophysin in the Prefrontal Cortex and Hippocampus

Stress is a multifarious ailment in which changes in neurotransmission occur. Expression of different monoamine receptor subtypes, e.g., dopamine D_1 and D_2, and synaptic vesicle release-regulating proteins, e.g., synaptophysin, is altered under conditions of stress and depression. Compared to the negative group (except D_2, HC in subacute condition), *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* treatment significantly ($p < 0.05$, $n = 5$) reversed the downregulation of mRNA expression of D_1, D_2, and synaptophysin in acute and sub-acute restraint-stressed mice (**Figures 6A–F**). Fluoxetine also significantly ($p < 0.05$, $n = 5$) upregulated stress-induced suppression of D_1, D_2, and synaptophysin mRNA expression in acute and subacute restraint-stressed mice (**Figures 6A–F**). In groups VI (LF) and VII (BC), the animals did not show significant ($p < 0.05$, $n = 5$) changes in the fold expression of D_1, D_2, and synaptophysin mRNA, both in acute and subacute probiotics fed mice, as compared to animals in the normal group. Results from dopamine receptor (D_1 and D_2) and synaptophysin expressions showed that *Bacillus clausii* treatment caused a greater increase in mRNA expression than *Lactobacillus fermentum* NMCC-14 treatment, both in acute and subacute restraint-stressed mice and LF and BC group animals.
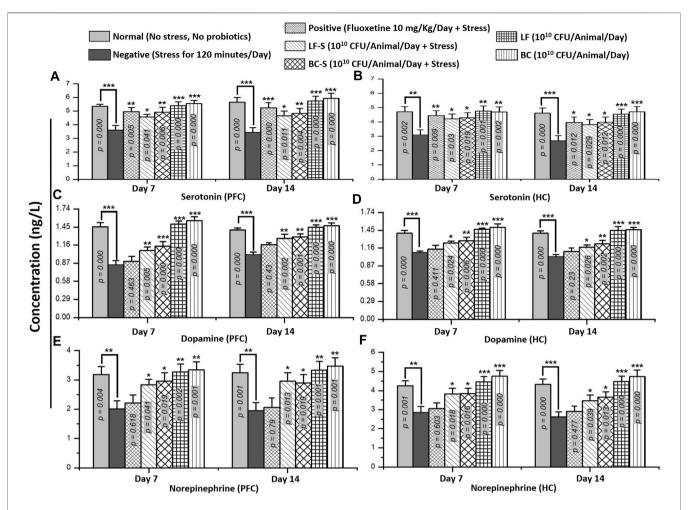
4 DISCUSSION

Stress is characterized by feeling overwhelmed and frustrated with a lack of interest in the things that once seemed interesting. Neurobiologists are exploring new entities to find the best, safest, and most definitive/targeted therapy for such a disorder. Probiotics are becoming prominent and gaining interest in molecular biology to unveil the possible mechanisms of action of microorganisms/microbiota in treating various neurological disorders. Studies mostly conducted on probiotics utilize chronic stress or the restraint-stressed model rather than any acute or subacute condition. Acute and subacute persistent stress mediate or promote depression and other complex psychopathologies. (Esch et al., 2002). Chronic pathologies can be prevented by correcting acute stress conditions early. For this reason, despite one time acute stressor condition, we focused on acute (7 days) and subacute (14 days) daily stress induction, which further can be considered a progressive or promoting factor to cause depression and other serious chronic neuropathological conditions. In this study, probiotic strains, *Lactobacillus fermentum* NMCC-14 and spores of *Bacillus clausii* (Enterogermina®), were evaluated for their role in treating acute and subacute restraint-stressed mice.

Our experimental results revealed that probiotics suppress symptoms of stress in acute and subacute restraint-stressed mice in behavioral paradigms, i.e., EPM, LDB, and OFT. Stress was reduced or suppressed by decreasing the serum cortisol and ACTH levels, increasing the monoamine (serotonin, dopamine, and NE) levels, and increasing the mRNA expression of dopamine (D_1 and D_2) receptors and synaptophysin. Moreover, enhancement in neurological arborization was also observed in the H&E slides on neurodegenerative effects and pyknosis caused by restraint stress in the mice HC.

Spores of *Bacillus clausii*, available with the trade name Enterogermina®, are registered as over-the-counter drugs and used as an antidiarrheal in humans. *Bacillus clausii*, spore-forming bacteria, has advantages over the *Lactobacillus* species as it is heat stable and can be stored at room temperature without any deleterious effects on its viability; it is also resistant to the acidic gastric environment. *Bacillus* species are extensively studied for their effects on dysbiosis and diarrhea. A study also showed that *Bacillus clausii* reduced the duration of respiratory tract infections in children (Marseglia et al., 2007). Shahgond et al. (2022) further reported that *Bacillus clausii* UBBC07 combined with *Lactobacillus plantarum* UBLP40 was effective in acute hepatic encephalopathy. A study conducted by Yunes and coworkers reported that *Bifidobacterium adolescentis* 150 and *Lactobacillus plantarum* 90sk showed a reduction in depressive behavior like that of fluoxetine in a force swimming test (Yunes et al., 2020). It is also reported that environmental enrichment suppresses stress behavior in an animal model and can greatly be approached to contribute to the treatment of mental disorders (Dandi et al., 2018). *Lactobacillus fermentum* has also been studied extensively for its role in treating many diseases, like inflammatory bowel disease (Bao et al., 2010), respiratory tract infections (Maldonado et al., 2012), and psychological abnormalities (Wang et al., 2015). In a similar manner, many studies have been conducted to test different species of *Lactobacilli* (*L. helveticus*, *L. rhamnosus*, *L. plantarum*, and *L. fermentum*) and *Bifidobacteria* (*B. longum* and *B. breve*) using EPM, LDB, OFT, fear conditioning, and step-down tests to evaluate stress (Bercik et al., 2010; Savignac et al., 2014; Liang et al., 2015; Wang et al., 2015; Liu WH et al., 2016; Liu YW et al., 2016) and depression (Desbonnet et al., 2008; Desbonnet et al., 2010; Bravo et al., 2011; Singh et al., 2012; Savignac et al., 2014; Liu WH et al., 2016; Liu YW et al., 2016) in animals. All these studies showed positive effects of probiotics in these behavioral paradigms, which greatly support the results of our study.

Sudo et al. (2004) demonstrated the elevation of ACTH and corticosterone levels in restraint-stressed mice. Another study showed that increased corticosterone levels were prevented by environmental enrichment in maternally separated stressed Wistar rat pups (Dandi et al., 2018). Fluoxetine was also found to normalize corticosterone serum levels (Wei et al., 2019). Neurotransmissions and their regulation contribute to all central nervous system physiology, including stress and depression. Like canonical gap junctions and mechanosensitive ion channels, synaptophysin is a transmembrane synaptic vesical release-regulating protein and marker for neuronal damage (Arthur and Stowell, 2007; Gudi et al., 2017; White and Stowell, 2021). Sze et al. (1997) observed a decrease in synaptophysin expression in Alzheimer's disease. Thome et al. (2001) also reported decreased synaptophysin and

FIGURE 5 | Effects of *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* on monoamine levels (serotonin, dopamine, and norepinephrine) in the prefrontal cortex and hippocampus of acute and subacute restraint-stressed and normal mice. Compared to the negative group ($n = 5$/group), a significant increase in PFC and HC monoamine levels—serotonin **(A,B)**, dopamine **(C,D)**, and norepinephrine **(E,F)**—was shown in LF-S and BC-S group restraint-stressed mice. Compared to the normal group ($n = 5$/group), no significant ($p < 0.05$) change was observed in monoamine levels—serotonin **(A,B)**, dopamine **(C,D)**, and norepinephrine **(E,F)**—in the PFC and HC of LF and BC group mice. Values expressed are mean ± standard error to the mean (SEM). Note that [#]$p = 0.05$, [*]$p < 0.05$, [**]$p < 0.01$, and [***]$p < 0.001$ versus the negative group by applying two-way ANOVA.

synaptotagmin mRNA expression in stressed rats. Stress can ameliorate or have detrimental effects on the brain if it persists in the long term and causes neurodegeneration in the HC (Esch et al., 2002). A study by Dandi et al. (2018) revealed that environmental enrichment increased the expression of brain-derived neurotrophic factors and synaptophysin in stressed Wistar rat pups. Heat-killed and live *Lactobacillus paracasei* PS23 was also found to reverse the corticosterone-induced reduction in the serotonin and dopamine levels in mice (Wei et al., 2019). Desbonnet and his colleagues reported enhancement of the neurotransmitters NE, dopamine, and serotonin and reduced neurotransmitter metabolites, i.e., dihydroxyphenylacetic acid and 5-hydroxy indole acetic acid, in probiotic-treated Sprague Dawley rats (Desbonnet et al., 2008). The dopaminergic neuronal system plays an important role in mental disorders, and studies from the literature reported that the activation of dopamine (D1 and D2) receptor subtypes is necessary to reduce symptoms of stress behaviors (Kamei et al., 1995; Sim et al., 2013).

Dopamine exerts stimulatory effects on HPA both through D_1 and D_2 receptors to provoke severe stress (Belda and Armario, 2009). A study conducted by Kamei and coworkers reported that the activation of both D_1 and D_2 receptors is necessary to attenuate fear-conditioned stress in mice (Kamei et al., 1995).

Humans harbor nearly 100 trillion bacteria in their gastrointestinal tract, which play an important role in maintaining normal health (Gill et al., 2006). *Bacillus*, including *Lactobacillus*, species are normal inhabitants of the human intestinal tract among the probiotics (Pereira et al., 2003). Probiotic use decreases several pathogenic gastrointestinal microorganisms, reduces bloating effects and flatulence, and improves bowel regularity. In humans, *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* as well as other species of gut microbiota can be used to reduce anxiety and to modulate the brain biochemical system. New insights are continuously emerging, and researchers strive to explore the human gut microbiota for a better alternative to
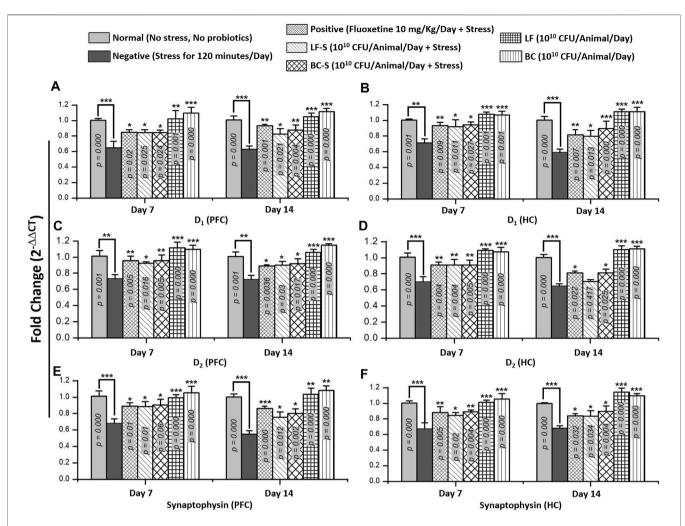
FIGURE 6 | Effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) on mRNA levels of dopamine receptors (D_1 and D_2) and synaptophysin in the prefrontal cortex and hippocampus of acute and subacute restraint-stressed and normal mice. Compared to the negative group, the mRNA expression of dopamine receptor subtypes (D_1 and D_2) and synaptophysin increased in the PFC **(A,C,E)** and HC **(B,D,F)** significantly (n = 5/group) by acute and subacute administration of *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* in LF-S (except D_2 in the HC, day 14) and BC-S mice. Compared to the normal group, no significant ($p < 0.05$) change was observed in the mRNA expression of dopamine receptor subtypes (D_1 and D_2) and synaptophysin in the PFC **(A,C,E)** and HC **(B,D,F)** of BC and LF mice. Values expressed are mean ± standard error to the mean (SEM). Note that $^\#p$ = 0.05, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the negative group by applying two-way ANOVA.

the already available remedies or to provide new strategies to treat stress and other neurological disorders.

The findings of these studies in the literature extend support in one way or another to our results, which further validate the role of probiotic use in stress and depression or any other neurological disorder. The mechanism of action is versatile for probiotics and not possible to define in a single way for any disease. Significant suppression of stress can be achieved by multimodal effects as indicated in our study. Probiotics may replace the pathogenic microbes, act as a digestive aid to improve nutritional contents, enhance the availability of more precursors of monoamines and finally to increase the level of serotonin, dopamine, and NE (**Figure 7**). Probiotics also upregulated dopamine receptor subtypes (D_1 and D_2) and synaptophysin mRNA expression and can also increase the cellular response and release of monoamines in

the HC and PFC neurons. Central modulation of the brain then decreased blood ACTH and cortisol levels. The major effects may also have occurred by enhancing the immune system and vagal stimulation either directly from the brain or through the enteric nervous system (**Figure 7**). All these positive alterations significantly improved the stress behavior of mice in the LF and BC groups.

Terminologies like "psychobiotics" (Sharma et al., 2021) to treat neurological diseases and "pharmabiotics" (Lee et al., 2018; Sharma and Im, 2018), in general, are emerging to explore new insights into probiotics for their role in different neurological disorders. Our study's evaluation of probiotics revealed that both spore-forming *Bacillus clausii* (Enterogermina®) and non-spore-forming *Lactobacillus fermentum* NMCC-14 strains significantly improved activity and performance and corrected biological functions in restraint-stressed mice. *Bacillus clausii* caused a greater
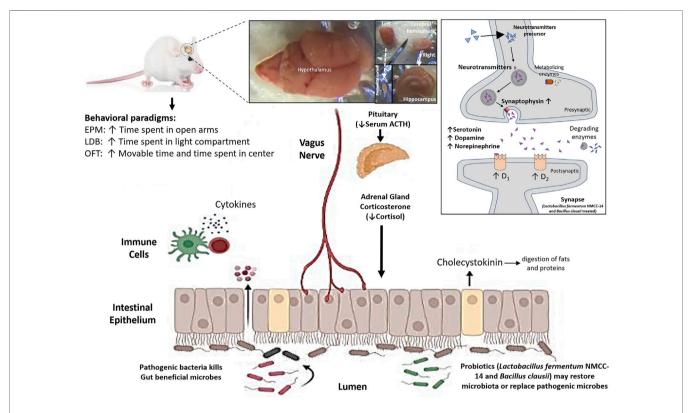
FIGURE 7 | Effects of *Lactobacillus fermentum* NMCC-14 (10^{10} CFU/day, p.o) and *Bacillus clausii* (10^{10} CFU/day, p.o) through the gut–brain axis on monoamine levels (serotonin, dopamine, and norepinephrine), mRNA expression of synaptophysin and dopamine receptor subtypes (D_1 and D_2) in the hippocampus and prefrontal cortex of mice brains, and blood ACTH and cortisol levels in restraint-stressed and normal mice. Note that ↑ shows increase and ↓ shows decrease, and the abbreviations used are **EPM**, elevated plus maze, **LDB**, light dark box, and **OFT**, open field test.

improvement in stressed and normal animals *than Lactobacillus fermentum* NMCC-14. No stress-inducing effects by *Lactobacillus fermentum* and *Bacillus clausii* (groups VI and VII) were observed compared to the normal group, but these probiotics even enhanced the activity of mice. *Lactobacillus fermentum* NMCC-14 and *Bacillus clausii* (Enterogermina®) can be a safer and better single therapeutic entity or in combination with already available drugs to treat stress and other mental disorders.

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 in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Bioethical Committee of Quaid-i-Azam University, Islamabad, approval number #BEC-FBS-QAU2021-266.

AUTHOR CONTRIBUTIONS

MR was responsible for experimental work throughout the study. He was also involved in conceptualizing, desingning, collection and processing of data. RH, JW, and WA are responsible for resources, validation, and formal analysis. S G was responsible for the isolation, identification, and confirmation of the strain and provision of bacterial cultures to be used in the study. SK was involved in supervising and performing all tests for behavioral parameters to assess the stress condition. SU performed the gene analysis for D1, D2, and synaptophysin in various brain areas. MT was involved in conceptualizing and designing the study, statistical analysis of data, and final revision/approval of the manuscript.

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Olanzapine-induced lipid disturbances: A potential mechanism through the gut microbiota-brain axis

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Objective: Long-term use of olanzapine can induce various side effects such as lipid metabolic disorders, but the mechanism remains to be elucidated. The gut microbiota-brain axis plays an important role in lipid metabolism, and may be related to the metabolic side effects of olanzapine. Therefore, we explored the mechanism by which olanzapine-induced lipid disturbances through the gut microbiota-brain axis.

Methods: Sprague Dawley rats were randomly divided into two groups, which underwent subphrenic vagotomy and sham surgery. Then the two groups were further randomly divided into two subgroups, one was administered olanzapine (10 mg/kg/day) by intragastric administration, and the other was administered normal saline by intragastric administration (4 ml/kg/day) for 2 weeks. The final changes in lipid parameters, gut microbes and their metabolites, and orexin-related neuropeptides in the hypothalamus were investigated among the different groups.

Results: Olanzapine induced lipid disturbances as indicated by increased weight gain, elevated ratio of white adipose tissue to brown adipose tissue, as well as increased triglyceride and total cholesterol. Olanzapine also increased the Firmicutes/Bacteroides (F/B) ratio in the gut, which was even aggravated by subphrenic vagotomy. In addition, olanzapine reduced the abundance of short-chain fatty acids (SCFAs) metabolism related microbiome and 5-hydroxytryptamine (5-HT) levels in the rat cecum, and increased the gene and protein expression of the appetite-related neuropeptide Y/agouti-related peptide (NPY/AgRP) in the hypothalamus.

Conclusion: The abnormal lipid metabolism caused by olanzapine may be closely related to the vagus nerve-mediated gut microbiota-brain axis.

1 Introduction

Schizophrenia is a complex and severe mental illness, that affects nearly 1% of the world's population, and it is one of the top ten causes of disability in the world (Marder and Cannon, 2019). Currently, atypical antipsychotic drugs (AAPDs) are the mainstay of treatment for schizophrenia, and the metabolic side effects have become a key factor in reduced quality of life and increased relapse of patients (Grajales et al., 2019). Olanzapine (OLZ), one of the representative AAPDs, is prone to causing weight gain, obesity, hyperglycemia and dyslipidemia after long-term use, and these side effects occur more frequently in children and women (Visconti et al., 2019). The metabolic side effects also greatly increase the risks in comorbidities of type two diabetes and cardiovascular disease (Anyanwagu et al., 2018). Administration of OLZ can promote food intake, alter energy expenditure and metabolic levels, and induce insulin resistance, ultimately having negative impact on the parameters of body weight, fasting blood glucose and triglyceride levels (Teff and Kim, 2011; Lord et al., 2017; Maruvada et al., 2017; Ballon et al., 2018; Bush et al., 2018; Skonieczna-Zydecka et al., 2019). Notably, overweight patients with schizophrenia are twice as likely to discontinue antipsychotic drugs as patients with normal weight, and discontinuation is a common cause of symptoms recurrence (Rummel-Kluge et al., 2008). Therefore, how to investigate the underlying mechanisms of AAPD-induced metabolic side effects and to form corresponding coping strategy are urgently needed.

The exact mechanisms by which AAPDs cause weight gain and lipid disturbances are complex. According to previous findings, AAPDs can accelerate peripheral adipogenesis by regulation of sterol-regulatory element binding protein (SREBP) (Ader et al., 2005). Another recent study further indicates that insulin-induced gene (INSIG), and progesterone receptor membrane component 1 (PGRMC1) as the upstream regulatory factors of SREBP, can be inhibited by AAPDs and consequently produce disturbances in lipid metabolism by affecting the PGRMC1/INSIG/SREBP pathway in the liver (Cai et al., 2015). Specifically, available evidence shows that altered levels of adenosine 5′-monophosphate-activated protein kinase (AMPK) and gastrointestinal peptide (such as glucagon-like peptide-1, GLP-1) can contribute to the lipid metabolic side effects induced by OLZ (Ikegami et al., 2013; Teff et al., 2013; Li et al., 2016). It has been suggested that OLZ significantly increased blood lipid levels and hepatic lipid accumulation by increasing the expression of mammalian target of rapamycin complex-1 (mTORC1) and p-mTORC1 (Liu et al., 2019). In addition, OLZ can increase body fat percentage in rats through oxidative stress signaling, which is reversable by antioxidants (Bilgic et al., 2017; Isaacson et al., 2020).

The metabolic disorders are also closely related to the central effects produced by OLZ (Yang et al., 2007). Current studies have shown that the possible mechanism is related to the increased antagonism of 5-HT$_{2C}$ and H$_1$ receptors in the hypothalamus (Casey and Zorn, 2015). Although OLZ exerts its therapeutic effects on various neurotransmitter systems (Huang et al., 2014), the changes in 5-HT seem to play a major role in appetite control, especially that the involvement of endogenous hypothalamic 5-HT can cause satiety during or after meals (Halford et al., 2005). Meanwhile, 5-HT suppresses appetite and promotes energy expenditure mainly by stimulating the sympathetic drive of brown adipose tissue (Tecott, 2007; Yabut et al., 2019), promotes the release of insulinotropic signals in white adipose tissue to reduce lipolysis, and alters the process of *de novo* adipogenesis in the liver (Crane et al., 2015; Oh et al., 2015). Furthermore, it has been shown that exogenous substances, such as glucose, fatty acids, and drugs, can alter intestinal 5-HT release in the duodenum by affecting the function of microbiota (Walther et al., 2003). In recent years, the gut microbiota has been found to be closely related to metabolic status (Zmora et al., 2019). Long-term administration of OLZ induces weight gain and lipid deposition in rats, whereas coadministration with antibiotics shows the opposite (Davey et al., 2013). In support, OLZ administration can increase the proportion of obesity-related bacteria in the and cause weight gain, and the combination with prebiotics can alleviate this side effect (Morgan et al., 2014; Kao et al., 2018; Kao et al., 2019; Abbas et al., 2022). In addition, targeting the *Akkermansia muciniphila* in intestinal microorganisms can partially correct abnormal blood glucose caused by OLZ (Huang et al., 2021). The abovementioned evidence reveals that the gut microbiota may play a pivotal role in the metabolic disorders caused by OLZ, but the specific mechanisms remain to be explored in depth.

The review by Coccurello and Moles (2010) has addressed comprehensively the clinical impact of AAPD-induced metabolic derangement and the hypotheses for related obesogenic and diabetogenic mechanisms. However, at that time, the key role of gut microbiota was largely undervalued or unknown, whereas the present attention towards gut microbiota-brain axis bidirectional communication allows the exploration of additional mechanistic hypotheses. The gut microbiota assists with the absorption of substances and the storage of energy (Eckburg et al., 2005; Duca and Lam, 2014), and can affect brain function through "The Microbiota-Gut-Brain Axis" (Mayer et al., 2015; Cryan et al., 2019). The gut microbiota and the brain can communicate with each other through signal conduction by various channels, including immunity, vagus nerve and enteric nervous system (Cryan et al., 2019). Among them, the most important one is the vagus nerve, which can innervate the entire intestine through vagus nerve afferent neurons and participate in the regulation of satiety, intestinal homeostasis and the inflammatory response (Singh et al., 2020). In addition, short-chain fatty acids (SCFAs) as the main metabolites produced by gut microbiota, have also been found to affect the colonic environment, blood sugar and blood lipid levels (Yamashita

et al., 2009; Canfora et al., 2015). It is noteworthy that SCFAs have also been proven to promote the secretion of 5-HT by stimulating enterochromaffin cells, and to stimulate the vagus nerve through 5-HT$_3$ receptors on the vagus nerve endings, thereby regulating the body's lipid metabolism (Browning, 2015; Reigstad et al., 2015). The vagus nerve serves as the physiological connection between the GM and the central nervous system, which provides the basis for regulating appetite through the Microbiota-Gut-Brain Axis (Cork, 2018). After receiving serotonergic signals from the gut, the brain regulates appetite through the expression of neuropeptide Y/ agouti-related peptide (NPY/AgRP) in the hypothalamus. As appetite-stimulating peptides, NPY and AgRP can induce food intake and reduce energy expenditure (Schwartz et al., 2000; Savontaus et al., 2002).

In the present study, we aimed to investigate one possible additional mechanism responsible of these important unwanted and highly deleterious metabolic side effects. It is hypothesized that gut microbiota-brain axis is involved in the metabolic effects induced by OLZ, and the changes in the gut microbiota can lead to a consequent increase in the expression of orexin-stimulating NPY/AgRP in the hypothalamus, and lipid disturbances in the periphery.

2 Materials and methods

2.1 Drugs and reagents

Olanzapine was purchased from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China) and dissolved in 0.9% saline at 2.5 mg/ml, adjusting to pH 6.5 with citric acid. CCK-8 was obtained from Sigma-Aldrich® (Shanghai, China) and freshly prepared in PBS solution (4 μg/ml). The rat 5-HT assay kits were provided by Jiangsu KETE Biotechnology Co., LTD. (Yancheng, China). BCA protein quantitative assay kit and radioimmunoprecipitation assay buffer containing phenylmethyl sulfonyl fluoride were purchased from Boster Biological Technology Co., Ltd. (California, United States). The primary antibody against NPY was purchased from Cell Signaling Technology Co., Ltd. (Danvers, United States). The primary antibody against AgPY was purchased from Santa Cruz Biotechnology Co., Ltd. (Shanghai, China). Primary antibody against β-actin, and all secondary antibodies were purchased from Proteintech Group, Inc. (Wuhan, China).
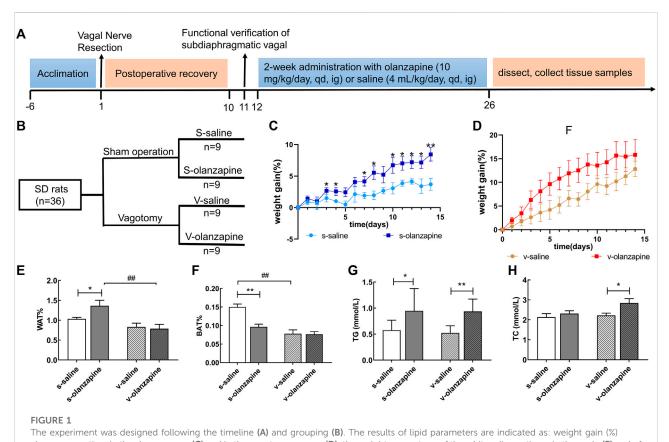
2.2 Animals and treatments

Adult female Sprague Dawley (SD) rats, weighing 200 ± 10 g, were provided by the Hunan STA Laboratory Animal Co., LTD. (Xinjiang China) [No. SCXK (xiang) 2019-0004]. The

experimental flow is depicted in Figure 1A. The rats were free to access to commercial rat chow (SLAC Laboratory Animal, Shanghai, China) and water, and were reared under 12-h light/ dark cycle environment at approximately 24–26°C and in relatively humidity of 40%–60%. All the experimental procedures conformed to the Declaration of Helsinki and were approved by the Experimental Animal Ethics Committee of the Department of Experimental Animals, Central South University (No. 2019sydw0259).

Forty female SD rats were randomly divided into a sham operation group (n = 22) and a vagotomy group (n = 18) according to body weight. After 1 week of acclimatization, rats in the sham operation group were sham-operated, and rats in the vagotomy group were performed with subphrenic vagotomy. The specific operation procedure of vagal nerve resection was as follows (Klarer et al., 2014): after being anesthetized by intraperitoneal injection of 3% sodium pentobarbital (1 ml/kg), the rat skin and abdominal wall were incised along the midline of the abdomen to expose the stomach and esophagus, in order to allow searching for the trunk of vagus nerve. The subphrenic vagus nerve trunk was resected and all vague nerve branches were transected. The wound was sutured and disinfected. The sham operation group underwent the same operation procedure except that the vagus nerve was not resected. After 10 days of postoperative recovery, to test the successfulness of vagotomy, rats were fasted overnight (12 h) and intraperitoneally injected with 4 μg/kg CCK-8 in PBS solution. After 2 h, the rats were given food and monitored for the extent of food intake (Klarer et al., 2014). Within a timeframe of 30 min after CCK-8 injection, the sham group with intact vagal nerve typically consumed 25%–40% less food than the vagotomy group (Klarer et al., 2014). Therefore, the rats in the vagotomy group which had less than 25% of food intake reduction in food intake in the first 30 min were excluded. Finally, 4 out of 22 vagotomy rat animals had to be culled. Then, each large group was randomly divided into two subgroups (each n = 9, Figure 1B), and the body weight of the rats was weighed every day. Each rat was administrated with olanzapine by oral gavage at 10 mg/kg/day or 0.9% saline for 2 weeks before sacrifice.

On Day 26, after fasting for 12 h, the rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (1 ml/kg). The truncal blood was collected using vacuum blood collection tubes and centrifuged for 10 min (3,000 r/min, 4°C) to obtain serum samples. Then the white adipose tissue (WAT) in the bilateral groin, the brown adipose tissue (BAT) between the scapulae, and the contents of the cecum and fecal samples were collected from the body and quickly frozen in liquid nitrogen. Rat heads were rapidly dissected and the tissue of hypothalamus was removed on ice trays and snap frozen in liquid nitrogen. All the above-mentioned rat samples were stored in −80°C refrigerator before analysis.

FIGURE 1
The experiment was designed following the timeline **(A)** and grouping **(B)**. The results of lipid parameters are indicated as: weight gain (%) changes over time in the sham groups **(C)** and in the vagotomy groups **(D)**; the weight percentage of the white adipose tissue in the groin **(E)** and of the brown adipose tissue between the scapula **(F)**; and the serum levels of **(G)** triglyceride (TG) and **(H)** total cholesterol (TC) in different groups. Data are represented as the mean $\pm$ SD, $*p < 0.05$, $**p < 0.01$ and $^{\#\#}p < 0.01$.

2.3 Lipid parameters, olanzapine concentration and microbiota metabolites

The rats were weighed every day, and the percentage of body weight gain was calculated as weight gain% (WG%) = (weight on the day-initial weight)/initial weight*100%. The percentage of two kinds of adipose tissue in the body weight was calculated as WAT% and BAT%. The serum levels of triglyceride (TG) and total cholesterol (TC) were determined by an automatic biochemical analyzer (Chemray 240/800, Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). Serum OLZ concentrations were determined by a high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–MS/ESI) method we previously established in our laboratory (Zhou et al., 2004).

For the microbiota metabolites in the content of the rat cecum, the determination for 5-HT was performed according to the protocol provided by the rat 5-HT assay kit. Moreover, the concentrations of SCFAs were measured using a gas chromatography-mass spectrometry (GC-MS) equipped with an electron ionization (EI) source. In brief, an appropriate amount of cecal content sample was added to 2 ml of

phosphoric acid-water (phosphoric acid: water = 1:3) solution and homogenized for 2 min. Two milliliters of ether were added to the sample for extraction for 10 min and centrifuged (4,000 r/min, 4°C), and the ether phase was removed and the aqueous phase was extracted again. The two extracts from ether phase were combined and evaporated to a constant volume of 2 ml, and then 2 μl of the sample was injected and analyzed. The analytes were separated with a capillary chromatographic column (HP-INNOWAX, 25 m × 0.20 mm × 0.40 μm). The sample was analyzed by GC-MS under the following conditions: the initial temperature was kept at 100°C for 5 min, linearly increased to 150°C at 5°C/min, and then fast increased to 240°C at 30°C/min, finally held at 240°C for 30 min. The mass spectrometer inlet temperature was 240°C, and the carrier gas flow rate was set at 1.0 ml/min. The EI source temperature was set to 200°C, and the collision energy was 70 eV.

The SCFAs content was calculated according to the formula:

$$w = (C * V * N)/(m * M)$$

In the formula: w, the content of SCFAs in the rat cecum, in mmol/kg; C, the concentration of SCFAs in the test solution of the sample (measured value of the test solution by GC-MS), in mg/L;

V, the constant volume, in the unit mL; N, dilution factor; m, weighed mass of the sample, in grams (g); M, molar mass of SCFAs, in g/mol.

2.4 16S rRNA high-throughput sequencing

DNA extraction from frozen fecal samples (180 mg) was subjected to use an PowerSoil® DNA Isolation Kit (MOBIO, United States) according to the company's protocols. After extracting the total DNA, specific primers with barcodes were synthesized according to the full-length primer sequences. After that, PCR amplification was performed and the products were purified, quantified and normalized to form a 16S rRNA sequencing library (SMRT Bell). After library quality inspection, qualified libraries were sequenced with PacBio Sequel. Data were analyzed by smart link analysis software. Then after exporting the PacBio off-board data as a CCS file, we first identified the CCS with a barcode, and obtained the Raw-CCS sequence data (lima v1.7.0). Using cut adapt 1.9.1, the raw sequence data were identified and removed of the primer sequence and filtered by length, to obtain a Clean-CCS sequences of primers. Finally, the chimera sequences were further identified and removed (UCHIME v4.2) to acquire an Effective-CCS sequences. The pretreated sequence files were quality-filtered by Trimmomatic (v 0.33) and FLASH (v 1.2.11). USEARCH (v10.0) was used to cluster the OTU, with 97% similarity truncation. The NCBI database was used for species annotation. The community richness (Chao1 richness estimator) and diversity (Shannon Index) were calculated using QIIME2. Beta diversity analysis was performed by the principal coordinate analysis (PCoA) method based on the R software package. The taxonomic groups (classes) and genera (subclasses) of bacteria represented by the differences between groups were identified by linear discriminant analysis (LDA) combined with an effector. The LEfSe criterion was set as LDA > 4 with $p < 0.05$. The t-test was performed on the species abundance data between groups using Metastats software.

2.5 Orexinogenic neuropeptides in the hypothalamus

2.5.1 Western blot

Protein extraction was performed using RIPA buffer and PMSF, and protein quantification was performed using a BCA protein quantification kit. Proteins were separated using 10% SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, United States). After blocking the membrane with 5% BSA for 1.5 h, the membrane was incubated with the corresponding primary antibodies (NPY and AgRP primary antibodies were diluted 1:1000 with antibody diluent, and β-actin was diluted 1:5000) for 12 h at 4°C. The membrane was then incubated with the secondary antibody for 90 min at

room temperature. Using β-actin as an internal reference, the protein bands were quantified with ImageJ software.

2.5.2 Quantitative real-time PCR

The samples were subjected to quantitative PCR using Applied Biosystems 7500/7500 Fast Real-Time PCR System and StepOnePlus Real-Time PCR System, and the primer sequences of the genes were as follows:

GAPDH, forward: 5′-ACAGCAACAGGGTGGTGGAC-3′, reverse: 5′-TTTGAGGGTGCAGCGAACTT-3′; NPY, forward: 5′-TACTCCGCTCTGCGACACTA-3′, reverse: 5′-TGGGGG CATTTTCTGTGCTT-3′; AgRP, forward: 5′-ACTCTGAAG CTGAATGCCCAC-3′, reverse: 5′-CCCACACGTGACTAC TTCCT-3′.

Total RNA was isolated from rat hypothalamus with TRIzol reagent according to the manufacturer's instructions. Gene expression was calculated as △CT using GAPDH as a reference and was expressed relative to the control group normalized to a value of 1.

2.6 Statistical analysis
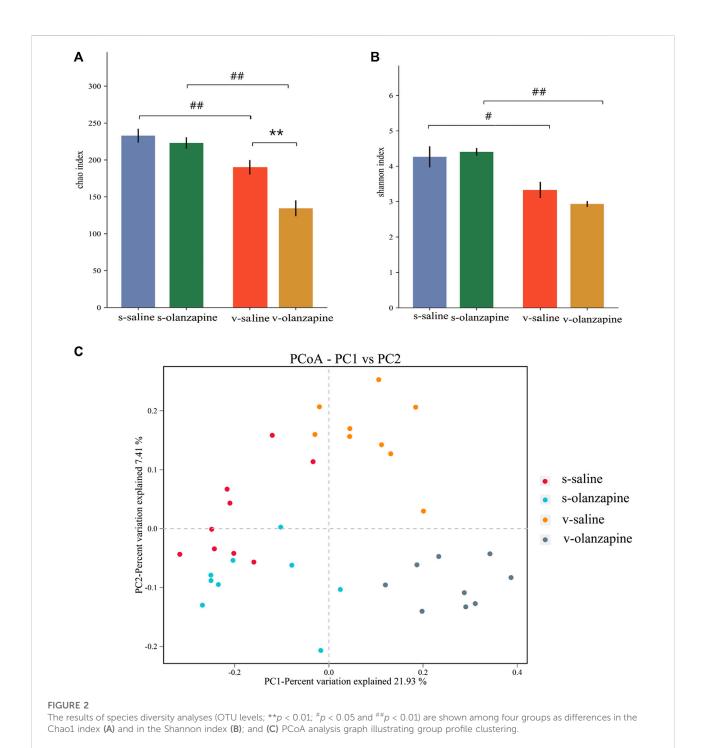
SPSS Statistics version 25.0 software (IBM Corp, Inc., Armonk, NY, United States) was used for data analysis. The results are expressed as the mean ± standard deviation (mean ± SD). The comparisons of repeated measures of WG% between saline and OLZ groups were made by multiple t-test with FDR determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. The abundance of SCFA metabolism-related microorganisms was compared in pairs of s-saline vs. s-olanzapine and v-saline vs. v-olanzapine, and serum levels of OLZ were compared between s-olanzapine and v-olanzapine groups, using Mann-Whitney U test. Before analyses, the Kolmogorov-Smirnov test was used to determine whether the relevant data are normally distributed. Then, Spearman's test was considered as the way of testing data for heteroscedasticity. If the data of the four subgroups passed the normality test and had equal variances, two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was utilized to compare the preselected pairs of groups (s-saline vs. s-olanzapine; v-saline vs. v-olanzapine; s-saline vs. v-saline; s-olanzapine vs. v-olanzapine). Otherwise, nonparametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons test was carried out. Graphs were made using GraphPad Prism 8 software.

3 Result

3.1 Olanzapine induces lipid disturbances in rats

The effects of olanzapine on body weight and metabolism of rats were analyzed by calculating WG%, WAT% and BAT% of
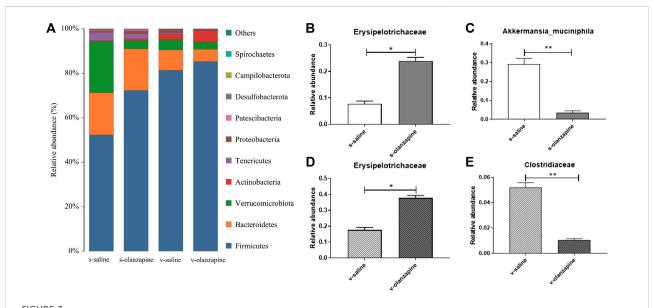
FIGURE 2
The results of species diversity analyses (OTU levels; **$p < 0.01$; #$p < 0.05$ and ##$p < 0.01$) are shown among four groups as differences in the Chao1 index **(A)** and in the Shannon index **(B)**; and **(C)** PCoA analysis graph illustrating group profile clustering.

rats. As shown in Figures 1C,D, in the sham-operated group, olanzapine administration from day 4 (t ratio = 2.304, $p = 0.035$) significantly increased the WG% relative to the saline group. However, under the condition of vagotomy, the olanzapine treatment had no significant effects on WG% compared with the saline group.

Under sham operation conditions, the olanzapine group had significantly increased rat WAT% ($p = 0.030$) and decreased BAT

% ($p = 0.005$) when compared with the saline group (Figure 1E). Under the condition of vagus nerve resection, the WAT% and BAT% of the olanzapine group did not change significantly (Figure 1F). In addition, comparing the two olanzapine groups, the WAT% of the rats in the vagotomy group was significantly lower than that in the sham group ($p = 0.001$). This experiment also found that, regardless of whether the gut microbiota-brain axis was intact, olanzapine significantly
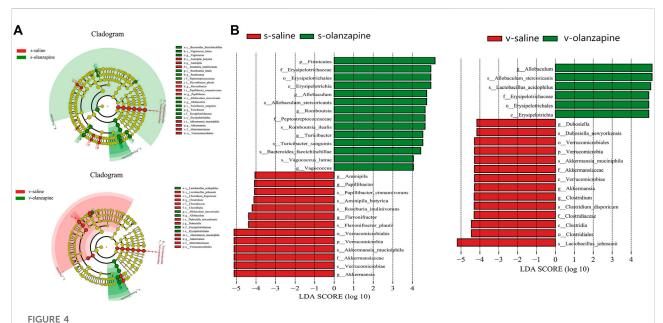
FIGURE 3
Gut microbial species distribution in the four groups: histograms showing species distribution at each phylum level **(A)**; difference analyses of Erysipelotrichaceae **(B)** and Akkermansia muciniphila **(C)** between the olanzapine-administered and saline groups under the sham-operated condition; and comparisons of Erysipelotrichaceae **(D)** and Clostridiaceae **(E)** between the olanzapine administrated and saline groups under the vagotomy condition. *$p < 0.05$ and **$p < 0.01$.

increased serum TG levels in rats on average (s-saline vs. s-olanzapine, $p = 0.015$; v-saline vs. v-olanzapine, $p = 0.002$; Figure 1G). The integrity of the gut microbiota-brain axis did not affect the level of TG in rat serum. Meanwhile, olanzapine administration significantly increased the serum TC content of the vagotomy-treated rats ($p = 0.022$) but did not affect the serum TC content of the sham-operated rats (Figure 1H).

3.2 Olanzapine alters the gut microbial composition

The effects of olanzapine and the vagus nerve on gut microbes were explored by analyzing the diversity and abundance of gut microbes in each group of rats. Under sham-operated conditions, olanzapine administration had no significant effect on the species abundance and β-diversity of gut microbes in rats. Under the condition of vagotomy, the olanzapine group significantly reduced the species abundance of rat gut microbes ($p = 0.002$, Figure 2A), and also showed a tendency to affect the β-diversity of rat gut microbes. In addition, the results indicated that gut microbial diversity and species abundance can be compromised by cutting off vagus nerve (Figures 2B,C), as revealed by comparisons of s-saline vs. v-saline (Chao1 index, $p = 0.002$; Shannon index, $p = 0.003$) and s-olanzapine vs. v-olanzapine (Chao1 index, $p = 0.012$; Shannon index, $p = 0.002$). Since the Firmicutes/Bacteroidetes ratio (F/B) was positively associated with obesity (Coras et al.,

2019), we analyzed the ratio of F/B in each group (Figure 3A). As depicted, under the sham-operated condition the OLZ group had a significantly increased F/B ratio, whereas OLZ administration had little effect on F/B in the setting of vagotomy. In addition, with only saline administration, vagotomy also significantly increased the F/B ratio of the rats compared with the sham-operated group. We further analyzed the abundance of SCFA metabolism-related microorganisms in the four groups of gut microbiota (Figures 3B–E). Under sham-operated conditions, the OLZ group had a significantly increased abundance of Erysipelotrichaceae ($U = 17$, $p = 0.040$) and a significantly decreased abundance of A. muciniphila ($U = 14$, $p = 0.019$). After vagotomy, OLZ treatment significantly increased the abundance of Erysipelotrichaceae ($U = 13$, $p = 0.014$) and significantly decreased the abundance of Clostridiaceae in the gut microbiota ($U = 7$, $p = 0.003$).

Figure 4A represents the LEfSe cladogram mapping the differential bacterial taxa between paired groups to taxonomic tree. Figure 4B shows the histogram of the LEfSe LDA scores computed for differential taxonomic clades between paired groups. The color (red or green) indicated the enrichment of the taxa within the corresponding groups. Under sham operation, the abundance of phylum Firmicutes was higher, and phylum Verrucomicrobiota was lower in the gut microbiota of OLZ treated group than saline group. However, when the rats were performed vagotomy, only the abundance of phylum Verrucomicrobiota was decreased in OLZ groups as compared with saline group.
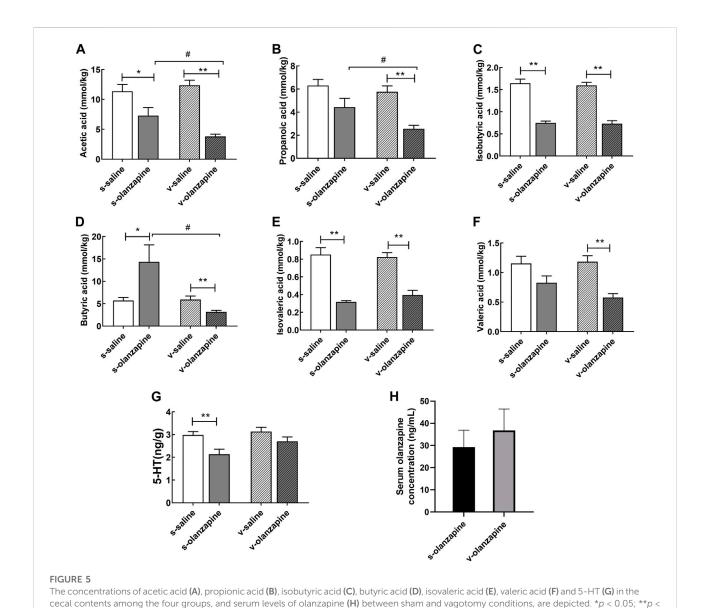
FIGURE 4
The LEfSe cladogram maps the distinct bacterial taxa between pairwise groups into a taxonomic tree **(A)**. The rings from inside to outside represent taxonomic levels from phylum to species. The diameter of each circle is proportional to the abundance of taxa. Colors of circles and shades indicate microbial lineages enriched in the corresponding samples, and p, c, o, f, g, and s stand for represent phylum, class, order, family, genus, and species, respectively. **(B)** Histograms of LEfSe LDA scores for different branches between groups. Only taxa with LDA values greater than 4 are shown. Colors (red or green) indicate that taxa are enriched in the corresponding taxa. The letters p, c, o, f, g, and s represent phylum, class, order, family, genus and species, respectively.

3.3 Olanzapine increases the levels of short-chain fatty acids and 5-hydroxytryptamine in the cecum

To explore the effects of OLZ administration on SCFAs, the differences in the levels of six main SCFAs in the cecum are summarized in Figures 5A–F. In the case of sham operation, OLZ administration significantly reduced the levels of acetic acid ($p = 0.036$), isobutyric acid ($p = 0.007$), and isovaleric acid ($p = 0.004$) in the cecum and significantly increased the level of butyric acid ($p = 0.033$). In the vagus nerve resection group, olanzapine administration significantly reduced the content of six SCFAs in the cecum (acetic acid, $p = 0.006$; propanoic acid, $p = 0.001$; isobutyric acid, $p = 0.005$; butyric acid, $p = 0.001$; isovaleric acid, $p = 0.009$; valeric acid, $p = 0.002$). Under olanzapine administration, the vagus nerve resection group had significantly reduced levels of acetic acid ($p = 0.019$), propionic acid ($p = 0.048$) and butyric acid ($p = 0.039$) compared with the sham operation group. However, under the condition of just saline administration, the vagus nerve resection group and the sham operation group showed no significant differences in these SCFAs. As shown in Figure 5G, in the case of the sham operation, OLZ administration significantly reduced the 5-HT content in the cecum ($p = 0.004$). Under the circumstance of vagus nerve resection, the effects of OLZ on 5-HT in the cecum was not statistically significant. Since the pharmacokinetics of OLZ may vary significantly, we compared the serum levels of OLZ between

s-olanzapine and v-olanzapine groups (Figure 5H) and no statistical difference was found ($U = 16$, $p = 0.818$).

3.4 Olanzapine regulates the expression of orexinogenic neuropeptides in the hypothalamus

To further explore the specific mechanisms by which OLZ induces abnormal lipid metabolism and obesity through the gut microbiota-brain axis, we measured the mRNA and protein levels of orexins in the hypothalamus of different groups. As shown in Figure 6, the olanzapine group significantly increased the mRNA expression of the hypothalamic orexin neuropeptides NPY ($H = 19.2$, post-hoc $p = 0.021$) and AgRP ($H = 30.9$, post-hoc $p = 0.042$) and only significantly increased the protein level of NPY under sham-operated conditions ($H = 24.6$, post-hoc $p = 0.016$). In both vagotomy groups, the olanzapine group had decreased NPY mRNA expression ($H = 19.2$, post-hoc $p = 0.049$) but still upregulated AgRP mRNA expression ($H = 30.9$, post-hoc $p = 0.033$), but also had no significant effects on NPY and AgRP protein expression. Moreover, both the mRNA and protein levels of NPY and AgRP were significantly increased in the v-saline group compared with the s-saline group (NPY mRNA: $H = 19.2$, post-hoc $p = 0.028$; NPY protein: $H = 24.6$, post-hoc $p = 0.006$; AgRP mRNA: $H = 30.9$, post-hoc $p = 0.015$; AgRP protein: $H = 27.2$, post-hoc $p = 0.034$).
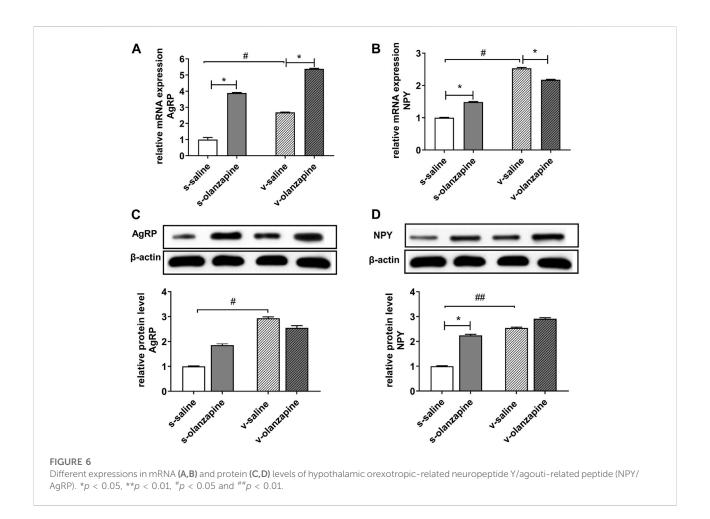
FIGURE 5
The concentrations of acetic acid **(A)**, propionic acid **(B)**, isobutyric acid **(C)**, butyric acid **(D)**, isovaleric acid **(E)**, valeric acid **(F)** and 5-HT **(G)** in the cecal contents among the four groups, and serum levels of olanzapine **(H)** between sham and vagotomy conditions, are depicted. *$p < 0.05$; **$p < 0.01$, #$p < 0.05$ and ##$p < 0.01$.

4 Discussion

In this study, the role of the gut microbiota-brain axis in olanzapine-induced lipid disturbances was systematically investigated for the first time in an animal model by severing the vagus nerve resection to disrupt gut-brain communication. We investigated the changes in lipid parameters induced by OLZ and inferred the effects of OLZ on gut microbiota by measuring the composition and abundance of gut microbes. We also discussed whether OLZ-induced alterations in SCFAs can activate the vagus nerve by increasing 5-HT, resulting in hypothalamic orexin expression.

The previous reports illustrate that even though OLZ promotes weight gain in humans, this effect seems to be gender-specific in rodent animal models, with OLZ-induced weight gain observed in female rats only, not male rats (Choi et al., 2007; Albaugh et al., 2011). Therefore, we chose female rats for our study. The results of this study found that the body weight of the rats in the v-olanzapine group had a tendency to increase, and olanzapine administration significantly increased the WG% of the sham-operated rats, which was consistent with the results in the literature (Kao et al., 2018). Olanzapine administration significantly increased WG% only when the vagus nerve was intact, suggesting that the intact gut microbiota-brain axis plays an important role in OLZ-induced weight gain in rats. Interestingly, the negative impact of the increase in WAT% and decrease in BAT% induced by OLZ administration was reversed by the disruption of the gut microbiota-brain axis

FIGURE 6
Different expressions in mRNA **(A,B)** and protein **(C,D)** levels of hypothalamic orexotropic-related neuropeptide Y/agouti-related peptide (NPY/AgRP). *$p < 0.05$, **$p < 0.01$, #$p < 0.05$ and ##$p < 0.01$.

(vagotomy), suggesting that OLZ-induced weight gain may require an intact gut microbiota-brain axis pathway. Previous studies have found that vagotomy reduces mitochondrial thermogenesis in brown adipose tissue (Andrews et al., 1985), indicating that the vagus nerve activation may have a certain inhibitory effect on brown adipose tissue mitochondrial function (Yang et al., 2007). Furthermore, OLZ significantly increased serum TG levels in rats regardless of whether the gut microbiota-brain axis was intact, consistent with another report (Chiu et al., 2006). The dyslipidemia caused by OLZ may due to a decrease in the metabolic rate or an increase in hepatic lipogenesis (Yang et al., 2007; Cai et al., 2015), which can be stratified to its peripheral mechanisms.

The abundance and diversity of gut microbes can be affected by OLZ, and the diversity of gut microbiota are more severely compromised when the vagus nerve was resected (Figures 2, 3). The results suggest that the vagus nerve allows the bidirectional communication between the gut and the brain, and has a key role in maintaining the homeostasis and diversity of gut microbes. In addition, we observed that OLZ increased the F/B of gut microbes by increasing the abundance of Firmicutes and decreasing the abundance of Bacteroidetes, which will become

non-significant when gut microbiota-brain axis was disrupted by vagotomy. Brain exerts regulatory effects on gut microbes, as evident by the fact that mood and stress can affect the composition of gut microbes (Kim and Shin, 2018). Therefore, the changes in F/B may largely owing to the feedback top-down regulation of gut microbes by the brain during the treatment with OLZ. Disrupting the integrity of the vagus nerve in this experiment hindered the regulation of the gut microbiota by the brain, thereby causing no F/B changes after OLZ administration. In addition, it has been reported that F/B is positively correlated with obesity, and it has been found that when a prebiotic (B-COS®) is add-on to OLZ, it reduces the abundance of Firmicutes and alleviates OLZ-induced weight gain (Indiani et al., 2018; Kao et al., 2018). The above results suggest that the abnormal lipid metabolism and the increase in body fat caused by olanzapine are related to the increase in the F/B of gut microbes. In addition, OLZ administration significantly decreased the abundance of *A. muciniphila* in the gut of sham-operated rats. *A. muciniphila* is considered to be a promising probiotic and mainly promotes the generation of acetic, propionic, butyric, isobutyric, and isovaleric acids (Li et al., 2021). A decrease in its abundance is thought to be associated with metabolic disorders and inflammatory diseases, including obesity, type 2 diabetes, and inflammatory bowel
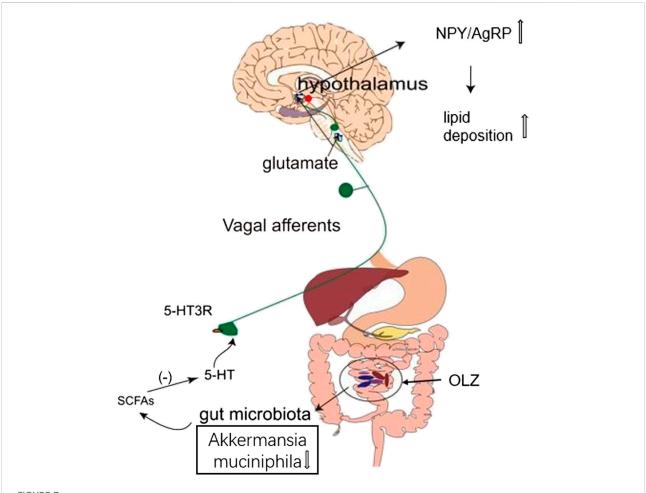
FIGURE 7
This study suggests a possible scenario that olanzapine can induce changes in the levels of short-chain fatty acids (SCFAs) by altering the abundance and composition of gut microbiota, thereby reducing 5-HT secretion in the gut and related glutamatergic signal transduction through vagus nerve, which increases the ratio of hypothalamic orexotropic-related neuropeptide Y/agouti-related peptide (NPY/AgRP), eventually in turn contributing to accumulated lipid deposition in rats.

disease (Zhang et al., 2019). Therefore, the results of this study suggest that OLZ-induced lipid disturbances are associated with a decrease in the abundance of *A. muciniphila* in the gut.

To further investigate whether OLZ affects 5-HT levels by altering the synthesis of SCFAs in the gut through the gut microbiota-brain axis, we determined the concentrations of SCFAs and 5-HT in the cecum content. Olanzapine can exert a negative impact on the gut microbiota to reduce the production of SCFAs, which in turn reduces 5-HT release and transmission from the vagus nerve to the brain. Therefore, the removal of the vagus nerve cuts off the bottom-up signal transmission of 5-HT related to the intestinal flora, but does not entirely affect the regulation of SCFAs by OLZ. Therefore, it is explainable that OLZ administration significantly reduced the levels of acetic acid, isobutyric acid, and isovaleric acid in the cecum of rats, no matter the rats were resected or not. However, among them, OLZ-induced reduction of acetic acid was aggravated when the vagus nerve was resected, reflecting

certain protective role of top-down regulation from brain to gut microbiota. In support, OLZ-associated changes in propanoic acid and butyric acid were more severe in vagotomy group than in sham group. Interestingly, the effect of OLZ on butyrate in the gut seems to be closely related to the integrity of the vagus nerve. It has been shown that exogenous supplementation of butyrate can antagonize weight gain by reducing food intake and increasing energy metabolism (Li et al., 2018). Olanzapine administration significantly increased the concentration of butyric acid in the cecum of the s-olanzapine group, whereas decreased this SCFA of the v-olanzapine group. This result may be partially owing to the imbalanced modulation of OLZ on the abundance of butyrate-producing bacteria Erysipelotrichaceae and Clostridiaceae as indicated in Figure 3, because OLZ significantly increased the abundance of Erysipelotrichaceae in the gut of the s-olanzapine group. Although OLZ also significantly increased the abundance of Erysipelotrichaceae in the v-olanzapine group, the abundance of

Clostridiaceae was simultaneously decreased in vagotomy group but not in the sham group, suggesting that the abundance of Clostridiaceae and related butyric acid yield may be specifically affected and more dependent on the integrity of the vagus nerve.

Changes in SCFA have been shown to affect 5-HT levels by affecting enterochromaffin cell activity. As shown in Figure 5, OLZ induced reductions in acetic acid and 5-HT in the sham-operated group, whereas only acetic acid reductions were observed in the vagotomy group. This suggests that olanzapine-induced reductions in acetic acid levels are mediated by the gut microbe-brain axis, further causing a decrease in 5-HT levels. 5-HT can stimulate vagal afferents through 5-HT$_3$ receptors at vagal afferent terminals, and activation of vagal afferents can inhibit food intake in rats by increasing satiety (Laskiewicz et al., 2003; de Lartigue, 2016). Therefore, the results suggest that OLZ may ultimately affect the regulation of energy intake in the hypothalamus by affecting the gut microbiota-brain axis. By measuring the mRNA and protein levels of orexin NPY and AgRP in the hypothalamus, we confirmed that OLZ further affects the levels of NPY and AgRP through the vagus nerve and may further aggravate the increase in body fat content in rats by affecting energy intake. It is worth noting that when the vagus nerve was resected, the OLZ-related changes of NPY and AgRP in protein levels were disappeared. The results suggest that impairment in the vagus nerve itself may also lead to dysregulation of energy metabolism, thereby aggravating the influence of external factors (e.g., AAPD) on lipid metabolism and energy intake in the body. Nevertheless, as regard to OLZ-induced lipid disturbances, there may be some differences between real schizophrenia patients and normal rats we used. Notably, both impaired vagal afferent and efferent signaling has been implicated in the pathophysiology of schizophrenia (Klarer et al., 2018). Because schizophrenia patients may experience disrupted function of vagus nerve and cannot properly regulate downstream gut microbiota, this factor may contribute to the predisposition of abnormal gut microbiota and metabolic disturbances in schizophrenia patients before AAPD treatment. Recently, the changes in gut microbiota in patients with schizophrenia taking OLZ have been studied (Pelka-Wysiecka et al., 2019). Using an animal model of vagotomy, herein we further explored how the impact of OLZ on the abundance and diversity of gut microbes can contribute to its lipidemia side effects through gut microbiota-brain axis.

There are several limitations of this study. First, the effects of OLZ on TG and TC seem to be independent of the gut microbiota-brain axis during the course of the study. Considering that TG and TC levels may be more related to hepatic lipid metabolism, the peripheral role of gut microbiota during AAPD treatment needs to be further investigated. Second, this study did not involve the treatment on gut microbiota, in future studies combinations of probiotics/prebiotics with OLZ

will be considered to explore the detailed mechanisms of and novel intervention strategy to AAPD related metabolic side effects. Third, the pharmacokinetics of OLZ can vary significantly in rats and may account for the differences between the two OLZ-treated groups. However, in our study, no significant difference in the serum concentrations of OLZ was found. The pharmacokinetics of OLZ cannot fully explain the differences in the indicators of weight gain, WAT%, the community richness and diversity of gut microbiota, and certain SCFAs levels (acetic acid, propanoic acid and butyric acid) between s-olanzapine and v-olanzapine groups.

In conclusion, OLZ administration is at least partially responsible for obesity by increasing F/B ratio, and this effect requires an intact gut microbiota-brain axis. In addition, OLZ may alter the levels of the microbial metabolite SCFAs by reducing the abundance of *A. muciniphila* in the gut. Its alterations lead to lower levels of 5-HT, which stimulate the activity of orexotropic neurons in the hypothalamus *via* the gut microbiota-brain axis to induce lipid disturbances (Figure 7). A better understanding of the peripheral and central mechanisms underlying OLZ-induced lipid disturbances could shed light on forming new strategy to ameliorate the metabolic side effects of antipsychotic treatment.

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/; PRJNA816822.

Ethics statement

The animal study was reviewed and approved by The Experimental Animal Ethics Committee of the Department of Experimental Animals, Central South University.

Author contributions

YG designed the study, collected and analyzed the data. ZZ contributed to data analyze and wrote the manuscript. CZ, MY, HY, and HC performed the experiment. BZ and HC supervised the study. All authors reviewed and approved the manuscript.

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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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The regulatory effects of second-generation antipsychotics on lipid metabolism: Potential mechanisms mediated by the gut microbiota and therapeutic implications

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Second-generation antipsychotics (SGAs) are the mainstay of treatment for schizophrenia and other neuropsychiatric diseases but cause a high risk of disruption to lipid metabolism, which is an intractable therapeutic challenge worldwide. Although the exact mechanisms underlying this lipid disturbance are complex, an increasing body of evidence has suggested the involvement of the gut microbiota in SGA-induced lipid dysregulation since SGA treatment may alter the abundance and composition of the intestinal microflora. The subsequent effects involve the generation of different categories of signaling molecules by gut microbes such as endogenous cannabinoids, cholesterol, short-chain fatty acids (SCFAs), bile acids (BAs), and gut hormones that regulate lipid metabolism. On the one hand, these signaling molecules can directly activate the vagus nerve or be transported into the brain to influence appetite *via* the gut–brain axis. On the other hand, these molecules can also regulate related lipid metabolism *via* peripheral signaling pathways. Interestingly, therapeutic strategies directly targeting the gut microbiota and related metabolites seem to have promising efficacy in the treatment of SGA-induced lipid disturbances. Thus, this review provides a comprehensive understanding of how SGAs can induce disturbances in lipid metabolism by altering the gut microbiota.

KEYWORDS

gut microbiome, SGAs, lipid disturbances, gut microbiota-brain axis, SCFAs, BAs

Introduction

The use of antipsychotic medications as a treatment for patients with schizophrenia is surging, and the incidence of schizophrenia is also rising dramatically worldwide (Hert et al., 2011; Gonçalves et al., 2015). However, long-term use of these drugs can cause numerous adverse effects on patients, especially the disruption of lipid levels, including high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), and total cholesterol (TC) (Jaberi et al., 2020). Although individuals with schizophrenia may exhibit dyslipidemia before the initiation of treatment, mounting evidence has shown that antipsychotics can independently induce further abnormalities. It has been noted that patients with first-episode schizophrenia have abnormal lipid profiles, and those with multiple-episode

schizophrenia are more likely to have dyslipidemia (Mackin et al., 2007; Vancampfort et al., 2015; Mhalla et al., 2018; Pillinger et al., 2019; Yang et al., 2022). Notably, second-generation antipsychotics (SGAs) have stronger associations with lipid abnormalities than first-generation antipsychotics (FGAs) have (Buhagiar and Jabbar, 2019). Metabolic abnormalities especially lipid metabolism disorders, are major risk factors contributing to cardiovascular events (Fan et al., 2013). They also play a role in the pathophysiological process of systemic organ damage and are causative factors in the development and progression of atherosclerotic cardiovascular disease. According to previous reports, patients with schizophrenia have a life expectancy that could be 15 years shorter than that of the general population. Additionally, more than two-thirds of patients with schizophrenia die from coronary heart disease, which is significantly higher than the mortality rate in the general population (Hennekens et al., 2005). A growing body of research indicated that SGA-induced disturbances of lipid metabolism and other metabolic abnormalities are the key factor linking to an increased risk of cardiovascular disease in patients with schizophrenia, in addition to some confounding risk factors such as smoking, physical inactivity, unhealthy lifestyle, and poor dietary habits (Arias et al., 2018).

Although the particular processes by which SGAs cause dysfunctional lipid metabolism are complex, a growing body of evidence suggests that the gut microbiota is involved in SGA-induced defects in lipid metabolism. From birth, humans have microbes in their digestive tract (Yatsunenko et al., 2012). Hundreds of millions of microorganisms, including bacteria, fungi, and viruses, exist in the healthy human gastrointestinal system, forming a microbial community that has a major impact on the body (Mirzaei and Maurice, 2017). A large number of these bacteria make up the collective intestinal flora. The intestinal flora contains 1000 to 1500 species of bacteria, which outnumber the body's cells by more than 10 times (Kim and Jazwinski, 2018) and have more than 100 times the total number of genes as humans (Cox et al., 2019). These bacteria play important roles in host metabolism, digestion, the immune system, and the central nervous system (John and Mullin, 2016; Rogers et al., 2016; Dinan and Cryan, 2017; Ipci et al., 2017; Kanji et al., 2018). The theory that the gut microbiota affects lipid metabolism has been extensively studied in mice. For example, germ-free (GF) mice on a chow diet showed lower fasting systemic TG, TC, HDL cholesterol, and portal vein TG (Martinez-Guryn et al., 2018), as well as higher liver cholesterol and lower TG levels, than conventionally raised (Conv-R) mice (Rabot et al., 2010). Rabot et al. found that Conv-R mice had increased blood TG, HDL, and TC levels after consuming a high-fat diet (Rabot et al., 2010). To maintain the same weight as Conv-R mice, GF mice had to increase their caloric intake by at least 30% (Hsiao et al., 2008). Further evidence that the intestinal flora affects lipid metabolism has been observed in fecal transplantation experiments. Peter et al. showed for the 1 time that the ability of the gut microbiota to harvest energy from the diet was a transmissible trait. GF mice colonized with an "obesity microbiota" had a much higher increase in total fat than GF mice colonized with a "lean microbiota" (Turnbaugh et al., 2006). Similarly, obese patients with reduced microbial gene abundance (40%) showed more pronounced metabolic disturbances and had increased total serum cholesterol and serum TG levels (Cotillard et al., 2013).

The sex and age of the host as well as the site in the gastrointestinal tract influence the makeup and variety of the intestinal flora (Kim and Jazwinski, 2018; Cox et al., 2019). Independent of host variables, diet,

lifestyle, and medicine can alter the composition of the gut flora (Kanji et al., 2018). Studies in recent years have shown that SGAs have some antibacterial activity and can alter the gut microbiota of patients with psychosis (Nehme et al., 2018; Ait Chait et al., 2020). Olanzapine can have direct antibacterial in vitro effects against the mammalian gut bacteria Escherichia coli and Enterococcus faecalis, which are the two most common species in the intestine (E. coli: Proteobacteria; E. faecalis: Firmicutes) (Morgan et al., 2014). Similarly, chlorpromazine (Kristiansen, 1979) has shown antibacterial effects against Mycobacterium tuberculosis in vitro, and thioridazine (Thorsing et al., 2013) acts against methicillin-resistant Staphylococcus aureus. These medications targeted a more comparable pattern of species than their degree of chemical similarity would suggest (Maier et al., 2018). This raises the possibility that direct bacterial inhibition by SGAs is not merely a side effect but also a part of their molecular mechanism.

The effect of gut microbes on lipid metabolism has been supported by many in vivo and in vitro studies, and evidence of the effect of SGAs on gut microbes is gradually emerging with the advancement of microbiological research techniques. Furthermore, positive results have been achieved with therapeutic strategies that directly target the gut microbiota and related metabolites, thereby ameliorating antipsychotic-induced disorders of lipid metabolism. This certainly identifies the gut microbiome as a potential target and establishes that the potential mechanism underlying lipid metabolism disturbances associated with antipsychotics is worthy of further investigation. However, the role of the gut microbiome in antipsychotic-induced disorders of lipid metabolism has not been systematically explained. This review aims to provide a comprehensive understanding of the potential for SGAs to alter the gut microbiota and promote adverse lipid metabolism events. Keyword search on PubMed is detailed in Figure 1, based on the Preferred Reporting Item Guidelines for Systematic Reviews and Meta-Analyses.

The critical impact of SGAs on the gut microbiota: Evidence from animals and humans

Research on the microbiota in schizophrenia patients treated with SGAs is very scarce within the general microbiota literature. Several studies have investigated the effect of the gut microbiome on animal and human models (Table 1; Table 2). Mice treated with risperidone (Ridaura et al., 2013; Bahr et al., 2015b; Riedl et al., 2021) and olanzapine (Morgan et al., 2014) have increased ratios of Firmicutes to Bacteroidetes, which is one of the distinguishing features of the microbiota of obese individuals (Turnbaugh et al., 2006; Schwiertz et al., 2010; Ferrer et al., 2013). Firmicutes and Proteobacteria are the two major phyla associated with the human intestinal microbiome, constituting the majority of intestinal bacteria (approximately 90%) (Human Microbiome Project Consortium, 2012). These studies have been well replicated in humans (Bahr et al., 2015a; Yuan et al., 2018; Ma et al., 2020). Exceptions to this rule are Kao et al. (Kao et al., 2018) and Pelka et al. (Pełka-Wysiecka et al., 2019). These studies showed no significant effects of olanzapine on the gut microbiota in female rats or in women with schizophrenia. The results of studies on changes in the phylum Actinomycetes are also inconsistent. Bahr et al. (Bahr et al., 2015b) found an increase in the relative abundance of the actinomycete clade in the feces of mice

FIGURE 1
Flow chart diagram delineating search method.
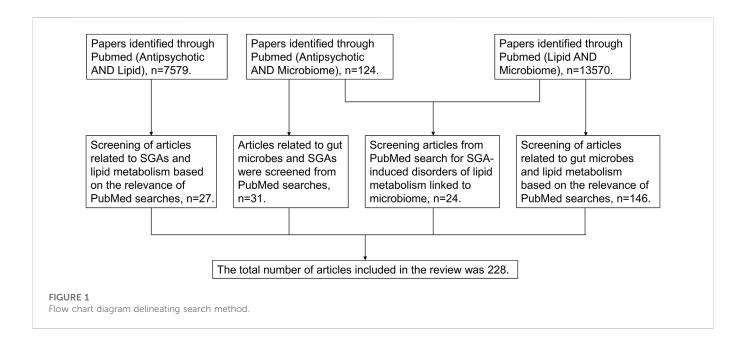
TABLE 1 Studies of SGAs and microbiota in rodents.

Subjects	Drugs	Composition	Body weight	Food intake	Key findings i: Adipose tissue ii: Liver iii: Plasma	References
Sprague Dawley rats	Olanzapine	↑: Firmicutes; ↓: Actinobacteria (significant in females), Proteobacteria, and Bacteroidetes	↑ (only in females)	↑ (mostly in females)	i: ↑ visceral fat; ↓ gene expression of SREBP-1c (in females); ↑ inflammation markers (IL-6 mRNA expression in females and 4-fold increase (insignificant) in males, CD68 expression in females and males); iii: ↓ circulating levels of ghrelin in females; ↑ hypothalamic expression of ghrelin 1a receptor mRNA in males; inflammation markers: ↑ IL-8 and IL-1β in females, ↓ IL-6 and TNFα in males	Davey et al. (2012)
Sprague Dawley rats	Olanzapine	↑: Firmicutes; ↓: Bacteroidetes	↑	↑	i: ↑ fat mass; ↑macrophage infiltration; ↑ inflammation markers (CD68 Mrna); ii: ↑ hepatic expression of FAS, SREBP-1c, and ACC iii: ↑ FFAs	Davey et al. (2013)
C57BL/6J mice	Olanzapine	↑: Erysipelotrichi, Actinobacteria, and Gammaproteobacteria; ↓: Bacteroidia	↑			Morgan et al. (2014)
C57BL/6J mice	Risperidone	↑phyla: Firmicutes and Actinobacteria; ↑genera: *Bacteroides*, Allobaculum, Turicibacter, and Aneroplasma; ↓phyla: Bacteroidetes and Proteobacteria; ↓ genera: Alistipes, *Lactobacillus*, and Akkermansia	↑			Bahr et al. (2015b)
Sprague Dawley rats	Olanzapine		↑		i: ↓ GPR43 mRNA; ii: ↑ hepatic expression of ACC mRNA; iii: ↑ acetate; inflammation markers: ↑ IL-1β and TNFα, ↓ IL-8	Kao et al. (2018)
Sprague Dawley rats	Aripiprazole	↑: Peptostreptococcaceae, Clostridiaceae, and Ruminococcaceae; ↓: Ruminococcus ₁			iii: ↑ acetate; ↑ isovalerate;	Cussotto et al. (2019)

SREBP-1c, sterol response element-binding protein-1c; CD68, Cluster of Differentiation 68; IL-6, interleukine-6; IL-8, interleukine-8; IL-1β, interleukine-1beta; TNF-α, tumor necrosis factor-alpha; FAS, fatty acid synthase; FFAs, Free fat acids; ACC, acetyl coenzyme A carboxylase; GPR43, G-protein-coupled receptor 43.

treated with risperidone, whereas Davey et al. (Davey et al., 2012) showed that the relative abundance of the actinomycete clade in mice administered olanzapine was decreased. During olanzapine treatment, the relative abundance of Erysipelotrichi and Gammaproteobacteria increased, while the relative abundance of Bacteroidia decreased (Morgan et al., 2014). Both Erysipelotrichi and Gammaproteobacteria are associated with non-alcoholic fatty liver disease (NAFLD) independent of weight gain (Spencer et al., 2011; Henao-Mejia et al., 2012). Risperidone treatment increased the relative abundance of *Allobaculum* spp. *Bacteroides* spp. *Bifidobacterium*

TABLE 2 Studies of SGAs and microbiota in humans.

Subjects	Drugs	Composition	Key findings	References
Male child with Mental Disorders	Risperidone	↓ Bacteroidetes than Firmicutes relative	i: Higher levels of KEGG-associated pathways for butyrate and propionate metabolism were found within the risperidone treatment group compared with psychiatric controls; ii: The microbiota of participants treated chronically with risperidone were enriched for KEGG orthologs affecting tryptophan metabolism	Bahr et al. (2015a)
Patients with Bipolar Disorder	Clozapine, olanzapine, risperidone, quetiapine, asenipine, ziprasodone, lurasidone, aripiprazole, paliperidone, and iloperidone	↑: Lachnospiraceae; ↓: Akkermansia and Sutterella	i: ↓ in species diversity for the SGA-treated cohort, a correlation that was stronger in SGA-treated females	Flowers et al. (2017)
Elderly (age ≥ 65) multimorbid (≥2 chronic diseases) patients	Multiple antipsychotics	strong associations with Prevotella; Desulfovibrionaceae family; Succinivibrionaceae family		Ticinesi et al. (2017)
Normal weight patients with first episode schizophrenia	Risperidone	↑: Bifidobacterium spp. And Escherichia coli; ↓: Clostridium coccoides group and *Lactobacillus* spp	i: It is speculated that the increased level of Bifidobacterium spp. could be a compensatory response to counteract weight gain and the upregulated inflammatory status; ii: Upregulated status of inflammation and oxidative stress	Yuan et al. (2018)
Patients with Mental Disorders	Clozapine, olanzapine, risperidone, quetiapine, or ziprasidone	↓: Alistipes	i: SGA-treated female patients exhibited less microbial diversity than those not treated with SGAs	Flowers et al. (2019)
Patients with Schizophrenia	Olanzapine	The microbiota in patients with the schizophrenia can be clustered into different taxonomical (Type 1, with a predominance of Prevotella, and Type 2 with a higher abundance of *Bacteroides*, Blautia, and Clostridium) and functional groups; the microbiota does not change during 6 weeks of treatment with olanzapine	i: Not associated with the weight gain that occurs in women treated. with olanzapine, as well as the treatment effectiveness; ii: Patients with schizophrenia were clustered at the level of KEGG genes, modules, and pathways	Pełka-Wysiecka et al. (2019)
Patients with Schizophrenia	Multiple antipsychotics	Both antipsychotic-naïve schizophrenics and Antipsychotic-experienced schizophrenics; ↑ family: Christensenellaceae and Enterobacteriaceae; ↑ genus: Escherichia from family Enterobacteriaceae; ↓ family: Turicibacteraceae and Pasteurellaceae. compared with Compare patients with antipsychotic-naïve schizophrenics, Antipsychotic-experienced schizophrenics: ↑ family: Peptostreptococcaceae and Veillonellaceae; ↑ genus: Megasphaera, *Fusobacterium* and SMB53		Ma et al. (2020)
Patients with Schizophrenia	Risperidone	↓: Lachnoclostridium; ↑: Rombutsia	i: Immune and inflammatory processes, such as increased levels of hs-CRP and HCY may be a compensatory response to counteract disruption of lipid metabolism	Yuan et al. (2021)
Patients with Schizophrenia	Amisulpride	↑: Dorea, Desulfovibrio, Butyricicoccus; ↓: *Actinomyces* and Porphyromonas	i: Increased IL-4 levels and decreased IL-6 levels could be a compensatory response to lipid metabolic disturbance; ii: Downregulation of butanoate metabolism might be a compensatory reaction to lipid metabolism dysfunction	Zheng et al. (2022)

KEGG, kyoto encyclopedia of genes and genomes; hs-CRP, high-sensitivity C-reactive protein; HCY, homocysteine; IL-4, interleukine-4; IL-6, interleukine-6.

spp. and *E. coli* and decreased the relative abundance of *Lactobacillus* spp. *Alistipes* spp. *Akkermansia* spp, and *Clostridium coccoides* groups (Ridaura et al., 2013; Bahr et al., 2015b; Yuan et al., 2018). It should be noted that the results of studies on the change in relative abundance of *Bacteroides* spp. Are inconsistent: one study showed an increase, while another showed no significant change (Bahr et al., 2015b; Yuan et al.,

2018). Furthermore, the abundance of *Bifidobacterium* spp. In the feces of mice treated with risperidone was negatively correlated with serum LDL levels; *E. coli* was negatively correlated with serum TG levels (Yuan et al., 2018). However, there is little evidence regarding the relationship between changes in lipid metabolism and alterations in the gut microbiota in humans following SGA treatment. Of particular interest is the search for potential probiotic bacteria such as *Akkermansia muciniphila*, which is a previously reported 'lean gut microbiota' species. *A. muciniphila*, a member of the phylum Verrucomicrobia, is the only species of the genus *Akkermansia*. *A. muciniphila* is a mucin degrader in the intestine and is significantly and negatively associated with altered fat metabolism and obesity (Henao-Mejia et al., 2012; Schneeberger et al., 2015). A significantly reduced abundance of fecal *A. muciniphila* was found in patients with bipolar disorder who were treated with a range of SGAs, such as clozapine, olanzapine, and risperidone, compared to controls (Flowers et al., 2017).

SGA-induced lipid disorders: An intimate involvement with microbiota

To describe the relationship between intestinal flora and SGA-induced lipid metabolism, Davey et al. investigated the effect of antibiotic-induced alterations in the gut microbiota on the metabolism of female rats treated with olanzapine (Davey et al., 2013). They found that clinically relevant doses of olanzapine accelerated metabolic disturbances and weight gain in C57BL/6J mice fed a high-fat diet. When the rats were treated with both olanzapine and a cocktail of broad-spectrum antibiotics, including oral neomycin, metronidazole, and polymyxin, the increases in the proportions of Firmicutes and Bacteroidetes bacteria were reversed, and this treatment reversed the olanzapine-induced metabolic disturbances and weight gain induced by high-fat diets in C57BL/6J mice. Thus, Morgan et al. conducted a further study and found that this phenomenon was consistent with a previously described study conducted under sterile conditions but that olanzapine-induced metabolic disturbances and weight gain occurred soon after gut microbial colonization (Morgan et al., 2014). Further experimental work has been conducted on mice treated with prebiotics in combination with SGAs. Coadministration of olanzapine and the prebiotic B-GOS led to a significant increase in circulating levels of TNFα in mice, which has been reported to affect lipid metabolism, elevate fecal *Bifidobacterium* spp. And reduce body weight, and these effects were not seen in response to olanzapine or B-GOS treatment alone (Kao et al., 2018). Similarly, the probiotic *A. muciniphila* was observed to have a similar effect (Huang et al., 2021). These studies suggest that intestinal microbes are necessary and sufficient for SGA-induced disruption of lipid metabolism. It is worth noting that none of these experiments were replicated in humans.

Sex differences in SGA-induced lipid disorders: A potential role of microbiota?

Accumulating evidence shows that female patients who take SGAs seem to have poorer lipid profiles than those of male patients, as well as a higher prevalence of metabolic syndrome and cardiovascular risk factors, including weight gain and dyslipidemia (Chen et al., 2020). It is noteworthy that sex-dependent differences in the host's metabolism may be associated with gut microbiota (Wu et al., 2007; Lange et al., 2017). For example, women usually have considerably higher Firmicutes:Bacteroidetes ratios as compared to men in a population-based cross-sectional investigation (Koliada et al., 2021). Given that SGA-induced lipid disturbances are frequently associated with an increased ratio of Firmicutes to Bacteroidetes, this finding raises the possibility that women are more susceptible than men to abnormal lipid metabolism (Morgan et al., 2014). Another substantial indication that men and women have different microbes is the fact that sex hormones can affect the composition of the host microbiome. Significant changes in the host gut microbiota, such as a drop in the abundance of butyrate-producing bacteria and a decline in alpha diversity, are linked to elevated levels of estrogen in pregnant women (Koren et al., 2012). These differentiations can result in a significant impact on SAG-induced changes in lipid metabolism between genders. Unfortunately, available studies are not enough to systematically explain the link between sex differences in gut microbes and sex differences in disorders of lipid metabolism caused by antipsychotics. However, this phenomenon might offer some guidance for future studies on sex differences regarding the side effects of SGAs.

Mechanisms of SGA-induced disorders of lipid metabolism mediated by the intestinal microbiota

Microorganisms and their metabolites are crucial in understanding how the gut microbiome is implicated in SGA-induced systemic lipid disorders (Skonieczna-Żydecka et al., 2019). Short-chain fatty acids (SCFAs), bile acids (BAs), and neurotransmitters are among the metabolites that the intestinal microbiota can create. Bacteroidetes and Firmicutes can create butyric acid, which accounts for approximately 20% and 60% of the total intestinal flora, respectively, while Proteobacteria and Actinobacteria produce very small amounts of SCFAs (5%–10% and 3%, respectively). Sulfate-reducing bacteria may use lactic acid to make acetic acid and hydrogen sulfide, while Veillonellaceae can convert it to propionic acid. Bacteroidetes is a phylum that can convert succinic acid to propionic acid, and its population density is related to the amount of propionic acid in the intestine (Karlsson et al., 2013). The dominant genera for BA production are *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Anaplasma*, and *Clostridium* (Krautkramer et al., 2021). In addition, *Candida*, *Streptococcus*, and *Escherichia* can produce 5-hydroxytryptamine (5-HT; serotonin) (Krautkramer et al., 2021). Approximately 36% of the small molecules in human blood are produced or modified by microbial metabolism. The total SCFA concentration in the colons of GF mice is 100 times higher than that of ordinary animals. Acetic acid is the most concentrated SCFA in organisms and is central to carbohydrate and lipid metabolic pathways (Kimura et al., 2020). Miller et al. used radioisotope analysis and showed that the main pathway for bacterial production of acetate is the Wood–Ljungdahl pathway (Miller and Wolin, 1996). Moreover, olanzapine treatment of patients with schizophrenia significantly increased plasma acetate concentrations (Kao et al., 2018). Increased levels of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways of butyric acid and propionic acid were found in a group of schizophrenia patients

treated with risperidone (Bahr et al., 2015a). Hepatocytes create primary BAs, which are then 7-dehydroxylated by intestinal bacteria to produce secondary BAs. The gut microbiome affects the composition of the BA pool, such as the primary BA/secondary BA ratio, and thus affects the function of BAs, especially the metabolism of lipids (Wahlström et al., 2016). SGAs can cause an increase in total serum BAs. Specific SGAs, including chlorpromazine (BREUER, 1965), olanzapine (Lui et al., 2009), haloperidol (Fuller et al., 1977), risperidone (Wright and Vandenberg, 2007), and quetiapine (Shpaner et al., 2008), have been reported to cause cholestasis in a small number of patients taking the medication and are unpredictable with no significant correlation between dose and the duration of administration.

The intestinal microbiota signals to enteroendocrine (EE) cells through metabolites in multiple ways, resulting in the secretion of a range of intestinal hormones, such as glucagon-like peptide 1 (GLP-1), 5-HT, gastrin, leptin, cholecystokinin (CCK) and peptide tyrosine-tyrosine (PYY) (Martin et al., 2019). First, the microbiota produces SCFAs, which signal to EE cells through free fatty acid receptors 2 or 3 (FFAR2/3) (Offermanns, 2014) or by activating nuclear histone deacetylase (HDAC) (Waldecker et al., 2008; Fellows et al., 2018; Larraufie et al., 2018). Second, secondary BAs signal to EE cells via the Takeda G-protein-coupled BA receptor TGR5 or the nuclear receptor known as farnesoid X receptor (FXR) (Wahlström et al., 2016). Numerous human and animal studies have demonstrated that leptin, ghrelin (Sentissi et al., 2008), and 5-HT levels (Bahr et al., 2015a) have a substantial positive link to aberrant lipid profiles and body mass index before and after SGA treatment in schizophrenia patients. This supports the idea that the intestinal flora and its metabolites play an important role in SGA-induced metabolic abnormalities.

Specific microorganisms can synthesize specific lipids

There appear to be distinct bacteria that are more or less related to specific classes of lipids. Gut commensal microorganisms (*Bacteroides*, *Prevotella* and *Porphyromonas*) are significantly altered by SGAs, and they can produce sphingolipids, including ceramide phospholipids and deoxy sphingolipids (Brown et al., 2019). Acute SGA treatment dramatically altered the homeostasis of central and peripheral sphingolipids (Castillo et al., 2016; Weston-Green et al., 2018). Notably, sphingolipids from bacteria were incorporated into the mammalian sphingolipid pathway (Johnson et al., 2020). The probiotic *Bacteroides* has also been shown to produce the endothelin-like molecule N-acyl-3-hydroxypalmitoyl-glycine (commendamide) (Cohen et al., 2015; Lynch et al., 2017). Furthermore, olanzapine-induced metabolic effects have been shown to be dependent on the endogenous cannabinoid system (Abolghasemi et al., 2021). Everard et al. showed that treating obese mice with *A. muciniphila* increased intestinal 2-oleoylglycerol (2-OG), 2-arachidonoylglycerol (2-AG) and 2-palmitoylglycerol (2-PG) levels (Everard et al., 2013). However, a recent study reported that *A. muciniphila* exerted its beneficial effects on metabolism independent of general changes in plasma endocannabinoidome mediators (Depommier et al., 2021). The gut microbiota–endocannabinoid axis is a key topic in the studies

listed above, and it is likely to be a new target for SGA-induced lipid metabolism disorders.

Central mechanism

Current evidence suggests that hyperphagic effects are responsible for a large percentage of the observed aberrations in lipid profiles, and there is a lack of satiety in both human and animal models in the presence of SGAs (Hartfield et al., 2003; Huang et al., 2020). The diversity of the gut flora is vital for appetite and metabolism regulation. Different gut bacteria and metabolites influence the gut's ability to perceive nutrients, influencing the host's appetite and energy metabolism (Oliphant and Allen-Vercoe, 2019). This is where the gut–brain axis becomes active. The gut–brain axis is a fundamental mechanism that links biochemical signals from the gastrointestinal tract to brain function (Carabotti et al., 2015). A sophisticated network of neurons regulates energy homeostasis in the host. Two types of neurons are particularly important for appetite control: neurons that express the neuropeptide proopiomelanocortin (POMC) (Baldini and Phelan, 2019) and those that express neuropeptide Y/agouti-related peptide (NPY/AgRP) (Han et al., 2018). These neurons interact with each other to form a switch that instantly adjusts appetite (Quarta et al., 2021). Among them, POMC neurons promote satiety, while AgRP neurons increase appetite.

On the one hand, gastrointestinal hormones affect the balance of the POMC/AgRP system, which controls appetite. Leptin (Endomba et al., 2020), CCK (Fan et al., 2004), PYY (Loh et al., 2015), GLP-1 (Teff et al., 2013), and 5-HT (Sohn et al., 2011; Bonn et al., 2013) activate POMC neuronal activity via receptors in the hypothalamus and inhibit NPY in AgRP neurons, sending appetite suppressant signals and regulating energy homeostasis and metabolism. Ghrelin is the only known gut hormone that promotes appetite by directly activating AgRP neurons and increasing the inhibitory effect of AgRP neurons on POMC neurons (Lage et al., 2010; Varela et al., 2011). On the other hand, the intestinal flora metabolites SCFAs and BAs can also influence appetite via the gut–brain axis. An increase in acetate production activates the parasympathetic nervous system, leading to an increase in gastrin secretion, which promotes host appetite (Perry et al., 2016). SCFAs also have the potential to enter the circulation, cross the blood–brain barrier, and directly affect the central nervous system (Morrison and Preston, 2016). In addition, BAs can reach the hypothalamus and are highly correlated with circulating BA levels, which can reach the hypothalamus via passive diffusion, causing a brief increase in hypothalamic BA concentrations and triggering the expression of the AgRP/NPY neuronal membrane receptor TGR5, which in turn regulates appetite (Perino et al., 2021). It is worth noting that an imbalance in the amount of proinflammatory pathogenic bacteria can compromise intestinal wall integrity, affecting brain–gut axis transmission (Küme et al., 2017; Tilg et al., 2020). A study showed that the mRNA levels of NPY and AgRP were significantly increased in the hypothalamus of olanzapine-administered rats and were considerably lower than those in normal animals (Zhu Z et al., 2022). Some notable causes include several of these mechanisms affecting neuronal function through the gut–brain axis, which leads to hyperphagia and results in abnormal lipid profiles. Interestingly, this study showed that olanzapine-induced increases in weight gain percentage (WG%) occurred only when the vagus nerve was intact, while the negative effects of olanzapine-induced increases in white

adipose tissue percentage (WAT%) and decreases in brown adipose tissue percentage (BAT%) were reversed by the disruption of the gut microbiota–brain axis (vagotomy), suggesting that an intact gut microbiota–brain axis may be necessary for olanzapine-induced disruption of lipid metabolism.

Lipopolysaccharide (LPS), a component of the outer membrane of most Gram-negative bacteria, is released upon bacterial cell death and enters the circulation through a "leaky gut", resulting in increased levels of LPS in the blood (known as endotoxemia, which is a leading cause of metabolic diseases, such as insulin resistance, and is promoted by increased IL-6 and tumor necrosis factor (TNF) (Tilg et al., 2020)), which acts as a powerful stimulator of host immunity (Park and Lee, 2013). LPS is detected by Toll-like receptor 4 (TLR4) on the immune cell surface, resulting in the release of numerous cytokines and chemokines (Rhee, 2014). LPS can also interact directly with lipid molecules. All lipoproteins can bind to LPS and neutralize its toxicity *in vitro* and *in vivo* (Barcia and Harris, 2005).

Peripheral tissue

SCFAs

SCFAs are used as a carbon source for the production of important endogenous host metabolites, such as fat and cholesterol (Besten et al., 2013). SCFAs produced by the intestinal flora are rapidly absorbed by colonic cells, due in part to monocarboxylate transporters, including the proton-coupled monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter 1 (SMCT1) (Dalile et al., 2019). The principal substrates for lipid synthesis in rat colonic epithelial cells, which convert SCFAs to acetyl coenzyme A (CoA), are acetate and butyrate (Zambell et al., 2003). CoA generates energy through the tricarboxylic acid cycle and produces palmitic acid under the action of the cytoplasmic enzyme system, which can be transferred to mitochondria to lengthen the carbon chain and form triglycerides with other substances stored in adipose tissue. In contrast, SCFAs that are not digested in colon cells enter the portal circulation of the liver through the basolateral membrane and provide substrates for hepatocyte energy metabolism. Carbohydrate-responsive element-binding protein (ChREBP) plays a key role in this process (Iizuka et al., 2020). A member of the acetyl-CoA synthetase short-chain family, encoded by Acss2, is induced by ChREBP and converts acetate to acetyl-CoA, which is used as a substrate for lipogenesis (BERG, 1956). Thus, regulating lipogenic gene expression and hepatic acetyl-CoA production from gut microbial acetate by inhibiting hepatic ChREBP is expected to prevent SGA-induced TG accumulation by inhibiting lipogenic gene expression and hepatic acetyl-CoA production. SCFAs are also involved in the biosynthesis of cholesterol and fatty acids in hepatocytes (Dalile et al., 2019). Chen et al. performed radiolabeling studies and showed that acetate was involved in the increase in *de novo* fat synthesis. Furthermore, antibiotic-treated mice showed reduced *de novo* fat synthesis (Kindt et al., 2018).

In addition, SCFAs are signaling molecules that regulate host-related functions mainly through two signaling pathways: the HDAC and G protein-coupled receptor signaling pathways. SCFAs have been shown to bind to the G protein-coupled receptors GPR43/FFAR2 and Gpr41/FFAR3 (Kimura et al., 2020), leading to further activation of downstream signaling cascades, including the phospholipase C (PLC), mitogen-activated protein kinase (MAPK), phospholipase A2 (PLA2) and nuclear factor-κB (NF-κB) pathways. Acetate inhibits insulin-mediated fat accumulation and improves lipid and glucose metabolism *via* GPR43.

Mice lacking GPR43 were obese on a normal diet, whereas mice specifically overexpressing GPR43 in adipose tissue remained lean even when fed a high-fat diet. Both types of mice recovered under sterile conditions or after being treated with antibiotics (Kimura et al., 2013). GPR41 has been shown to regulate host energy homeostasis in a gut microbiota–dependent manner. Mice with knockout of the GPR41 gene exhibited a leaner body weight, but this difference was not observed in GF mice (Samuel et al., 2008). SCFAs also activate AMP-activated protein kinase (AMPK), a downstream signal of the G-protein-coupled receptor signaling pathway, and AMPK activation increases peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) expression in adipose tissue and skeletal muscle (Taylor et al., 2005; Wan et al., 2014; Yan et al., 2016). In addition, PGC-1α regulates the transcriptional activity of peroxisome proliferator-activated receptor α (PPARα) and peroxisome proliferator-activated receptor γ (PPARγ) (Muoio et al., 2002; Lin et al., 2005). Butyrate and propionate can activate PPARγ (Alex et al., 2013). Activation of liver and adipose tissue PPARγ by SCFAs regulates lipid metabolism by increasing energy expenditure, reducing inflammation in adipose tissue, improving insulin sensitivity, reducing body weight, and decreasing hepatic TG accumulation (Besten et al., 2015). A study in fish showed that the effects of olanzapine on lipid metabolism may be related to the regulation of the gut microbiota–SCFA–PPAR signaling pathway (Chang et al., 2022). The gut microbiome was significantly altered in carp that were administered olanzapine, as evidenced by an increase in the abundance of SCFA-producing bacteria, which led to an increase in the production of SCFAs. In addition, many genes that are components of the PPAR signaling pathway were significantly altered; specifically, the mRNA levels of genes related to lipid synthesis (including PPARγ, fatty acid synthase (FAS), and SREBP1) were significantly increased, and lipolysis-related genes (such as hormone-sensitive lipase (HSL) and PPARα) were significantly decreased. The activated AMPK signaling pathway can also promote the expression of HSL and adipose triglyceride lipase (ATGL), which promote lipolysis (Cantó and Auwerx, 2010; Deng et al., 2020; Guo et al., 2020; Tang et al., 2020). Jocken et al. performed *in vitro* experiments with a human white adipocyte model (human multipotent adipose tissue-derived stem (hMADS) cells). Acetate was found to be the main driver of the antilipolytic effect of SCFAs and attenuated HSL phosphorylation in hMADS adipocytes in a Gi-coupled manner (Jocken et al., 2017). This is reminiscent of the fact that the effect of SGAs on AMPK may also be an indirect consequence of the activation of AMPK by SCFAs in peripheral tissues. Indeed, olanzapine can reduce AMPK phosphorylation and activation in hepatocytes and 3T3-L1 cells, accompanied by a concomitant increase in SREBP-dependent lipid synthesis (Oh et al., 2011; Li et al., 2016). Interestingly, acetate supplementation did not attenuate olanzapine-induced weight gain in mice but appeared to increase it (Kao et al., 2019a). This concept of SCFA-induced weight gain appears to be consistent with the olanzapine-induced increase in plasma acetate (Kao et al., 2018).

BAs

BAs bind to FXR and TGR5 in the host and regulate lipid and energy metabolism (Chiang and Ferrell, 2019). FXR is a transcription factor that binds to the promoter region and induces the expression of multiple target genes and is expressed in the liver, ileum, kidney, and other tissues (Lefebvre et al., 2009; Teodoro et al., 2011). The most potent ligand for FXR is chenodeoxycholic acid (CDCA), followed by cholic acid (CA), deoxycholic acid (DCA), and lithocholic acid (LCA), all of which are FXR agonists. CDCA is converted to ursodeoxycholic

acid in humans through a sequence of processes, and it does not activate FXR but rather inhibits FXR activity (Wang et al., 1999; Mueller et al., 2015). In addition, Sayin et al. identified two natural FXR antagonists: the taurine-conjugated murine BAs tauro-α-muricholic acid (TαMCA) and tauro-β-muricholic acid (TβMCA) (Sayin et al., 2013). TGR5 is a binding G-protein-coupled receptor expressed in tissues such as the intestine, liver, and brown–white adipose tissue. TGR5 is mainly activated by the secondary BAs LCA and DCA (Maruyama et al., 2002; Kawamata et al., 2003).

FXR-deficient animals had increased hepatic and serum TG and cholesterol levels (Sinal et al., 2000). This finding indicates that FXR is required for lipid metabolism and energy homeostasis (Trauner et al., 2010). Reduced sterol-response element-binding protein-1c (SREBP-1c) expression caused by natural or synthetic FXR agonists *via* the FXR-SHP (small heterodimer partner) pathway could explain the inhibitory effect of BAs on TG production (Watanabe et al., 2004). In addition, Caron et al. used immortalized human hepatocyte (IHH) and HepaRG cell lines, which are glucose-responsive human hepatocyte lines, to show that the activation of FXR inhibits the transcriptional activity of ChREBP in human hepatocytes (Caron et al., 2013). BAs can also induce the expression of the human PPARα gene, which is a nuclear receptor that controls lipid and glucose metabolism and exerts anti-inflammatory effects *via* FXR (Pineda Torra et al., 2003). The activation of FXR has been shown to induce a decrease in serum apolipoprotein (Apo) CIII concentrations, leading to the amelioration of TG-rich remnant lipoprotein metabolism to reduce serum TG levels and cardiovascular risk profiles (Claudel et al., 2003). The FXR signaling pathway in mice and humans is significantly affected by SGAs. At present, pharmacological therapies that target FXR in combination with SGAs are still needed to translate the positive findings of these studies into practical outcomes. Exposure of a mouse precision-cut liver slice (PCLS) model to chlorpromazine significantly altered cholesterol and BA cellular transport regulated by FXR and BA regulation of glucose and lipid metabolism *via* FXR (Szalowska et al., 2013). In addition, a study also showed the downregulation of FXR targets such as Bsep, Mdr3, Ntcp, and Cyp8b1. This finding was consistent with that observed in chlorpromazine-treated HepaRG cells (Anthérieu et al., 2013). As a next step, more experiments on the effect of SGAs on FXR are needed to further understand the beneficial effects of chlorpromazine.

TGR5 has also been shown to be a BA-responsive receptor involved in host lipid metabolism. In muscle and brown adipose tissue, TGR5 may play a role in energy homeostasis by promoting intracellular thyroid hormone activity and thereby increasing energy expenditure (Watanabe et al., 2006). In addition, TGR5 has been shown to activate PPARα and PGC-1α to increase mitochondrial oxidative phosphorylation and energy metabolism (Chiang and Ferrell, 2020). However, there are limited data on changes in TGR5 receptor activity in schizophrenia patients during the use of SGAs.

GLP-1

Ishøy et al. published the first clinical data supporting the use of the GLP-1 agonist liraglutide to treat clozapine-induced lipid profile disturbances and weight gain in schizophrenia (Ishøy et al., 2013). Consistent with this study, Larsen et al. and Siskind et al. demonstrated that GLP-1 agonists could be effective in reducing clozapine- or olanzapine-induced lipid metabolism disorders (Kouidrat and Amad, 2019). GLP-1, a glucose-dependent incretin, plays a crucial role in lipid metabolism and body weight maintenance

by binding to the GLP-1 receptor (GLP-1R). Many human tissues, including the pancreas, liver, muscle, fat, gastrointestinal tract, heart, and brain, express GLP-1R (Campbell and Drucker, 2013). When bound to GLP-1, GLP-1R acts through its coupled G protein (Gαs) in pancreatic β-cells, activating adenylyl cyclase and increasing the intracellular levels of cyclic adenosine monophosphate (cAMP) (Doyle and Egan, 2007); this increase in cAMP exerts a series of effects. The activation of factors (protein kinase A (PKA) (Béguin et al., 1999) and exchange protein directly activated by cAMP (EPAC) (Kang et al., 2008)) leads to calcium influx, increased transcription of the proinsulin gene, and the stimulation of insulin secretion. In addition, GLP-1R may regulate pancreatic β-cell metabolism by activating the phosphoinositide 3-kinase (PI3K)/AKT (protein kinase B)/mTOR (mammalian target of rapamycin) and MAPK signaling pathways (Rowlands et al., 2018). The binding of GLP to GLP-1R in adipocytes activates the adenylyl cyclase (AC)/cAMP signaling pathway, regulates the apoptosis and proliferation of preadipocytes through various cellular signaling pathways, such as extracellular signal–regulated kinase (ERK), protein kinase C (PKC), and AKT, and alters the expression of PPARγ and its target genes (Challa et al., 2012; Chen et al., 2017). Furthermore, by reducing macrophage infiltration in adipose tissue, GLP-1 can directly block the inflammatory signaling pathway, improving insulin resistance, lowering liver fat levels, and considerably alleviating NAFLD (Blaslov et al., 2014). This explains how GLP-1R might decrease hepatic substrate supply (e.g., glucose and non-esterified fatty acids (NEFAs)) by affecting adipose tissue, which may be partially responsible for the overall effect. GLP-1R-based treatment of metabolic diseases has been reported to act on hepatocyte lipid metabolism through PI3K, type 1 protein phosphatase (PP-1), and PKC (Redondo et al., 2003). Interestingly, a study showed that liraglutide ameliorated hepatocyte steatosis by inducing autophagy through the AMPK/mTOR pathway (He et al., 2016). Additionally, GLP-1 may promote hepatocyte survival by downregulating microRNA-23, resulting in increased expression of PGC-1α and uncoupling protein 2 (UCP2) (Wang C et al., 2015). Recent studies have shown that GLP1/GLP-1R signaling is involved in the effect of brexpiprazole, a new multitarget antipsychotic drug (APD) approved by the US FDA in 2015 that induces disorders of glucose and lipid metabolism (Li et al., 2021). Brexpiprazole administration significantly reduced the protein and mRNA levels of GLP1 in the pancreas and small intestine by inhibiting Ca^{2+}/calmodulin-dependent kinase IIα (CaMKIIα), AMPK, and β-catenin. Brexpiprazole administration also caused islet dysfunction and decreased GLP-1R, PI3K, and IRβ expression in the pancreas. Cotreatment with liraglutide and brexpiprazole is an effective strategy for certain aberrant metabolisms.

Leptin

Leptin was found to be involved in lipid metabolism and energy balance by mediating certain signaling pathways. Leptin inhibits acetyl-CoA carboxylase (ACC) activity by activating AMPK in skeletal muscle, thereby stimulating the oxidation of fatty acids (Minokoshi et al., 2002). Consistently, another study revealed that the activation of AMPK can have a therapeutic effect on metabolic syndrome only if leptin is present and active (Stockebrand et al., 2013). In addition, p38 MAPK may also contribute to the effect of leptin on fatty acid oxidation (Dardeno et al., 2010). In non-adipose tissues, leptin may promote fatty acid oxidation by activating PPARα-induced CoA expression *via* signal transducer and activator of transcription 3

(STAT3) (Unger et al., 1999). Maya et al. found that leptin could regulate lipid metabolism and inflammation by modulating the PI3K/Akt/mTOR pathway (Maya-Monteiro and Bozza, 2008). Consistent with this report, Schmidt et al. found that olanzapine simultaneously upregulated the mTOR pathway and downstream signaling cascades, including the activation of mTORC1, in mice (Schmidt et al., 2013). mTORC1 activation interferes with lipid and energy metabolism, leading to the upregulation of lipid biosynthesis and the accumulation of TGs. Furthermore, activation of the mTOR pathway inhibits autophagy, thereby increasing intracellular lipid accumulation (Zhuo et al., 2022). Enhanced mTOR activity disrupts hepatic lipid homeostasis by regulating the expression of the transcription factor SREBP-1c (Takashima et al., 2009).

Strategies for modifying the gut microbiome to ameliorate SGA-induced disorders of lipid metabolism

Pharmacological interventions

To date, the mechanisms of SGA-induced metabolic changes have not been thoroughly investigated. However, in clinical treatment, side effects of SGAs on lipid metabolism can usually be suppressed by other drugs, and some interventions have yielded significant results. Researchers found that metformin, a biguanide antihyperglycemic agent, had a positive effect on the lipid profile, insulin resistance, and body weight in patients with schizophrenia, which has been supported by animal (Zhu W et al., 2022) and human models (Wu et al., 2016; Vancampfort et al., 2019; Jiang et al., 2020). Interestingly, the intestinal flora plays a vital role in the positive effects of metformin. Luo et al. (Luo et al., 2021) and Wang et al. (Wang et al., 2021) found that metformin not only prevented olanzapine-induced disruption of the lipid profile and hepatic histopathological changes but also partially reversed olanzapine-induced alterations in the gut microbiota and helped correct peripheral and central satiety-related neuropeptide disorders. This finding demonstrated that the gut–brain axis is a mediator by which metformin ameliorates SGA-induced metabolic dysfunction. Statins are also considered a potential preventive and therapeutic approach to reduce SGA-induced weight gain and dyslipidemia in patients with schizophrenia. It has been reported that pravastatin (Vincenzi et al., 2014), atorvastatin (Ojala et al., 2008), lovastatin (Ghanizadeh et al., 2014), rosuvastatin (Hert et al., 2006), or simvastatin (Tajik-Esmaeeli et al., 2017) in combination with SGAs can reduce TC, LDL cholesterol, and TG levels in patients with schizophrenia. Animal studies have shown that statins improve SGA-induced metabolic disturbances partly due to statin-mediated modulation of BAT activity (Liu et al., 2020) and inhibition of the hepatic mTOR signaling pathway (Liu et al., 2019). Interestingly, statins were also recently shown to improve the gut microbiota, which seems to partially explain the associated clinical improvements (Kim et al., 2019; Vieira-Silva et al., 2020).

Non-pharmacological interventions

New biological therapeutic strategies, including probiotics, prebiotics, gut hormone, and fecal microbiota transplantation (FMT), are being explored to directly target the gut microbiota and its metabolite products

to improve SGA-induced dyslipidemia. Probiotics have been shown to play a vital role in lipid homeostasis in the host (Table 3). However, there have been few studies on the effects of probiotics and prebiotics on SGA-induced changes in lipid metabolism and energy. Tomasik et al. discovered that probiotics and prebiotics could alleviate SGA-induced gastrointestinal distress (Tomasik et al., 2015). However, the effects of probiotics and prebiotics on SGA-induced changes in lipid metabolism are unclear and controversial because the effects of these factors on lipid metabolism are strain and population specific. For example, the probiotic A. muciniphila (Huang et al., 2021) or prebiotic B-GOS (Kao et al., 2018) can partially reverse olanzapine-induced disturbances in the gut microbiota and lipid metabolism in rats. The probiotic mixture VSL#3, a mixture of eight different bacterial probiotic species, was shown to attenuate olanzapine-induced body weight gain, uterine fat deposition, and dyslipidemia (Dhaliwal et al., 2019). Importantly, while their effectiveness has been relatively well documented in animal studies, translation to humans has sometimes shown controversy. Kao et al. found that B-GOS supplementation did not affect SGA-induced weight gain or changes in circulating metabolic markers, contrary to their observations in rats (Kao et al., 2019b). Yang et al. reported that the addition of probiotics, including Bifidobacterium and Lactobacillus, was not sufficient to reduce weight gain in patients with schizophrenia, nor did it significantly improve lipid profiles (Yang et al., 2021). In comparison, the combined use of probiotics and dietary fiber was effective in reducing olanzapine-induced weight gain without any apparent adverse effects while maintaining the desired psychopathological effect (Liu et al., 2021; Huang et al., 2022a; Huang et al., 2022b). Therefore, more randomized controlled trials in humans are needed to translate beneficial findings in animals. Indeed, A. muciniphila has been shown to be safe and effective in human trials, and pasteurized A. muciniphila is more effective than live A. muciniphila (Depommier et al., 2019). In addition, the gut hormone GLP-1 has demonstrated the potential to improve SGA-induced disorders of lipid metabolism. The combination of liraglutide, a GLP-1 receptor agonist, and SGAs has potential benefits on body weight and lipid metabolism in patients with schizophrenia, but patients must receive daily subcutaneous injections and have a relatively high rate of adverse events (Whicher et al., 2019). In contrast, FMT, which is being researched as an alternative to SGAs (Settanni et al., 2021), lacks experimental data to demonstrate its potential in SGA-induced metabolic disorders.

Future perspectives

Long-term use of SGAs can cause weight gain and increase lipids, which can lead to an increased chance of patients suffering from metabolic syndrome, thereby increasing the risk that they will develop hypertension and cardiovascular and cerebrovascular diseases. During this process, the microbiome is both essential and sufficient, and several pathways involved in lipid metabolism have been postulated (Figure 2). First, SGAs directly inhibit the growth of microbial species that produce specific lipids (e.g., endogenous cannabinoids, and cholesterol). Second, SCFAs and BAs produced by the gut microbiota can regulate gut hormones such as CCK, PYY, GLP-1, and 5-HT. On the one hand, these signaling molecules can stimulate

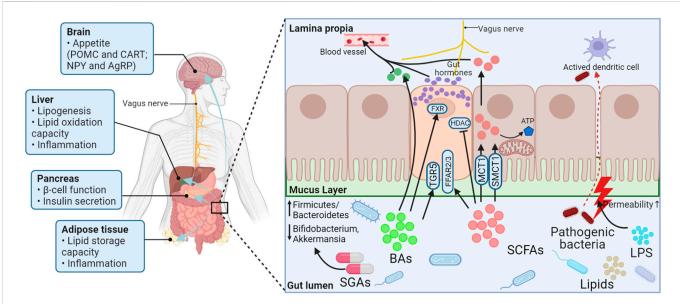**TABLE 3 Effects of prebiotic supplementation on host lipid metabolism.**

Subject	Models	Probiotics	Implicated microbiota	Changes in lipid profile			References
				Serum	Liver	Fecal	
Sprague-Dawley rats	HCD	*Lactobacillus* plantarum MA2	↑: Lactic acid bacteria and Bifidobacterium	↓: TC, LDL-C, and TG	↓: TC and TG	↑: TC and TG	Wang et al. (2009)
Sprague-Dawley rats	HCD	*Lactobacillus* acidophilus 4356	↑: Lactobacilli and Bifidobacteria	↓: TC, LDL-C, TAG	↓: TC and TAG		Huang et al. (2010)
Sprague-Dawley rats	HFD	Bifidobacteria L66–5, L75–4, M13-4 and FS31-12		↓: TC and TG	↓: TC and TG		Yin et al. (2010)
Sprague-Dawley rats	HCD	*Lactobacillus* plantarum 9–41-A, *Lactobacillus* fermentum M1-16	↑: *Lactobacillus* and Bifidobacterium	↓: TC, LDL-C, TG	↓: TC and TG	↓: TC	Xie et al. (2011)
Sprague-Dawley rats	HFD	Bifidobacterium pseudocatenulatum SPM 1204, Bifidobacterium longum SPM 1205, and Bifidobacterium longum SPM 1207	↑: Lactobacilli	↓: TC, LDL-C, HDL-C, and TG			An et al. (2011)
Wistar rats	HFD	Lactobacilli LIP-1, MG9-2, and E7301		↑: HDL-C; ↓: TC, LDL-C, and TG	↓: TG	↑: TC	Wang et al. (2012)
Sprague-Dawley rats	HFD	*Lactobacillus* plantarum LS/07, *Lactobacillus* plantarum Biocenol LP96		L. LS/07; ↓: TC and LDL-C; L.LP96; ↓: TG and VLDL	no significant change	↑: TC and TG	Salaj et al. (2013)
Sprague-Dawley rats	HCD	*Lactobacillus* plantarum NS5, *Lactobacillus* delbrueckii subsp	↑: *Bacteroides*; ↓: Clostridium	↑: ApoA-I; ↓: TC, HDL-C, Apo-B, FFAs	↓: TC and TG		Hu et al. (2013)
C57BL/6J mice	HFD + HCD	*Lactobacillus* plantarum KY1032, *Lactobacillus* curvatus HY7601		L. KY1032; ↑: TG; L. HY7601; ↑: TG; ↓: TC; L. KY1032 + L. HY7601; ↑: TG; ↓: TC and LDL-C	L. HY7601; ↓: TC, TG, and FFAs; L. KY1032 + L. HY7601; ↓: TC, TG, and FFAs	↑: TC and TG	Yoo et al. (2013)
C57BL/6J mice	HFD	*Lactobacillus* curvatus HY7601 and *Lactobacillus* plantarum KY1032	↓: Proteobacteria	↓: TC			Park et al. (2013)
C57BL/6J mice	HFD	*Lactobacillus* rhamnosus GG		ORO staining	ORO staining		Kim et al. (2013)
Human	overweight children	Synbiotic capsules	↑: *Lactobacillus*	↓: TC, LDL-C, and TG			Safavi et al. (2013)
C57BL/6J mice	HFD	*Lactobacillus* casei NCDC 19		↓: TC and LDL-C			Rather et al. (2014)
Albino rats	HCD	*Lactobacillus* reuteri LR6		↓: TC, LDL, TG			Singh et al. (2015)
Wistar rats	HCD	Kluyveromyces marxianus M3		↑: HDL-C; ↓: TC, LDL-C, and TG	↑: HDL-C; ↓: TC, LDL-C, and TG		Xie et al. (2015)
C57BL/6J mice	HFD	*Lactobacillus* paracasei CNCM I-4270, L. rhamnosus I-3690 and Bifidobacterium animalis subsp. lactis I-2494	↑: *Lactobacillus* paracasei CNCM I-4270, L. rhamnosus I-3690 and Bifidobacterium	HE staining	HE staining		Wang J et al. (2015)
Human	overweight adults	*Lactobacillus* curvatus HY7601 and *Lactobacillus* plantarum KY1032		↑: ox-LDL			Jung et al. (2015)
Sprague-Dawley rats	HCD	*Lactobacillus* plantarum Lp3	↑: *Lactobacillus* and Bifidobacterium; ↓: Escherichia coli	↓: TC, LDL-C, and TG	↓: TC and TG	↑: TC and TBA	Ding et al. (2017)
Wistar rats	HFD	*Lactobacillus* plantarum YS5		↑: HDL-C; ↓: TC, LDL-C, and TG			Nami et al. (2019)
Wistar rats	HCD			↓: TC, LDL-C, and TG	↓: TC		

TABLE 3 (*Continued*) Effects of prebiotic supplementation on host lipid metabolism.

Subject	Models	Probiotics	Implicated microbiota	Changes in lipid profile			References
				Serum	Liver	Fecal	
		Lactobacillus fermentum PD2 and PH5	↑: *Lactobacillus*; ↓: coliforms				Thakkar et al. (2020)
Sprague-Dawley rats	HFD	*Lactobacillus* plantarum LS/07	↑: Lactobacilli; ↓: Coliforms	↓: TC, LDL-C, ox-LDL			Hijova et al. (2020)
Human	hypercholesterolemia	Lactoplantibacillus plantarum strains (CECT7527, CECT7528, and CECT7529)		↓: TC and LDL-C			Guerrero-Bonmatty et al. (2021)
Human	overweight adults	*Lactobacillus* plantarum K50	↑: *Lactobacillus* plantarum; ↓: Actinobacteria	↓: TC and TG			Sohn et al. (2021)
Human	overweight adult women	Bifidobacterium lactis UBBLa-70		↑: arginine, glutamine, and 2-oxoisovalerate; ↓: glycerol			Crovesy et al. (2021)

HCD, high cholesterol diet; HFD, high fat diet; TC, total cholesterol; TG, triglycerides; LDL-C, low density liptein cholesterol; HDL-C, high density liptein cholesterol; ox-LDL, oxidized low-density lipoprotein; LDL, low-density lipoprotein; TBA, total bile acids; TAG, triacylglycerols; VLDL, very-low-density lipoprotein; ORO, staining, Oil Red O staining; HE, staining, hematoxylin-eosin staining; Apo-B, apolipoprotein B; ApoA-I, apolipoprotein A-I; FFAs, Free fat acids.
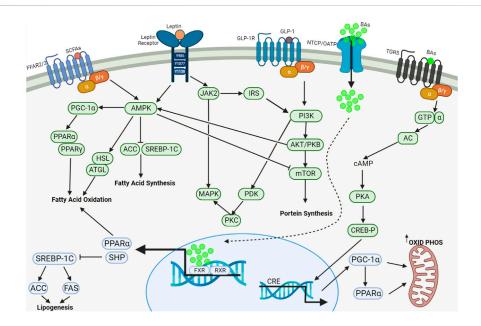

FIGURE 2
Schematic presentation of the potential mechanism of lipid metabolism disorders secondary to SGA treatment based on the gut microbiota. Treatment with SGAs may increase the relative ratio of Firmicutes to Bacteroidetes bacteria, As well as decrease the relative abundance of Bifidobacterium and Akkermansia muciniphila. The products of the gut microbiota (lipids, LPS, SCFAs, and BAs) change as a result of this transformation. SCFAs activate FFAR2/3 or HDAC. BAs send signals to EE cells through TGR5 or nuclear FXR, allowing EE cells to synthesize and secrete various gut hormones. LPS, SCFAs, BAs, and gut hormones are important players in interorgan crosstalk by affecting appetite, regulating gut integrity, and improving liver, pancreas, and adipose tissue function and lipid metabolism. POMC, Proopiomelanocortin; CART, Cocaine- and amphetamine-regulated transcript; NPY, Neuropeptide Y; AgRP, Agouti-related peptide; FXR, Farnesoid X receptor; HDAC, Histone deacetylase; ATP, Adenosine triphosphate; TGR5, Takeda G protein-coupled receptor 5; FFAR2/3, Free fatty acid receptors 2/3; MCT1, Proton-coupled monocarboxylate transporters 1; SMCT1, Sodium-coupled monocarboxylate transporters 1; BAs, Bile acids; SCFAs, Short-chain fatty acids; LPS, Lipopolysaccharide; SGAs, Second-generation antipsychotics.

the vagus nerve or be carried into the brain to affect appetite *via* the gut–brain axis; on the other hand, they can regulate lipid metabolism *via* peripheral signaling pathways (Figure 3).

However, many unanswered questions remain. Which components of the gut microbiota and host metabolism are chiefly associated with schizophrenia? Are the microbiota changes observed in schizophrenia treated with SGAs secondary to SGA treatment? What are the metabolic side effects of SGAs and their impact on the microbiota? Do changes in the gut microbiota affect the efficacy of SGAs? To answer these questions, further experimental data are needed. This will lead to improved schizophrenia treatment options, individualized therapy, and the prediction and mitigation of side effects.

FIGURE 3
Regulation of lipid metabolism by SCFAs, BAs, leptin and GLP-1. The major signaling pathways including AMPK, MAPK, PI3K/Akt/mTOR and cAMP/PKA/CREB-P work systematically in concert to regulate fatty acid oxidation, fatty acid synthesis, protein synthesis and mitochondrial oxidative phosphorylation and energy metabolism. SCFAs: Short-chain fatty acids; GLP-1: Glucagon-like peptide 1; BAs, Bile acids; FFAR2/3, Free fatty acid receptors 2/3; GLP-1R, GLP-1 receptor; NTCP, Na$^+$-taurocholate cotransporting polypeptide; OATP, Organic anion transporting polypeptide; TGR5, Takeda G protein-coupled receptor 5; AMPK, AMP-activated protein kinase; PGC-1α, Peroxisome proliferator-activated receptor-γ coactivator 1α; PPARα, Peroxisome proliferator-activated receptor α; PPARγ, Peroxisome proliferator-activated receptor γ; HSL, Hormone-sensitive lipase; ATGL, Adipose triglyceride lipase; ACC, Acetyl coenzyme A carboxylase; SREBP-1c, Sterol response element-binding protein-1c; JAK2, Janus kinase-2; MAPK, Mitogen-activated protein kinase; IRS, Insulin receptor substrate; PDK, Phosphoinositide-dependent protein kinase; PKC, Protein kinase C; PI3K, Phosphoinositide 3-kinase; AKT, Protein kinase B (PKB); mTOR, Mammalian target of rapamycin; GTP, Guanosine triphosphate; AC, Adenyl cyclase; cAMP, Cyclic adenosine monophosphate; PKA, Protein kinase A; CREB-P, Phosphorylated CREB (cAMP-response element-binding protein); FXR, Farnesoid X receptor; RXR, Retinoid X receptor; CRE, cAMP response element; SHP, Small heterodimer partner; FAS, Fatty acid synthase.

Current strategies to modulate the gut microbiome to improve SGA-induced lipid metabolism disturbances are particularly promising. Prebiotics, probiotics, and FMT have achieved certain curative effects in animal experiments. As a next step, more randomized controlled trials into humans are needed to translate the beneficial findings in animals. It is important to observe changes in host lipid metabolism after concurrent administration of SGAs and the abovementioned treatments. Compared with prebiotic therapy and other drug interventions, probiotic treatment offers superior specificity and safety. To develop this specific microbial therapeutic approach, a better understanding of the precise role of microbes in SGA-related lipid metabolism and elucidation of the linkages between specific microbiota and lipid profiles of the gastrointestinal tract will be needed. Furthermore, proper exercise and diet must not be overlooked.

Author contributions

HC collected the literature and wrote the article. TC conducted the preliminary research. BZ, HC supervised the study. All authors reviewed and approved the manuscript.

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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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