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Animal models, gut microbiota and brain diseases

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Animal models, gut microbiota and brain diseases

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Table of contents

06 Editorial: Animal models, gut microbiota and brain diseases
Yu Wang, Jin Song, Chang Liu, Niraj Kumar Jha and Kavindra Kumar Kesari

11 Causal relationship between gut microflora and dementia: a Mendelian randomization study
Jinjie Fu, Yuan Qin, Lingyong Xiao and Xiaoyu Dai

25 The role of the peripheral system dysfunction in the pathogenesis of sepsis-associated encephalopathy
Jingyu Zhang, Shuangli Chen, Xiyou Hu, Lihong Huang, PeiYong Loh, Xinru Yuan, Zhen Liu, Jinyu Lian, Lianqi Geng, Zelin Chen, Yi Guo and Bo Chen

42 Association between sleep-related phenotypes and gut microbiota: a two-sample bidirectional Mendelian randomization study
Xiaoqiu Wang, Chi Wang, Kai Liu, Qingyun Wan, Wenzhong Wu and Chengyong Liu

53 Fecal microbiota transplantation unveils sex-specific differences in a controlled cortical impact injury mouse model
Tulasi Pasam and Manoj P. Dandekar

66 Alteration of the gut microbiota profile in children with autism spectrum disorder in China
Hui Li, Wei Guo, Sijie Li, Bishao Sun, Ningshan Li, Dongjing Xie, Zongming Dong, Dan Luo, Wei Chen, Weihua Fu, Ji Zheng and Jingzhen Zhu

78 Electroacupuncture at ST25 corrected gut microbial dysbiosis and SNpc lipid peroxidation in Parkinson's disease rats
Xuan-ming Hu, Li-zhe-xiong Song, Zhi-zi Zhang, Xi Ruan, Hai-chang Li, Zhi Yu and Lin Huang

95 Microbiota profiling reveals alteration of gut microbial neurotransmitters in a mouse model of autism-associated 16p11.2 microduplication
Zhang Fu, Xiuyan Yang, Youheng Jiang, Xinliang Mao, Hualin Liu, Yanming Yang, Jia Chen, Zhumei Chen, Huiliang Li, Xue-Song Zhang, Xinjun Mao, Ningning Li, Dilong Wang and Jian Jiang

107 Electroacupuncture regulates gut microbiota to reduce depressive-like behavior in rats
Junying Wang, Haohan Zhu, Xingke Song, Jun Zhao, Jianliang Zhang, Jinling Zhang, Shaoyuan Li and Peijing Rong

120 Improving precision management of anxiety disorders: a Mendelian randomization study targeting specific gut microbiota and associated metabolites
Ming-Min Xu, Wen-Hui Qiu, Qing-Yu Ma, Zhi-Yun Yu, Wen-Miao Yang, Tian-Nuo Hu, Yu Guo and Xiao-Yin Chen

134 Anorexia nervosa and bulimia nervosa: a Mendelian randomization study of gut microbiota
Zongliang Yu, Manping Guo, Binyang Yu, Yiming Wang, Zian Yan and Rui Gao

147 Gut microbiota and serum metabolomic alterations in modulating the impact of fecal microbiota transplantation on ciprofloxacin-induced seizure susceptibility
Shangnan Zou, Yinchao Li, Qihang Zou, Man Yang, Huifeng Li, Ruili Niu, Huanling Lai, Jiaoyang Wang, Xiaofeng Yang and Liemin Zhou

167 Baseline gut microbiota profiles affect treatment response in patients with depression
Yingjing Xie, Hanwen Zhu, Yanling Yuan, Xuan Guan, Qinglian Xie and Zaiquan Dong

178 Gut microbiota from patients with Parkinson's disease causes motor deficits in honeybees
Jiaqi Zeng, Yiyuan Li, Jingshuang Yan, Ruqi Chang, Mengqi Xu, Guanzhou Zhou, Jie Meng, Di Liu, Zhiqi Mao and Yunsheng Yang

189 Duodenal microbiota dysbiosis in functional dyspepsia and its potential role of the duodenal microbiota in gut–brain axis interaction: a systematic review
Xueping Zhang, Lei Chen, Tao Zhang, Ryu Gabo, Qianying Wang, Zhuotai Zhong, Mengxi Yao, Wei Wei and Xiaolan Su

209 Role of inflammatory cytokines and the gut microbiome in vascular dementia: insights from Mendelian randomization analysis
Yihan Yang, Ting Rao, Sheng Wei, Jing Cheng, Ying Zhan, Teng Lin, Jincheng Chen, Xiaoling Zhong, Yijing Jiang and Shanli Yang

225 Exploring the pathogenesis of insomnia and acupuncture intervention strategies based on the microbiota-gut-brain axis
Jia Guo, Jixing Guo, Xiang Rao, Rongni Zhang, Qiang Li, Kun Zhang, Shanbo Ma, Jingyu Zhao and Changchun Ji

235 Fecal microbiota transplantation alleviates cognitive impairment by improving gut microbiome composition and barrier function in male rats of traumatic brain injury following gas explosion
Xinwen Dong, Yaguang Su, Zheng Luo, Cuiying Li, Jie Gao, Xiaofeng Han, Sanqiao Yao, Weidong Wu, Linqiang Tian, Yichun Bai, Guizhi Wang and Wenjie Ren

255 The gut-brain-metabolic axis: exploring the role of microbiota in insulin resistance and cognitive function
Gulshara Zh Abildinova, Valeriy V. Benberin, Tamara A. Vochshenkova, Alireza Afshar, Nadiar M. Mussin, Asset A. Kaliyev, Zhanna Zhussupova and Amin Tamadon

268 Total Alkaloids of Rhizoma Corydalis regulates gut microbiota and restores gut immune barrier to ameliorate cognitive dysfunction in diabetic rats
Yazhi Qi, Jun Li, Ya Tang, Rui Cao, Yishu Gao, Qiang Xu and Yusheng Han

282 Gut microbiota has important roles in the obstructive sleep apnea-induced inflammation and consequent neurocognitive impairment
Mingxing Tang, Yongliang Wu, Junyi Liang, Shuai Yang, Zuofeng Huang, Jing Hu, Qiong Yang, Fei Liu and Shuo Li

290 Influential articles in autism and gut microbiota: bibliometric profile and research trends
Jiangbo Ying, Melvyn Weibin Zhang, Ker-Chiah Wei, Sunny H. Wong and Mythily Subramaniam

311 Investigating the causal impact of gut microbiota on trigeminal neuralgia: a bidirectional Mendelian randomization study
Chuan Zeng, Chaolong Zhang, Yuxuan Jia, Huaiyu Zhou, Chunming He and Haimin Song

321 Microbiota-gut-brain-axis: a new target of acupuncture therapy for post-stroke cognitive impairment
Te Ba, Rui Niu, Jianjun Gao, Wenfang Li, Mengwei Dong, Jingjing Duan, Yinan Gong, Sinuo Wu, Zhongxi Lyu, Yang Liu and Ningcen Li

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Editorial: Animal models, gut microbiota and brain diseases

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Editorial on the Research Topic
Animal models, gut microbiota and brain diseases

Introduction

Brain diseases, including neurological and psychiatric disorders such as Alzheimer's, Parkinson's, epilepsy, depression, anxiety, autism, insomnia, etc., significantly impact human health. These conditions are mainly characterized by abnormal thinking patterns, cognition, emotional states, and behavior (Gao et al., 2023). Research primarily relies on animal models, but the pathophysiology remains incompletely understood. Determining whether findings are causal, related, or irrelevant is critical for advancing effective treatments.

There is a need for updated insights from preclinical animal studies. Since 2011, research has shown that germ-free mice exhibit reduced anxiety-like behavior and altered neurochemicals, sparking interest in the gut microbiota's role in health (Neufeld et al., 2011). The concept of the microbiota-gut-brain axis (MGBA), formalized in 2012, highlights the connection between peripheral systems and the brain via gut microbiota (Cryan and Dinan, 2012). Over the past decade, animal models have been crucial in investigating the gut microbiota's impact on brain diseases and potential therapies. However, applying these findings to human brain disease diagnosis and treatment remains challenging.

The aim of this Research Topic was to compile new studies on animal models, gut microbiota, and brain diseases. It features 23 articles: 15 original research papers, 4 reviews, 2 systematic reviews, and 2 mini reviews.

Neurodegenerative disorders

Dementia and cognitive impairment

Researchers have long debated the link between gut microflora and dementia. Fu J. et al. conducted a Mendelian randomization (MR) study and identified 21 gut microbiota taxa linked to dementia subtypes. *Desulfovibrionaceae* was associated with a higher risk

of Alzheimer's, while *Butyricimonas* was linked to a lower risk of Parkinson's dementia. A bidirectional relationship was observed between Ruminococcus gnavus and Lewy body dementia. Sensitivity analyses confirmed these results, though the study's limitations include relaxed SNP thresholds and a lack of ethnic diversity, as most data came from European populations. These findings indicate gut microbiota could be key in diagnosing and treating dementia. Yang et al. examined the impact of inflammatory cytokines and gut microbiota on the risk of vascular dementia (VaD) and their causal links. Their MR study found causal links between specific gut microbiota, like *Negativicutes* (beneficial) and *Melainabacteria* (detrimental), and inflammatory cytokines, such as IL-18 and MIF (risk-increasing) and IL-4 (beneficial), with different types of vascular dementia. Multivariable analyses confirmed the microbiome effects were independent of factors like body mass index, while mediation MR analyses dismissed cytokines as intermediaries between the gut and VaD. These results highlight new microbial and immune targets for VaD treatment and improve our understanding of the gut-brain connection in cerebrovascular cognitive decline.

The above two MR studies highlight the significant role of intestinal microbiota in the pathogenesis of dementia. This raises the question of what therapeutic interventions are available for dementia and cognitive impairment within this framework. Dong et al. demonstrated that fecal microbiota transplantation (FMT) improved cognitive function in rats with traumatic brain injury (TBI) from gas explosions by restoring gut microbial balance (e.g., *Clostridium_T*, *Allobaculum*) and strengthening the gut-brain barrier. FMT reduced neuroinflammation, increased tight-junction proteins like Claudin-1, Occludin, and ZO-1, and affected Treg-related factors. Multi-omics analyses revealed fatty acid biosynthesis activation as a key mechanism. These results highlight FMT's potential as a TBI treatment by modulating the MGB axis. Similarly, Qi et al. found that Total Alkaloids of Rhizoma Corydalis (TAC) ameliorated cognitive function by balancing gut microbiota, enhancing intestinal barrier integrity, and reducing neuroinflammation. TAC decreases *Lachnoclostridium*, increases *Bacteroides*, upregulates ZO-1 and occludin, and inhibits hippocampal NLRP3 inflammasome activation and neuronal ferroptosis.

Additionally, three reviews summarize research on gut microbiota and cognitive impairment in dementia. Tang et al. linked intermittent hypoxia from obstructive sleep apnea (OSA) to gut dysbiosis, marked by fewer short-chain fatty acid (SCFA) producers and more *Prevotella* species, as well as to systemic inflammation and cognitive decline. FMT from OSA-model mice into healthy mice replicated these effects, confirming a causal link. They suggest prebiotic, probiotic, or SCFA treatments could help mitigate OSA-related neural damage. Abildinova et al. emphasized the role of microbiota-derived short-chain fatty acids, like butyrate, in regulating insulin sensitivity and cognitive function. Dysbiosis can lead to metabolic endotoxemia, marked by lipopolysaccharide translocation, systemic inflammation, and brain insulin resistance, which may speed up cognitive decline. Potential therapies include multi-strain probiotics, personalized nutrition, and microbiota-derived exosomes. While FMT and probiotics show promise in restoring metabolic-cognitive balance, challenges such as outcome variability and long-term safety remain. Future research should aim to clarify mechanisms and develop personalized microbial therapies. Ba et al. identify the MGBA as a promising target for treating post-stroke cognitive impairment (PSCI), emphasizing acupuncture's role in enhancing intestinal barrier function, gut microbiota, and reducing neuroinflammation. Clinical studies demonstrated that acupuncture at specific points (e.g., GV20, ST36) improves cognitive scores, and research indicates its modulatory effects on metabolites, immune markers, and HPA axis activity. However, the lack of high-quality trials and standardized protocols remains a significant challenge. Future research should focus on integrating multi-omics and refining acupuncture techniques for precise interventions in PSCI.

Parkinson's disease

Zeng J. et al. investigated transplantation of gut microbiota from individuals with Parkinson's disease (PD) and healthy controls (HC) were into germ-free honeybees. The results indicate that fecal microbiota transplants from PD patients result in motor impairments, reduced expression of tyrosine hydroxylase, and compromised gut barrier integrity in honeybees, paralleling the pathology observed in rotenone-induced PD models. This "humanized bee model" exhibited enrichment of PD-associated genera (e.g., *Dorea*, *Collinsella*), and altered microbial pathways involved in hydrogen sulfide and methane production, thereby underscoring the significance of the MGBA in PD pathogenesis. Despite certain translational limitations, this innovative invertebrate model offers a rapid and ethical approach to exploring the mechanistic aspects of gut-brain interactions.

As for therapy, emerging evidence indicates that electroacupuncture (EA) may benefit PD, though its mechanisms are not well understood. Hu et al. demonstrated that EA at ST25 improved motor function and reduced neuron loss in a PD rat model induced by rotenone. EA also corrected gut dysbiosis by decreasing harmful bacteria and increasing beneficial ones, lowering inflammation and lipid peroxidation in the brain. These results highlight the GMBA as a potential non-drug treatment pathway for PD.

Epilepsy

Using ciprofloxacin-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models, Zou et al. elucidated that extended administration of ciprofloxacin results in gut microbiota dysbiosis. This dysbiosis, characterized by increased *Akkermansia* and *Bacteroides* abundance, is associated with enhanced seizure susceptibility in rats. Critically, FMT reversed both the microbial dysbiosis and the pro-epileptogenic effects. Furthermore, reduced serum indole-3-propionic acid levels were identified as a potential driver of neuroinflammation. These findings provide compelling evidence supporting the role of the MGBA in the pathogenesis of epilepsy and suggest that gut microbiota modulation represents a

therapeutically viable. Although clinical translation requires further validation, this research provides crucial mechanistic insights into the risks associated with antibiotic-induced seizures and potential microbial-targeted intervention.

Psychiatric disorders

Depression

Xie et al.'s review highlights the pivotal role of gut microbiota in modulating antidepressant treatment responses, associating baseline microbial profiles (e.g., *Firmicutes/Bacteroidetes* ratio) with clinical outcomes via immune-neural interactions. Although these findings are promising, limitations including small sample sizes constrain generalizability and require further validation. Microbiota-targeted therapies offer potential for advancing personalized approaches to depression management. In this regard, the study by Wang J. et al. illustrates that electroacupuncture at points ST36 and ST25 alleviates depressive-like behaviors in rats subjected to chronic unpredictable mild stress by modulating gut microbiota (e.g., increased *Bacteroidetes* and decreased *Firmicutes*) and neuropeptides (VIP/CGRP). These findings implicate the MGBA in the antidepressant effects of acupuncture, while the precise neural mechanisms require further elucidation.

Anxiety

Xu et al. conducted a Mendelian randomization study revealing that certain gut microbiota genera affect anxiety disorders. The *Eubacterium nodatum* group and *Ruminococcaceae* UCG011 are protective, while *Lachnospiraceae* UCG010 elevates risk. These effects are linked to neurotransmitter-related metabolites like tyrosine, phenylalanine, glycine, and cortisol. The study highlights the influence of modifiable factors, such as diet, smoking, and physical activity, on microbiota-anxiety interactions, suggesting potential for precision anxiety management through targeted lifestyle interventions.

Insomnia

Wang X. et al. conducted a bidirectional Mendelian randomization study establishing causal links between specific gut microbiota and sleep traits. The analysis revealed that class *Negativicutes* and order *Selenomonadales* elevate severe insomnia risk, whereas phylum *Lentisphaerae* is causally associated with longer sleep duration, and genus *Senegalimassilia* reduces snoring propensity. Reverse MR analysis demonstrated that sleep patterns reciprocally alter microbiota composition. The study's strengths include multivariable sensitivity analyses and STROBE-MR guideline adherence, though limitations encompass ethnic homogeneity and relaxed SNP thresholds. Collectively, these findings suggest targeting microbiota could help manage sleep disorders. Guo et al.'s review further implicates the MGBA as key in insomnia's pathogenesis, focusing on dysbiosis-related disruptions in various signaling pathways. It identifies acupuncture as a potential therapeutic strategy effective in restoring microbial homeostasis, modulating neurotransmitters like serotonin and GABA, and reducing neuroinflammation. While clinical evidence supports acupuncture's benefits for sleep quality and relapse reduction, more detailed studies are needed to standardize treatments and validate integrative approaches for insomnia.

Anorexia nervosa/bulimia nervosa

Yu et al.'s bidirectional MR analysis established causal relationships between 18 gut microbial taxa and anorexia nervosa (AN)/bulimia nervosa (BN). Family *Lachnospiraceae* exhibits opposing effects: specific genera increase AN risk yet confer protection against BN. These findings underscore microbiota's complex role in eating disorders and offer novel targets for probiotic therapies, notwithstanding limitations in microbial genome-wide association studies that require further validation.

Neurodevelopmental disorders

Autism spectrum disorder

Li et al. conducted a study employing 16S rRNA sequencing to examine 957 children with autism spectrum disorder (ASD) aged 2–12 years, alongside 161 HC within the same age range in China. This extensive research elucidates gut dysbiosis among Chinese children with ASD, demonstrating: reduced α-diversity, altered microbial abundances (*Faecalibacterium* enrichment and *Prevotella_9* depletion), and disrupted metabolic pathways, including folate synthesis. These findings substantiate the involvement of the gut-brain axis in ASD pathogenesis and underscore the potential of microbiota as biomarkers, necessitating further longitudinal studies for validation. Using a 16p11.2 microduplication mouse model of autism, Fu Z. et al. identified gut dysbiosis and disrupted microbial neurotransmitter networks. Key findings comprise the following: reduced microbial biodiversity, depletion of *Faecalibaculum*, elevated histaminergic metabolites, and correlations between specific bacterial shifts and autism-like phenotypes. The research highlights the gut-brain axis's role in neurodevelopmental disorders and proposes targeting histamine metabolism as a potential therapy for 16p11.2 microduplication-associated ASD. Ying et al.'s bibliometric analysis delineates the evolving ASD-gut microbiota research landscape (2000–2021). Analysis of 100 foundational publications reveals: surging annual citations peaking in 2021, leading contributions from the United States and Ireland, and dominant themes of short-chain fatty acids and MGBA mechanisms. Although 62% of the publications are reviews, pivotal experimental studies, notably Hsiao's 2013 *Cell* paper, demonstrate therapeutic promise. The analysis also maps collaborative networks and emerging research areas, despite being limited to one database, offering a roadmap for future research.

Other brain diseases or related diseases

Pasam and Dandekar studied the impact of controlled cortical impact (CCI) injury on gut microbiota in male and female mice. Post-CCI dysbiosis was characterized by significant reductions in *Lactobacillus helveticus* and *L. hamsteri* in female. Cross-sex FMT indicated that female recipients of male microbiota exhibited enriched neuroprotective *Lactobacillus*, whereas male recipients shoed increased abundance of *Alistipes* and *Ruminococcus*. These results highlight sex-specific microbial responses to traumatic brain injury and suggest *Lactobacillus*-targeted interventions as a mechanisnm-driven strategy for sex-specific neurorecovery.

Using a Mendelian randomization study, Zeng C. et al. established causal effects of specific gut microbiota on trigeminal neuralgia (TN). The analysis identified *Butyricimonas*, *Bacteroidales* S24.7 group, and an unclassified genus as significant risk of TN, contradicting the presumed exclusively beneficial role of *Butyricimonas*. Sensitivity analyses supported these results, and reverse MR analysis excluded reverse causation. Despite limited generalizability from Finnish GWAS data, this study suggests a gut-brain connection in TN's pathogenesis, emphasizing the need for randomized trials to explore mechanisms and therapeutic options.

Zhang J. et al.'s review highlights the crucial role of peripheral dysfunction, including gut microbiota imbalance, liver metabolite changes, and cholinergic pathway issues, in sepsis-associated encephalopathy (SAE). The work elucidates novel mechanisms by which neuroinflammation, blood-brain barrier disruption, and inter-organ crosstalk exacerbate SAE progression. The study emphasizes multiorgan interactions as therapeutic targets, suggesting mechanism-based interventions including fecal microbiota transplantation, short-chain fatty acids, and vagus nerve modulation. This comprehensive view moves SAE management beyond traditional central nervous system-focused approaches.

While primarily a digestive disorder, functional dyspepsia (FD) exhibits neuropsychiatric linkages. Zhang X. et al.'s systematic review highlights duodenal microbiota dysbiosis as a key factor in FD pathogenesis and gut-brain axis dysfunction. Analysis of 391 FD patients revealed symptom-associated alterations: increased *Streptococcus* abundance coupled with decreased *Prevotella* and *Selenomonas*. *Staphylococcus*-driven inflammation may exacerbate FD progression. Paradoxically, despite stable α-diversity, microbial imbalances correlated with symptom severity and quality of life. The authors advocate for expanded studies to validate the therapeutic targets and elucidate underlying mechanisms.

Final remarks

This Research Topic highlights the causal involvement and therapeutic potential of gut microbiota in brain disorders through animal models. Mendelian randomization studies identified disease-specific microbial signatures, notably *Desulfovibrionaceae* elevating Alzheimer's disease risk and *Butyricimonas* attenuation Parkinson's dementia risk. Interventions including fecal microbiota transplantation and electroacupuncture improved cognitive/motor deficits by restoring microbial homeostasis, enhancing gut-brain barrier integrity, and suppressing neuroinflammation. The MGBA emerges as a central pathway in epilepsy, depression, and autism spectrum disorder. Current limitations encompass population homogeneity, therapeutic standardization deficits, and translational gaps. Future priorities include multi-omics integration to elucidated metabolite-neural circuits, longitudinal biomarker validation, and randomized trials for personalized microbial therapeutics.

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Causal relationship between gut microflora and dementia: a Mendelian randomization study

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Background: Numerous pertinent investigations have demonstrated a correlation between gut microflora (GM) and the occurrence of dementia. However, a causal connection between GM and dementia and its subtypes has not yet been clarified.

Objective: To explore the causal association between GM and dementia, including its subtypes, a two-sample Mendelian randomization (TSMR) analysis was used.

Methods: Our data comes from the Genome-Wide Association Study (GWAS). The principal approach employed for the Mendelian randomization study was the inverse-variance weighted method, supplemented by four methods: MR-Egger, weighted median, simple mode, and weighted mode. This was followed by Cochrane's Q test, MR-Egger intercept test, MR-PRESSO global test, and leave-one-out as sensitivity analysis validation.

Results: Twenty-one GMs associated with any dementia, Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson's disease, and dementia under other disease classifications were derived from the analysis, and 21 passed sensitivity tests.

Conclusion: We confirmed the causal relationship between GM and dementia and its subtypes, derived specific flora associated with increased or decreased risk of dementia, and provided new ideas for preventive, diagnostic, and therapeutic interventions for dementia mediated by gut microbiota.

KEYWORDS

dementia, gut microflora, Mendelian randomization, causality, Alzheimer's disease

1 Introduction

Dementia is a prevalent neurodegenerative disorder clinically distinguished by cognitive impairment and a gradual deterioration in one's ability to function autonomously (Liu et al., 2019). According to a WHO report (Cheah et al., 2022), dementia has now become the seventh leading cause of death globally, and it is expected that the number of dementia patients worldwide will reach 139 million by 2050. At the same time, the prevention and treatment of dementia bring a substantial economic and healthcare burden to society and countries, and the global investment in dementia will reach 2.8 trillion dollars by 2030 (World Alzheimer Report, 2015). Alzheimer's disease represents the prevailing form of dementia, comprising approximately 50 to 70% of cases. Other frequently encountered kinds comprise vascular dementia, Lewy body dementia, Parkinson's disease, and dementia in other diseases as classified

elsewhere (Aarsland, 2020; Wilbur, 2023). To date, the underlying mechanisms leading to dementia have not been clarified, and the medical requirements of individuals with dementia are not well met (Zagórska et al., 2023). Therefore, clarification of dementia-related risk factors, and thus dementia prevention, intervention, and care, can significantly help enhance the well-being and survival rates of individuals with dementia (Livingston et al., 2020).

Gut microbiota (GM) generally refers to bacteria in the human gut. It is involved in regulating a wide range of physiological functions in the host organism and protecting the host from pathogenic bacteria (Álvarez et al., 2021; Kuziel and Rakoff-Nahoum, 2022). Increasingly, GM has been found to fulfill an essential role in the nervous system through the brain-gut axis and has even been implicated in neurodegenerative diseases (Cryan et al., 2020; Mitrea et al., 2022). Studies have demonstrated that GM metabolites, molecules, and endotoxins may affect the central nervous system through the bloodstream or the vagus nerve, affecting brain function and cognitive behavior (Chen et al., 2021). This is undoubtedly a complementary approach to diagnosing and treating dementia, and many scholars have endeavored to address neurodegenerative illnesses through the manipulation of gut microbiota (Möhle et al., 2016; Sasmita, 2019). A systematic evaluation based on dementia studies showed that probiotic supplements improved memory in patients with dementia, as well as elevated levels of brain-derived neurotrophic factor (Ruiz-Gonzalez et al., 2021). In addition, some studies have found differences in GM composition between healthy people and people with cognitive impairment or different types of cognitive impairment, attempting to use this information to make a diagnosis of the disease (Guo et al., 2021; Hung et al., 2022). Therefore, clarifying the influence of different flora on dementia is essential for ascertaining new therapeutic targets for dementia and diagnosing dementia using microbial profiles (Liu et al., 2019; Guo et al., 2021; Cuartero et al., 2023).

Mendelian randomization (MR) is a study method that explores causal relationships between exposure factors and outcomes using single nucleotide polymorphisms (SNPs) as instrumental variables (IVs; Burgess et al., 2017), which is consistent with the principle of random allocation of genetic variation during meiosis, avoiding the influence of confounding variables and the potential for reverse causality (Sekula et al., 2016). This work employed GWAS summary statistics of GW taxa associated with dementia and their subtypes for MR analysis in order to evaluate the risk relationship between genetically determined GW taxa and dementia and its subtypes, which provides evidence for existing findings, new research ideas for pathogenesis that has not yet been clarified, and new directions for the early diagnosis, avoidance, and therapy of all types of dementia.

2 Materials and methods

2.1 Study design

TSMR was employed to analyze the association between GM and dementia (any dementia, Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson's disease, dementia in other diseases classified elsewhere) with regard to causality. The overall design of the study is illustrated in Figure 1. In order to carry out a TSMR study, it is imperative that three fundamental assumptions are satisfied: (1) Strong correlation between IVs and exposure; (2) No correlation between IVs and confounders; (3) IVs can only affect outcomes through exposure (EPIC-InterAct Consortium et al., 2015). IVs that fulfill these three assumptions were included in this MR study (Figure 2). This study followed the most updated guidelines (STROBE-MR; Skrivankova et al., 2021).

2.2 Sources of data on exposure

The GWAS data for gut microbes were acquired through the MiBioGen consortium[1] from genomic statistical research by Kurilshikov et al. of 18,340 individuals of European ethnicity from 11 countries (24 cohorts), and the data contained 211 gut microbes with 122,110 variant loci (Kurilshikov et al., 2021). From this GWAS, we screened IVs of gut bacterial taxa in five ranks.

2.3 Source of data on endings

GWAS statistics for any dementia, Alzheimer's disease, vascular dementia, Parkinson's disease, and dementia in other diseases classified elsewhere were derived from the FinnGen study program.[2] GWAS statistics for Lewy body dementia were derived from the GWAS Catalog.[3] The diagnostic standards for dementia are according to F03 in the ICD-10 criteria, where the GWAS dataset has 16,380,466 variant loci from 5,933 patients and 212,859 controls. The diagnostic standards for Alzheimer's disease are according to G30.901 of the ICD-10 criteria, which contains 16,380,451 variant loci from 2,191 cases and 209,487 controls. The diagnostic standards for Lewy body dementia are according to G31.805 of the ICD-10 criteria, which contains 7,593,175 variant loci from 2,591 cases and 4,027 controls. The diagnostic standards for vascular dementia are according to F01 of the ICD-10 criteria and contain 16,380,453 variant loci from 98 cases and 211,300 controls. The diagnostic standards for Parkinson's disease are according to the G20 of the ICD-10 criteria and contain 16,380,459 variant loci from 267 cases and 216,628 controls. The diagnostic standards for dementia in other diseases classified elsewhere are according to F02.8 of the ICD-10 criteria and contain 16,380,450 variant loci from 581 cases and 209,487 controls. In addition, three datasets related to any dementia were added as three validation groups (Table 1).

2.4 Selection of IVs

We screened the relevant IVs according to the following standards: (1) a significant threshold ($p < 5 \times 10^{-8}$) for IVs was associated with exposure and outcome, but the quantity of eligible IVs (exposure) was low, so a more appropriate threshold ($p < 1 \times 10^{-5}$) was used to acquire a larger quantity of IVs (Lv et al., 2021; Zeng et al., 2023); (2) the chain imbalance coefficient $r^2 < 0.001$, distance = 10,000 kb was set to remove the presence of chain imbalance among IVs; (3) to avoid the effects of horizontal pleiotropy, IVs linked to dangerous elements of dementia were eliminated by the utilization of PhenoScanner (Kamat et al., 2019); (4)

1 https://mibiogen.gcc.rug.nl
2 https://r5.finngen.fi/
3 https://www.ebi.ac.uk/gwas/home

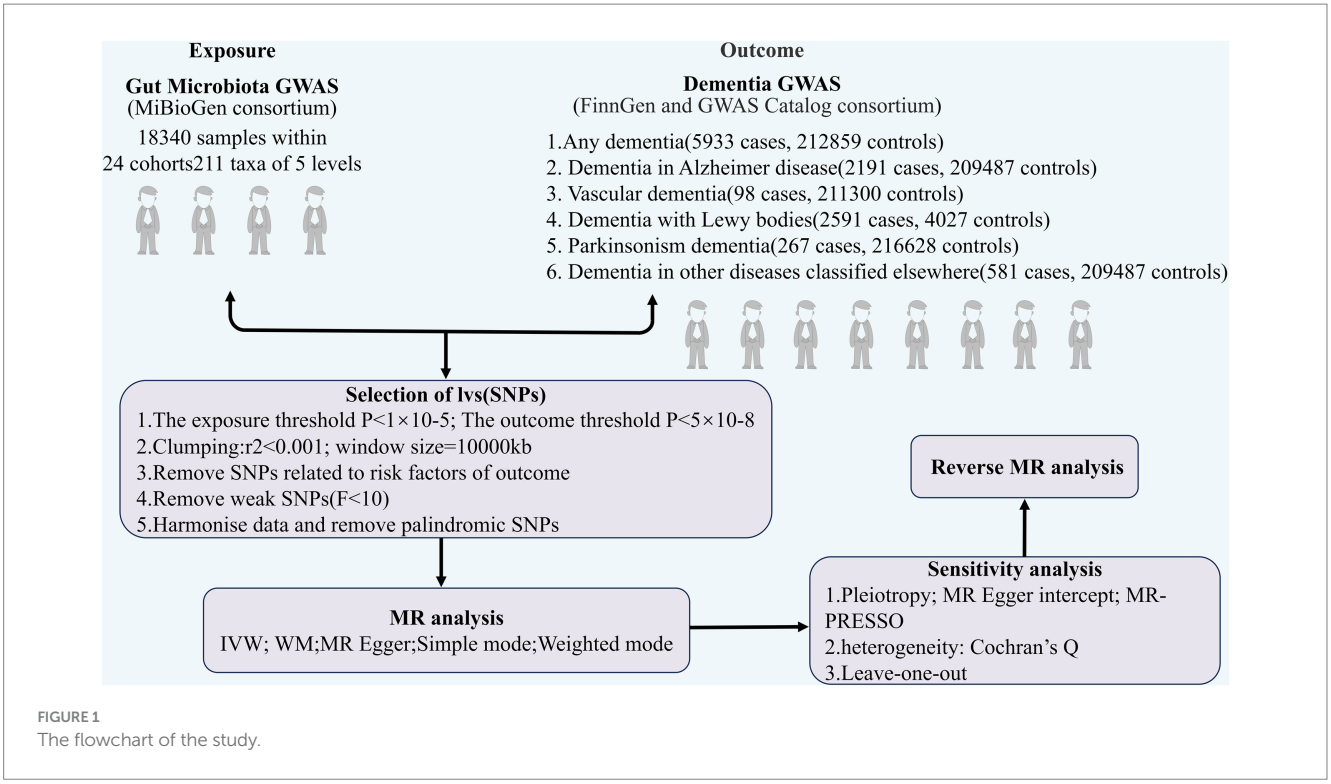

FIGURE 1
The flowchart of the study.

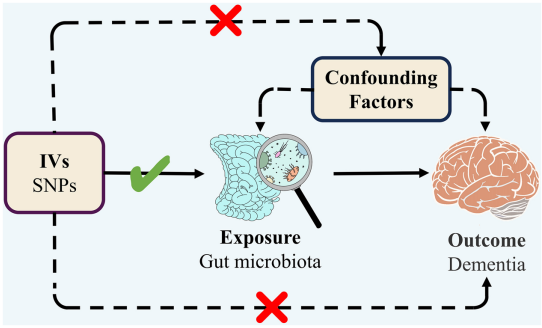

FIGURE 2
Three assumptions of MR.

palindromic SNPs were removed from IVs; and (5) to avoid bias from weak instrumental variables, we removed IVs with $F < 10$ (Burgess et al., 2011).

2.5 Statistical analysis

In TSMR analysis of gut microbes and dementia (any dementia, Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson's disease, dementia in other diseases classified elsewhere) in causality, the fixed-effects IVW method and the random-effects IVW method were the main methods (Burgess et al., 2013). The choice between the two is determined by the heterogeneity between IVs, if there is heterogeneity in Cochrane's Q test ($p < 0.05$), then random-effects IVW method was used, otherwise fixed-effects IVW method or random-effects IVW method was used. Therefore, in this TSMR analysis, We chose the random-effects IVW method as the main method (Greco et al., 2015). In addition, MR-Egger, weighted median, simple mode, and weighted mode can complement IVW (Bowden et al., 2015, 2016), and ORs and 95% confidence intervals were also obtained. A causal relationship between gut microbes and dementia was considered likely if the outcome of one TSMR method was remarkable ($p < 0.05$; Jin et al., 2023), and the causal relationship was considered reliable if the results of two or more TSMR methods were significant (Ni et al., 2021).

Sensitivity analyses took place to verify the robustness of the findings, and Cochrane's Q test was used to test for heterogeneity. The IV was considered heterogeneous if $p < 0.05$. The MR-Egger method's intercept term indicates horizontal multiplicity in the IVs, and if this intercept term is significantly different from 0, it indicates the presence of horizontal multiplicity (Burgess and Thompson, 2017). MR-PRESSO is also commonly used to test for horizontal multiplicity (Verbanck et al., 2018). Finally, the validation of the data was conducted using the leave-one-out procedure (Xiang et al., 2021). The investigation was carried out utilizing R program (version 4.3.0). The "Two SampleMR" R package[4] and "MRPRESSO" R package[5] were used for our MR study.

2.6 Reverse MR analysis

Assuming that there are relevant GMs that can have an effect on dementia and its subtypes in the final findings, we will further conduct a reverse MR analysis to explore the effect of dementia on GMs, with

4 https://mrcieu.github.io/TwoSampleMR/
5 https://github.com/rondolab/MR-PRESSO

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TABLE 1 Details of the datasets included in this study.

	Trait	Year	Sex	Population	Case	Control	Number of SNPs	PMID/URL (Data download)
Exposure	Any dementia	2021	Male and female	European	5,933	212,859	16,380,463	https://gwas.mrcieu.ac.uk/datasets/finn-b-KRA_PSY_DEMENTIA/
	Alzheimer's disease	2021	Male and female	European	2,191	209,487	16,380,451	https://gwas.mrcieu.ac.uk/datasets/finn-b-F5_ALZHDEMENT/
	Vascular dementia	2021	Male and female	European	98	211,300	16,380,453	https://gwas.mrcieu.ac.uk/datasets/finn-b-VD_MX/
	Lewy body dementia	2021	Male and female	European	2,591	4,027	7,593,175	33,589,841
	Parkinson's disease	2021	Male and female	European	267	216,628	16,380,459	https://gwas.mrcieu.ac.uk/datasets/finn-b-PD_DEMENTIA/
	Dementia in other diseases classified elsewhere	2021	Male and female	European	581	209,487	16,380,463	https://gwas.mrcieu.ac.uk/datasets/finn-b-F5_DEMINOTH/
Outcome	Gut microbe	2021	Male and female	European	-	-	122,110	33,462,485
Validation group	Any dementia	2021	Male and female	European	5,933	166,584	16,380,199	https://gwas.mrcieu.ac.uk/datasets/finn-b-KRA_PSY_DEMENTIA_EXMORE/
	Any dementia	2021	Male and female	European	7,284	209,487	16,380,450	https://gwas.mrcieu.ac.uk/datasets/finn-b-F5_DEMENTIA/
	Any dementia	2021	Male and female	European	7,395	211,397	16,380,465	https://gwas.mrcieu.ac.uk/datasets/finn-b-F5_DEMENTIA_INCLAVO/

dementia as the exposure and GMs as the outcome to avoid reverse causality interfering with the results of this study.

3 Results

3.1 IV details

After screening the above entries, 605 IVs associated with dementia were finally obtained, involving 60 GMs. Detailed information can be found in Supplementary Table 1. All IVs involved had *F* values greater than 10 (range 16.91–85.37), so there were no weak instrumental variables. These IVs were categorized into five classes: phylum, class, order, family, and genus, comprising two phylum (14 IVs), four classes (40 IVs), nine orders (90 IVs), 13 families (134 IVs), and 32 genera (327 IVs). Because of the inclusionary relationship between gut microbial classifications, there may be a substantial overlap of SNPs and their associated orders contained in various types of enterobacteria.

3.2 Results of the TSMR analysis

Causal relationships between the 60 GMs screened and dementia were analyzed using five TSMR methods: IVW, MR-Egger, weighted median, simple mode, and weighted mode (Supplementary Table 2). Potential causal relationships between the 60 GMs and dementia were determined using two TSMR methods, in which six GMs associated with dementia, four GMs associated with Alzheimer's disease, two GMs associated with vascular dementia, three GMs associated with Lewy body dementia, two GMs associated with Parkinson's disease, and four GMs associated with other diseases under the classification of dementia-associated GMs, and cross-validation was performed (Table 2; Figure 3). Our attention was directed toward the 21 causal associations that have a relatively steady nature.

Causal relationships were obtained for six related GMs in any dementia using the IVW method, and all six relationships were more stable under IVW and WM cross-validation. Among them, family Desulfovibrionaceae (OR: 1.481, 95% confidence interval (CI): 1.064–2.062, $p = 0.020$), family Lactobacillaceae (OR: 1.216. CI: 1.040–1.422, $p = 0.014$), genus Ruminococcusgnavus group (OR: 1.196, CI: 1.033–1.385, $p = 0.016$), genus Lactobacillus (OR: 1.304, CI: 1.115–1.525, $p = 0.001$), and order Desulfovibrionales (OR: 1.408, CI: 1.057–1.875, $p = 0.019$) were related to increased risk of dementia. Genus Defluviitaleaceae UCG011 (OR: 0.735, CI: 0.553–0.977, $p = 0.034$) was related to a reduced risk of dementia.

Causal relationships were obtained for nine related GMs in Alzheimer's disease using the IVW method, and four relationships were more stable under IVW and WM cross-validation. Among them, family Desulfovibrionaceae (OR: 1.682, CI: 1.102–2.568, $p = 0.016$), genus Sellimonas (OR: 1.273, CI: 1.068–1.518, $p = 0.007$), and order

Desulfovibrionales (OR: 1.592, CI: 1.011–2.507, $p = 0.045$) were associated with increased risk of Alzheimer's disease. Order Bacillales (OR: 0.738, CI: 0.608–0.896, $p = 0.002$) was related to a decreased risk of Alzheimer's disease.

Causal relationships were obtained for 14 relevant GMs in vascular dementia using the IVW method, and two relationships were more stable under IVW and WM cross-validation. Among them, genus Lachnospiraceae NK4A136 group (OR: 0.197, CI: 0.046–0.851, $p = 0.030$) and order Victivallales (OR: 0.350, CI: 0.125–0.980, $p = 0.030$) were related to a reduced risk of vascular dementia.

Causal relationships were obtained for nine relevant GMs in Lewy body dementia using the IVW method, and three relationships were more stable under IVW and WM cross-validation. Among them, class Alphaproteobacteria (OR: 1.970, CI: 1.320–2.940, $p = 0.001$) and order Bacillales (OR: 1.378, CI: 1.116–1.703, $p = 0.030$) were associated with increased risk of Lewy body dementia. Genus Ruminococcusgnavus group (OR: 0.678, CI: 0.523–0.878, $p = 0.003$) was related to a reduced risk of Lewy body dementia.

Causal relationships were obtained for 10 related GMs in Parkinson's disease using the IVW method, and two relationships were more stable under IVW and WM cross-validation. Among them, genus Butyricimonas (OR: 0.314, CI: 0.134–0.737, $p = 0.008$) and phylum Lentisphaerae (OR: 0.500, CI: 0.255–0.980, $p = 0.044$) were related to a reduced risk of Parkinson's disease.

Causal relationships were obtained for 12 relevant GMs in dementia in other diseases classified elsewhere using the IVW method, and four relationships were more stable under IVW and WM cross-validation. Among them, genus Ruminococcusgnavus group (OR: 1.707, CI: 1.125–2.591, $p = 0.012$) and genus Hungatella (OR: 1.697, CI: 1.026–2.809, $p = 0.040$) were associated with an increased risk of dementia in other diseases classified elsewhere. Order Burkholderiales (OR: 0.500, CI: 0.250–0.998, $p = 0.049$) and genus Oscillibacter (OR: 0.538, CI: 0.344–0.841, $p = 0.007$) were related to decreased risk of dementia in other diseases classified elsewhere.

Finally, we utilized a heat map to causally present the results of the study in the form of various types of GMs and any dementia, Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson's disease, and dementia in other diseases classified elsewhere (Figure 4).

3.3 Sensitivity analysis

Cochrane's Q test showed no heterogeneity among the 20 colonies except for the genus Defluviitaleaceae UCG011 ($p = 0.031$) in dementia, which had a value of p greater than 0.05 (Supplementary Table 3). Genus Defluviitaleaceae UCG011 had $p < 0.05$ ($p = 0.002$) in the fixed-effects IVW model, suggesting the presence of causality, and also $p < 0.05$ ($p = 0.034$), OR: 0.735, CI: 0.553–0.977 in the random-effects IVW model, and a cause-and-effect link was also present. The MR-Egger regression intercepts for the 21 GMs showed no horizontal pleiotropy, with p-values greater than 0.05 (Supplementary Table 4). The MR-PRESSO Global test value of $p > 0.05$ also demonstrated no horizontal pleiotropy (Supplementary Table 4). Leave-one-out results showed that phasing out any of the SNPs did not affect the overall results, so this MR analysis has good robustness (Supplementary Figure S1).

3.4 Reverse MR analysis results

Out of the 211 GMs, a total of 50 GMs affected by dementia and its subtypes were finally obtained, including 13 GMs affected by overall dementia, eight GMs by Alzheimer's disease, five GMs by vascular dementia, seven GMs by Lewy body dementia, nine GMs by Parkinson's disease, and eight GMs by dementia under the classification of other diseases. Five TSMR methods—IVW, MR-Egger, weighted median, simple mode, and weighted mode—were used to analyze the causal relationships between the different types of dementia and the 50 GMs (Supplementary Table 5). Forest plots were drawn using IVW and WM cross-validation (Figure 5). Upon comparison with the positive MR results, it was found that among the 21 GMs we focused on for causality with dementia, there was only a reverse causality between Lewy body dementia and genus Ruminococcusgnavus group (id: 14376), and no reverse causality was found between the remaining 20 GMs and dementia. Further sensitivity analysis of MR results between Lewy body dementia and genus Ruminococcusgnavus group (Table 3) was performed, and the test showed no heterogeneity or horizontal pleiotropy in this result.

3.5 Validation group MR analysis results

The first validation dataset obtained 121 IVs of GMs associated with dementia involving 10 GMs; the second obtained 77 IVs of GMs associated with dementia involving eight GMs; and the third obtained 82 IVs of GMs associated with dementia involving eight GMs. The MR analysis methodology was consistent with the above studies, and detailed information on the results can be found in Supplementary Table 6. All results passed sensitivity tests. Similarly, a forest plot of the IVW and WM cross-tests was plotted and is shown in Figure 6. Compared to the six GMs associated with the formal group any dementia, five overlapping GMs were in the first validation group and four in the second and third validation groups. Three validation groups had four GMs overlapping with the formal group any dementia, accounting for 66.7% of the formal any dementia group, 40% of the first validation group, and 50% of the second and third validation groups. Therefore, the selected formal group dataset is representative.

4 Discussion

In this study, by combining MR analysis and sensitivity analysis, 21 GMs were identified as being causally associated with dementia (any dementia, Alzheimer's disease, vascular dementia, Lewy body dementia, Parkinson's disease, and dementia in other diseases classified elsewhere). Among them, Desulfovibrionaceae, Lactobacillaceae, Ruminococcusgnavus group, Lactobacillus, Desulfovibrionales, Sellimonas, Bacillales, and Hungatella were positively associated with the risk of outcome disease, and therefore, there may be a risk for the corresponding types of dementia. Defluviitaleaceae UCG011, Bacillales, Lachnospiraceae NK4A136 group, Victivallales, Alphaproteobacteria, Ruminococcusgnavus group, Butyricimonas, Lentisphaerae, Oscillibacter, and Burkholderiales were negatively correlated with the risk of outcome disease, and they may be protective against the corresponding types of dementia.

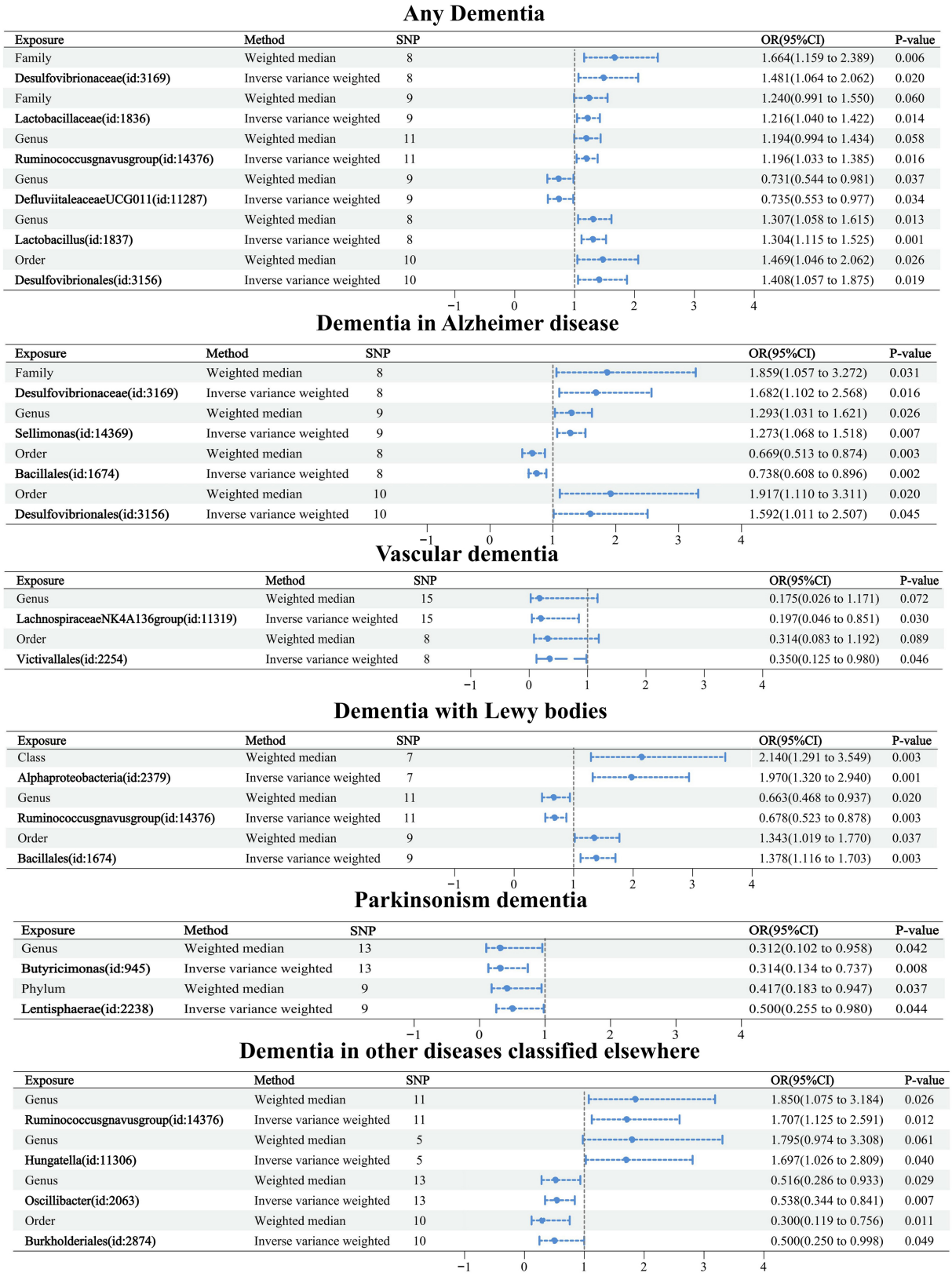

FIGURE 3
Forest plot of causal relationships between 21 GMs and dementia under cross-validation.

Understanding the pathogenesis of dementia and the role GM plays in this process is critical to preventing and intervening in dementia. The gut-brain axis is the pathway of communicating among the nervous system and the gastrointestinal tract, which mainly includes the central nervous system (CNS), enteric nervous system (ENS), hypothalamic–pituitary–adrenal axis (HPA), and

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TABLE 2 Causal relationship between intestinal flora and dementia and its classified diseases.

Outcome	Exposure	Method	SNPs	*p*-value	OR	95%CI
Any dementia	Family Desulfovibrionaceae (id:3169)	WM	8	0.006	1.664	1.159–2.389
		IVW		0.02	1.481	1.064–2.062
	Family Lactobacillaceae (id:1836)	WM	9	0.06	1.240	0.991–1.550
		IVW		0.014	1.216	1.040–1.442
	Genus Ruminococcusgnavus group (id:14376)	WM	11	0.058	1.194	0.994–1.434
		IVW		0.016	1.196	1.033–1.385
	Genus Defluviitaleaceae UCG011 (id:11287)	WM	9	0.037	0.731	0.544–0.981
		IVW		0.034	0.735	0.553–0.977
	Genus Lactobacillus (id:1837)	WM	8	0.013	1.307	1.058–1.615
		IVW		0.001	1.304	1.115–1.525
	Order Desulfovibrionales (id:3156)	WM	10	0.026	1.469	1.046–2.062
		IVW		0.019	1.408	1.057–1.875
Alzheimer's disease	Family Desulfovibrionaceae (id:3169)	WM	8	0.031	1.057	1.859–3.272
		IVW		0.016	1.102	1.682–2.568
	Genus Sellimonas (id:14369)	WM	9	0.026	1.031	1.293–1.621
		IVW		0.007	1.068	1.273–1.518
	Order Bacillales (id:1674)	WM	8	0.003	0.513	0.669–0.874
		IVW		0.002	0.608	0.738–0.896
	Order Desulfovibrionales (id:3156)	WM	10	0.02	1.110	1.917–3.311
		IVW		0.045	1.011	1.592–2.507
Vascular dementia	Genus Lachnospiraceae NK4A136 group (id:11319)	WM	15	0.072	0.026	0.171–1.175
		IVW		0.03	0.046	0.197–0.851
	Order Victivallales (id:2254)	WM	8	0.089	0.083	0.314–1.192
		IVW		0.046	0.125	0.350–0.980
Lewy body dementia	Class Alphaproteobacteria (id:2379)	WM	7	0.003	1.291	2.140–3.549
		IVW		0.001	1.320	1.970–2.940
	Genus Ruminococcusgnavus group (id:14376)	WM	11	0.02	0.468	0.663–0.937
		IVW		0.003	0.523	0.678–0.878
	Order Bacillales (id:1674)	WM	9	0.037	1.019	1.343–1.770
		IVW		0.003	1.116	1.378–1.703
Parkinson's disease	Genus Butyricimonas (id:945)	WM	13	0.042	0.102	0.312–0.958
		IVW		0.008	0.134	0.314–0.737
	Phylum Lentisphaerae (id:2238)	WM	9	0.037	0.183	0.417–0.947
		IVW		0.044	0.255	0.500–0.980
Dementia in other diseases classified elsewhere	Genus Ruminococcusgnavus group (id:14376)	WM	11	0.026	1.075	1.850–3.184
		IVW		0.012	1.125	1.707–2.591
	Genus Hungatella (id:11306)	WM	5	0.061	0.974	1.795–3.308
		IVW		0.04	1.026	1.697–2.809
	Genus Oscillibacter (id:2063)	WM	13	0.029	0.286	0.516–0.933
		IVW		0.007	0.344	0.538–0.841
	Order Burkholderiales (id:2874)	WM	10	0.011	0.119	0.3–0.756
		IVW		0.049	0.250	0.5–0.998

autonomic nervous system (ANS; Cryan et al., 2019). Moreover, it has been found that GM can influence the pathophysiological processes of diseases such as Alzheimer's disease and Parkinson's through ENS (Glinert et al., 2022). For example, it activates the ENS and uses the vagus nerve as a pathway to communicate with the brain (Wang et al., 2020). In addition, GM triggers the

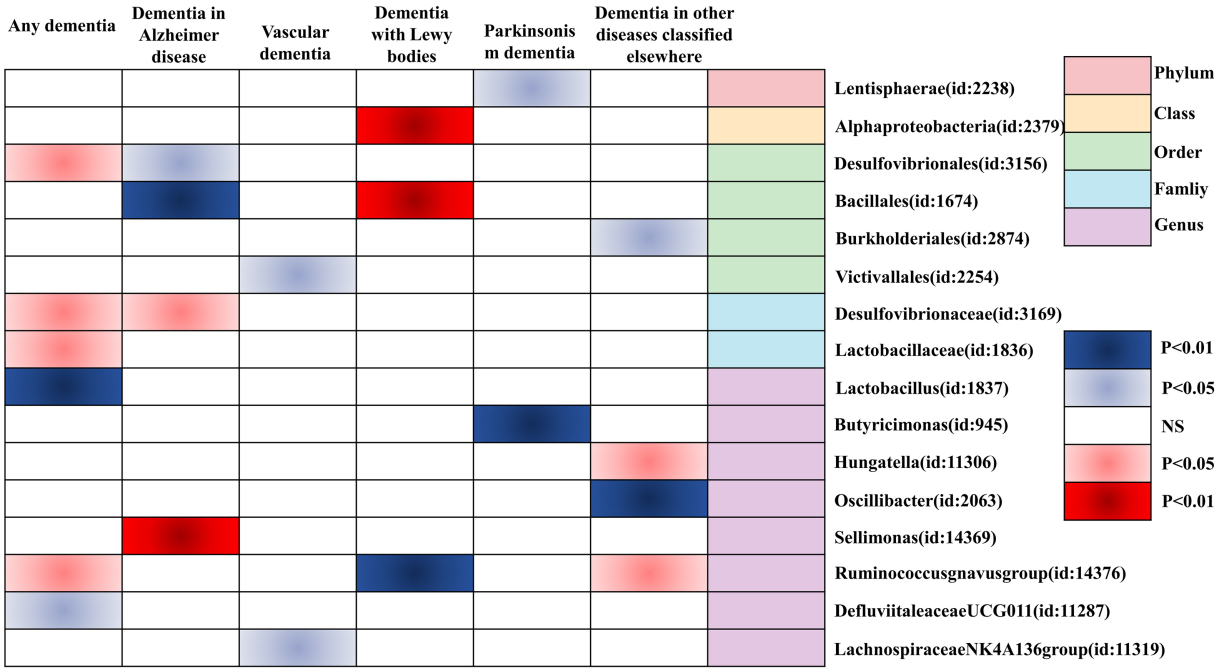

FIGURE 4
Heatmap of GM causally associated with dementia identified by the IVW method. Red represents risk factors, and blue represents protective factors.

progression of a metabolic, inflammatory response that promotes neuroinflammation by engaging in processes that disrupt the blood–brain barrier (BBB), activating astrocytes and microglia, and leading to the deposition of β-amyloid (Aβ), which is now recognized as a significant contributing factor in neurodegenerative diseases (Cryan et al., 2019; Wei et al., 2020; Mou et al., 2022). Relevant scholars have proven that the majority of the variable risk factors for dementia are associated with GM alterations by studying the different variable risk factors for each type of dementia and the different roles of GM for each factor (Cabrera et al., 2021). However, the constitution of GM is subject to the effect of numerous causes, and the diversity of GM may vary due to inconsistencies in gender, ethnicity, and environment.

In our study, we found that order Desulfovibrionales (OR: 1.592, CI: 1.011–2.507, $p = 0.045$) and family Desulfovibrionaceae (OR: 1.682, CI: 1.102–2.568, $p = 0.016$) were strongly related to a high risk of Alzheimer's disease. The results of related experiments showed that Desulfovibrionaceae abundance at the family and genus levels was significantly higher in amyloid precursor protein transgenic mice than in wild mice (Shen et al., 2017). Abnormal production and processing of Aβ and hyperphosphorylation of tau proteins are the molecular signatures of Alzheimer's disease (He et al., 2020). GM has been shown to reduce Aβ load in patients with Alzheimer's disease (Li et al., 2019), and related researchers have found that brain Aβ accumulation is negatively correlated with the family Desulfovibrionaceae (Sheng et al., 2022). *Tetragonia Tetragonioides* Kuntze (TTK) ameliorates memory by decreasing Aβ deposition and modulating GM, with more Desulfovibrionales in the AD-Control group than AD-TTK (Kim et al., 2020). The above studies mentioned the family/order Desulfovibrionales as clinically significant for Alzheimer's disease. However, the results of the two studies on Aβ deposition in the brain conflicted. In the present study, we found with MR analysis that the family/order Desulfovibrionaceae was associated with an increased risk of developing dementia and Alzheimer's disease. A growing body of research has been able to demonstrate that altering GMs can attenuate microglia-mediated neuroinflammation and reduce Aβ deposition in the brain, thereby improving cognition (Abraham et al., 2019; Sun et al., 2020; Benichou Haziot and Birak, 2023). Supporting the above findings, we propose that the effect of family/order Desulfovibrionaceae on patients of dementia or Alzheimer's disease might be related to brain Aβ deposition. However, the exact mechanism of action has not yet been confirmed. Inhibition of patient-specific family/order Desulfovibrionaceae and further study of its pathogenesis based on this may become a new way of intervention to prevent or delay Alzheimer's disease.

Probiotics are non-pathogenic microorganisms and are beneficial to the organism's health, with a great capacity to rebuild the microbiota and restore health (Den et al., 2020). Notably, probiotic treatment attenuates age-related learning and memory deficits by reducing microglia activation (Go et al., 2021). Therefore, it has been used as a potential treatment to alleviate psychiatric disorders, including cognitive impairment (CI; Azad et al., 2018). Lactobacillales as a probiotic has been widely used in various CI-related studies, and a study was conducted to induce the expression of brain-derived neurotropic factor (BDNF), inhibit NF-κB activation, and regulate GM in mice to alleviate CI accompanied by systemic inflammation through Lactobacillus griseus (Yun et al., 2023). A systematic evaluation showed increased levels of brain-derived neurotrophic factor, improved inflammatory profile, and cellular biomarker modulation in patients with dementia taking probiotic Lactobacillus (Ruiz-Gonzalez et al., 2021). In addition, the AD-Control group with

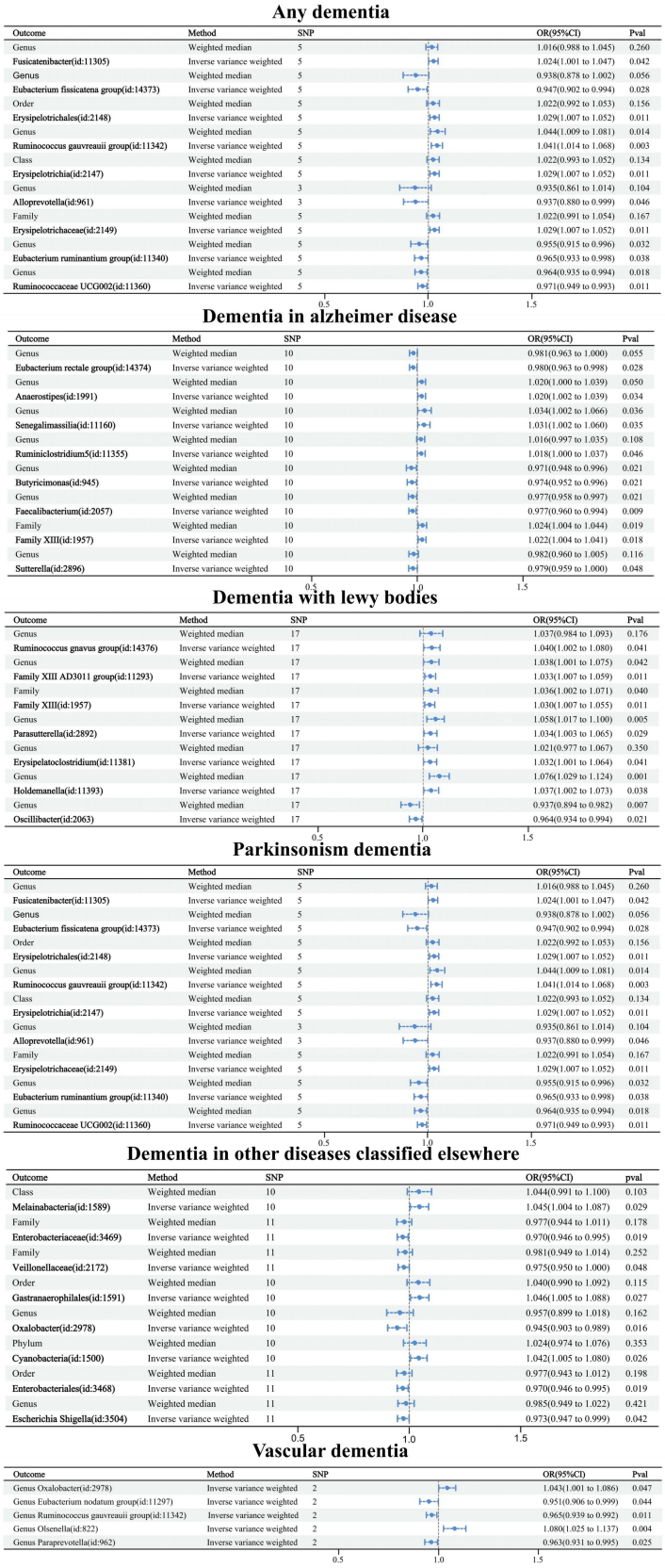

FIGURE 5
Forest plot of causal relationships between dementia and 50 GMs under cross-validation.

TABLE 3 Sensitivity test of DLB with genus Ruminococcusgnavus group.

Exposure	Outcome	Method	Q	Value of *p*	MR-Egger intercept test			MR-PRESSO global test	
					Egger-intercept	SE	Value of *p*	RSS obs	Value of *p*
Lewy body dementia	Genus Ruminococcusgnavus group	MR-Egger	14.264	0.506	0.003	0.010	0.792	15.438	0.635
		IVW	14.336	0.574					

excessive brain Aβ deposition decreased in the order Lactobacillales (Lactobacillales) compared to the AD-TTK group (Kim et al., 2020). A Mediterranean diet (MeDi) containing very high amounts of Lactobacillales has also been highly effective in preventing Alzheimer's disease (Trichopoulou and Lagiou, 1997; Walker, 2000; Alfawaz and Aljumah, 2012). It is believed that the evidence that Lactobacillus reduces blood ammonia levels not only offers a connection between Alzheimer's disease and the MeDi but also lays the groundwork for hyperammonemia and the pharmacology of various neurological disorders (Alfawaz and Aljumah, 2012; Jin et al., 2018). The above research demonstrated the protective function of Lactobacillales in CI from different angles of action. However, our results showed that the family Lactobacillaceae and genus Lactobacillus were weakly correlated with the increased risk of dementia. The reason may be related to sample size, genetics, and research scope.

In addition, our results also showed causal associations with outcomes for probiotics, including Defluvititaleaceae UCG011 associated with dementia, Bacillale associated with Alzheimer's disease, Ruminococcusgnavus group associated with Lewy body dementia, Lachnospiraceae NK4A136 group and Victivallales strongly associated with vascular dementia, Butyricimonas and Lentisphaerae strongly associated with Parkinson's disease, and Oscillibacter and Burkholderiales strongly associated with dementia in other diseases classified elsewhere. Some of these results are consistent with existing research findings where Bacillussubtilis was shown to have a protective effect on neurons and behavior in the *Caenorhabditis elegans* AD model and can help alleviate Alzheimer's disease (Cogliati et al., 2020). Butyricimona has also been shown to be strongly associated with the reduced hippocampal volume associated with cognitive disorder. Jang hypothesized that acupuncture alleviated inflammation in mice with Parkinson's disease due to an increase in Butyricimonas (Jang et al., 2020; Liang et al., 2022). Neoagarotetraose (NAT) was shown to modulate GM and thereby attenuate brain damage in mice with Alzheimer's disease, with a remarkable rise of intestinal bacterial genera (Lactobacillus, Butyricimonas, and Akkermansi) observed after NAT treatment (Li et al., 2023). Our study clarified the beneficial bacterial genera for dementia, Alzheimer's disease, Parkinson's disease, and Lewy body dementia. This might be a novel research line for the clinical therapy of various types of dementia.

Short-chain fatty acids (SCFAs), which mainly include acetate, propionate, and butyrate, are metabolites produced by GM. Butyrate in SCFAs has anti-inflammatory effects (Mirzaei et al., 2021) and can improve cognitive function by mediating inflammatory responses and inducing Aβ phagocytosis in microglia (Xie et al., 2023). It has been found that Alzheimer's disease may occur when butyrate is deficient (Tran et al., 2019). Interestingly, propionate induced higher levels of microglia activation than butyrate (Hou et al., 2021), and this hyperactivated state may reduce their ability to phagocytose Aβ, which may have a differential effect on the disease (Xie et al., 2021). When excessive propionate is ingested, there is an increased risk of developing Alzheimer's disease (Killingsworth et al., 2021). Ruminococcaceae can promote the production of SCFAs and can be associated with diseases of cognitive dysfunction by affecting the expression of proteins involved in neurotransmission (D'Amato et al., 2020). This study showed that the Ruminococcusgnavus group was associated with a risk of dementia, Lewy body dementia, and dementia in other diseases classified elsewhere. However, its high and low risk of different outcome diseases was inconsistent, and we hypothesized that this might be related to the metabolite SCFAs it produces. The different types and doses of SCFAs might be the influencing factors. In addition, the results of the reverse MR analysis done in this study suggested that elevated levels of Ruminococcusgnavus group were associated with an increased risk of Lewy body dementia. Therefore, the present study provides possible mechanism points of SCFAs for dementia at the microbial level, and its specific role and association need to be further explored.

In addition, this study found a strong risk association between Alphaproteobacteria and Lewy body dementia [OR = 1.97 (95% CI: 1.320–2.940) $p = 0.001$]. It has been shown that GM is associated with Lewy body dementia, a pathology of dementia characterized by aggregation of α-synuclein, in which the microbe-gut-brain axis plays a vital role through a variety of potential mechanisms (Ryman et al., 2023). However, research into the relationship between Alphaproteobacteria and Lewy body dementia is scarce; Alphaproteobacteria is usually associated with depression, and antidepressants can reduce their abundance (Lukić et al., 2019). Therefore, the conclusion of this study provides suggestions for future research areas with regard to Alphaproteobacteria for the treatment of Lewy body dementia, which may be the key mechanism of its pathogenesis or a potential therapeutic target.

Current research on GM and various types of dementia is both a hot topic and a great challenge at the same time. Since there is no method of preventing, reversing, or eradicating Alzheimer's disease, medications licensed for the therapy of Alzheimer's disease have only been able to slow progression to improve symptoms (Breijyeh and Karaman, 2020). Therefore, in terms of GM and dementia, future research should focus on identifying specific GM bacteria with the pathogenesis of dementia. On the one hand, different GM taxa may have diagnostic value for various types of dementia. On the other hand, the risk of dementia can be reduced through the development of new drugs, disease prevention, treatment, and other aspects.

The limitations of this study are as follows: (i) since the number of IVs satisfying the strict threshold ($p < 5 \times 10^{-8}$) was minimal, a relatively loose threshold ($p < 1 \times 10^{-5}$) was used to screen the IVs; (ii) in this study, part of the data for dementia was obtained in 2021 from

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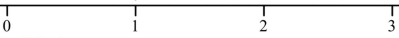
First validation group

Exposure	Method	SNP	OR(95%CI)	pval
Phylum	Inverse variance weighted	12	1.228(1.004 to 1.502)	0.046
Tenericutes(id:3919)	Weighted median	12	1.319(1.014 to 1.715)	0.039
Class	Inverse variance weighted	19	1.189(1.014 to 1.393)	0.033
Actinobacteria(id:419)	Weighted median	19	1.178(0.937 to 1.480)	0.161
Class	Inverse variance weighted	11	1.339(1.009 to 1.777)	0.043
Deltaproteobacteria(id:3087)	Weighted median	11	1.425(1.012 to 2.007)	0.042
Class	Inverse variance weighted	12	1.228(1.004 to 1.502)	0.046
Mollicutes(id:3920)	Weighted median	12	1.319(1.013 to 1.717)	0.040
Order	Inverse variance weighted	10	1.426(1.067 to 1.905)	0.016
Desulfovibrionales(id:3156)	Weighted median	10	1.433(1.009 to 2.035)	0.044
Family	Inverse variance weighted	8	1.521(1.094 to 2.116)	0.013
Desulfovibrionaceae(id:3169)	Weighted median	8	1.680(1.155 to 2.442)	0.007
Genus	Inverse variance weighted	11	1.188(1.024 to 1.380)	0.023
Ruminococcusgnavusgroup(id:14376)	Weighted median	11	1.193(0.982 to 1.450)	0.076
Genus	Inverse variance weighted	18	1.173(1.002 to 1.374)	0.048
Bifidobacterium(id:436)	Weighted median	18	1.116(0.891 to 1.398)	0.339
Genus	Inverse variance weighted	8	1.287(1.098 to 1.509)	0.002
Lactobacillus(id:1837)	Weighted median	8	1.237(0.994 to 1.540)	0.057
Genus	Inverse variance weighted	12	0.793(0.648 to 0.970)	0.024
RuminococcaceaeUCG003(id:11361)	Weighted median	12	0.776(0.597 to 1.008)	0.057



Second validation group

Exposure	Method	SNP	OR(95%CI)	Pval
Order	Inverse variance weighted	10	1.405(1.083 to 1.825)	0.0110
Desulfovibrionales(id:3156)	Weighted median	10	1.596(1.166 to 2.185)	0.0040
Order	Inverse variance weighted	7	0.763(0.589 to 0.988)	0.0400
Enterobacteriales(id:3468)	Weighted median	7	0.744(0.531 to 1.043)	0.0860
Family	Inverse variance weighted	8	1.465(1.074 to 1.998)	0.0160
Desulfovibrionaceae(id:3169)	Weighted median	8	1.797(1.298 to 2.486)	0.0004
Family	Inverse variance weighted	7	0.763(0.589 to 0.988)	0.0400
Enterobacteriaceae(id:3469)	Weighted median	7	0.744(0.535 to 1.035)	0.0790
Family	Inverse variance weighted	16	1.282(1.053 to 1.560)	0.0130
Lachnospiraceae(id:1987)	Weighted median	16	1.237(0.962 to 1.591)	0.0970
Genus	Inverse variance weighted	15	0.832(0.703 to 0.983)	0.0310
Eubacteriumhalliigroup(id:11338)	Weighted median	15	0.870(0.696 to 1.087)	0.2210
Genus	Inverse variance weighted	6	1.377(1.006 to 1.884)	0.0460
Lachnospira(id:2004)	Weighted median	6	1.333(0.908 to 1.956)	0.1430
Genus	Inverse variance weighted	8	1.251(1.083 to 1.445)	0.0020
Lactobacillus(id:1837)	Weighted median	8	1.184(0.967 to 1.451)	0.1030

Third validation group

Exposure	Method	SNP	OR(95%CI)	pval
Order	Inverse variance weighted	10	1.358(1.037 to 1.778)	0.026
Desulfovibrionales(id:3156)	Weighted median	10	1.517(1.116 to 2.064)	0.008
Order	Inverse variance weighted	7	0.757(0.585 to 0.979)	0.034
Enterobacteriales(id:3468)	Weighted median	7	0.755(0.540 to 1.055)	0.100
Family	Inverse variance weighted	8	1.397(1.010 to 1.932)	0.044
Desulfovibrionaceae(id:3169)	Weighted median	8	1.720(1.203 to 2.459)	0.003
Family	Inverse variance weighted	7	0.757(0.585 to 0.979)	0.034
Enterobacteriaceae(id:3469)	Weighted median	7	0.755(0.539 to 1.056)	0.101
Family	Inverse variance weighted	16	1.278(1.052 to 1.554)	0.014
Lachnospiraceae(id:1987)	Weighted median	16	1.337(1.018 to 1.755)	0.037
Genus	Inverse variance weighted	15	0.836(0.707 to 0.987)	0.035
Eubacteriumhalliigroup(id:11338)	Weighted median	15	0.882(0.696 to 1.118)	0.299
Genus	Inverse variance weighted	8	1.244(1.077 to 1.436)	0.003
Lactobacillus(id:1837)	Weighted median	8	1.200(0.988 to 1.457)	0.066
Genus	Inverse variance weighted	11	1.211(1.005 to 1.459)	0.044
RuminococcaceaeUCG014(id:11371)	Weighted median	11	1.075(0.831 to 1.392)	0.580

FIGURE 6
Forest plot of causal relationships between GMs and three validation groups under cross-validation.

the FinnGen database version R5, the most recent online data for the IEU data. Nevertheless, there are still limitations regarding the duration of data collection and the quantity of available data. Further supplementation of the results of this study is warranted in the future through the ongoing updating of online data; and (iii) the number of cases of strictly defined as vascular dementia and Parkinson's disease

is relatively low, so a more significant amount of GWAS pooled data is needed for future analysis.

5 Conclusion

Altogether, we confirmed a causal relationship between GM and dementia and its subtypes based on Mendelian randomization, including family Desulfovibrionaceae (id: 3169), family Lactobacillaceae (id: 1836), genus Ruminococcusgnavus group (id: 14376), genus Defluviitaleaceae UCG011 (id: 11287), genus Lactobacillus (id: 1837), order Desulfovibrionales (id: 3156), family Desulfovibrionaceae (id: 3169), genus Sellimonas (id: 14369), order Bacillales (id: 1674), order Desulfovibrionales (id: 3156), genus Lachnospiraceae NK4A136 group (id:11319), order Victivallales (id: 2254), class Alphaproteobacteria (id: 2379), genus Ruminococcusgnavus group (id: 14376), order Bacillales (id: 1674), genus Butyricimonas (id: 945), phylum Lentisphaerae (id: 2238), genus Ruminococcusgnavus group (id: 14376), genus Hungatella (id: 11306), genus Oscillibacter (id: 2063), and order Burkholderiales (id: 2874). These 21 GMs hold promise as novel markers for the future diagnosis of dementia and its subtypes, as well as new targets for therapy.

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.

Author contributions

JF: Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. YQ: Formal analysis, Investigation, Visualization, Writing – original draft. LX: Funding acquisition, Project administration, Writing – review & editing. XD: Funding acquisition, Project administration, Resources, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1306048/full#supplementary-material

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The role of the peripheral system dysfunction in the pathogenesis of sepsis-associated encephalopathy

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Sepsis is a condition that greatly impacts the brain, leading to neurological dysfunction and heightened mortality rates, making it one of the primary organs affected. Injury to the central nervous system can be attributed to dysfunction of various organs throughout the entire body and imbalances within the peripheral immune system. Furthermore, central nervous system injury can create a vicious circle with infection-induced peripheral immune disorders. We collate the pathogenesis of septic encephalopathy, which involves microglial activation, programmed cell death, mitochondrial dysfunction, endoplasmic reticulum stress, neurotransmitter imbalance, and blood–brain barrier disruption. We also spotlight the effects of intestinal flora and its metabolites, enterocyte-derived exosomes, cholinergic anti-inflammatory pathway, peripheral T cells and their cytokines on septic encephalopathy.

KEYWORDS

sepsis-associated encephalopathy, glial cells, intestinal flora, peripheral immune cells, exosomes

1 Introduction

The brain is often considered the first organ to be exposed to inflammation. Sepsis, a condition characterized by an uncontrolled immune response to infection, is defined as life-threatening organ dysfunction. Within this context, the occurrence of brain injury emerges as a frequently observed complication (Singer et al., 2016). Sepsis-associated encephalopathy (SAE) refers to a condition characterized by widespread cognitive impairment resulting from the systemic inflammatory reaction triggered by diverse infections. The main manifestation is an altered state of consciousness (Molnár et al., 2018). High prevalence of SAE is observed, reaching 70%, in patients suffering from severe systemic infection while admitted to the intensive care unit (Gofton and Young, 2012). SAE is closely associated with increased mortality, high hospital costs and prolonged hospital stays, persistent cognitive impairment and limited physical function (Ren et al., 2020). The underlying mechanism of SAE remains

undetermined. Current studies suggest that SAE may be caused by inflammation activated by endothelial/ microglia, increased blood–brain barrier permeability, hypoxia, neurotransmitter imbalance, loss of axons and neurons. The clinical treatment of SAE mainly centers on the rapid optimization of antibiotics, such as statins, levodopa/ benserazide, and bactericidal non-dissolving antibiotics. Animal experiments focus on regulating inflammation, stabilizing of the blood–brain barrier and rehabilitating mitochondrial function (Tauber et al., 2021).

The central nervous system (CNS) is widely recognized as an essential component in maintaining the proper functioning of the immune system. It can manipulate systemic inflammatory signals and immune regulation through multiple pathways, including cholinergic anti-inflammatory pathway (Olofsson et al., 2012). SAE-induced brain dysfunction can lead to abnormal responses of multiple neuroendocrine immune networks. Brain injury is a crucial factor in the prognosis and survival of sepsis patients and should be acknowledged not only as an affected organ but also as a significant contributor to the compromised immune regulation resulting from sepsis (Lee et al., 2010). In SAE research, two main models are commonly used. The first model involves cecal ligation and puncture (CLP), while the second model involves intraperitoneal injection of a specific dose of lipopolysaccharide (LPS). The brain regions that have received the most attention in these models are the cerebral cortex and hippocampus. Therefore, we have summarized the changes observed in these brain regions, as well as in the intestines, liver, and peripheral immune system, in the SAE model. Furthermore, relevant clinical studies are also discussed. In short, this paper summarizes the existing studies on the pathogenesis of sepsis on the one hand, and explores the interactions between the brain, intestine, liver and peripheral immune system on the other hand. Furthermore, it can provide theoretical basis for the clinical treatment of SAE.

2 Pathogenesis of sepsis-associated encephalopathy

2.1 Neuroinflammation

Sepsis-induced central nervous system (CNS) neuroinflammation has been considered as an underlying mechanism of delayed cognitive impairment (Xing et al., 2018). Systemic inflammation during sepsis spreads to the brain through damaged blood–brain barrier (BBB) (Obermeier et al., 2013), thereby activating resting glial cells. Activated glial cells can be found even under circumstances where there is no obvious BBB damage (Griton et al., 2020). These cells release inflammatory factors, which aggravate neuroinflammation, thus forming a vicious circle (Faraco et al., 2007). Microglia, which are crucial components of the innate immune system in the brain, serve as significant contributors to the production of pro-inflammatory cytokines. Consequently, they gain primary attention in the investigation of neuroinflammation. Microglia can be swiftly triggered by diverse stimuli, encompassing infectious or pathological stimuli, along with Aβ peptides. Once activated, microglia have the capability to release substantial quantities of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β. This augmented neuroinflammation within the brain exacerbates neuronal harm, leading to the manifestation of SAE's behavioral and psychological symptoms (Ye et al., 2019). A prospective autopsy study was conducted to compare microglia numbers in specific brain regions between 16 well-characterized patients with septic shock and 15 controls. The study revealed that patients who died during septic shock and had systemic inflammation exhibited higher numbers of CD-68 microglia in the putamen, cerebellum, and hippocampus. This finding suggests that severe systemic inflammation triggers a neuroinflammatory response characterized by the activation of microglia (Westhoff et al., 2019).

The receptors on the surface of neuroglial cell have been found to be involved in the occurrence of neuroinflammation. The study found that both astrocytes and microglia (increased Iba-1 and C3) were activated in SAE. Intracerebroventricular administration of BRL-44408, a specific antagonist of α2A adrenergic receptors, to CLP mice led to the upregulation of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, in the hippocampus. Neuroinflammation was effectively suppressed by dexmedetomidine, a remarkably selective α2-adrenoceptor agonist. However, the therapeutic effect of dexmedetomidine remained intact even after depleting microglia. This indicates the crucial involvement of astrocytes, particularly in the expression of α2A-adrenoceptors, rather than microglia in neuroinflammation (Mei et al., 2021). In SAE, the NLRP3 inflammasome in microglia is activated and promotes cleavage of procaspase-1. Mature caspase-1 cleaves pro-IL-1β to IL-1β, thereby promoting neuroinflammation (Swanson et al., 2019). A neuroprotective molecule, indole-3-propionic acid (IPA), inhibited the levels of NLRP3 and IL-1β in the cortex. Vitro experiments found that IPA can inhibit the increase in levels of NLRP3 and IL-1β in primary microglial cells stimulated by LPS. The therapeutic effect of IPA was weakened by an aryl hydrocarbon receptor (AhR) inhibitor (CH223191). This suggests that AhR on microglia may play a crucial role in neuroinflammation (Fang et al., 2022). Furthermore, microglia are the major resident immune cells in the CNS. These unique cells possess the remarkable capability to undergo morphological and functional adjustments in response to alterations in their surrounding microenvironment. In instances of sepsis, a transition from a state of "surveillance" to a pro-inflammatory M1 phenotype occurs. This change prompts the release of inflammatory signals, as evidenced by observations in the hippocampus of CLP mice and in primary microglia cultures stimulated with LPS (Shen et al., 2021). Moreover, in addition to glial cells, many immune cells play regulatory roles in infection-induced neuroinflammation. The brain of mice displayed the infiltration of neutrophils and T cells following sepsis for 10 days. This infiltration was accompanied by an augmentation of regulatory T cells (Treg) and Th2 cells, which played a crucial role in the mitigation of SAE (Saito et al., 2021).

The interplay between oxidative stress and inflammatory responses is evident. The inflammatory response triggers oxidative stress, which in turn intensifies the inflammatory response (Liu et al., 2023). Notably, oxidative stress plays a pivotal role in the pathophysiology of SAE. Analysis of brain tissue sections of CLP mice using fluorescent double staining exhibits the co-localization of the microglia marker protein IBA-1 with the protein inducible nitric oxide synthase (i-NOS) and Cyclooxygenase-2 (COX-2). The Nrf2/HO-1 pathway is a crucial mechanism for combating oxidative stress. When there is oxidative stress, Nrf2 is activated to regulate the gene expression of antioxidant proteins in cells. HO-1, a downstream target protein of Nrf2, also functions as an antioxidant (Ali et al., 2018; Korytina et al., 2019). The Western blot analysis revealed a moderate

upregulation of Nrf2 and HO-1 expression in primary microglia treated with LPS. However, the levels of Nrf2 and HO-1 were significantly increased upon administration of sodium butyrate. These findings suggest that the Nrf2/HO-1 pathway plays a crucial role in regulating oxidative stress in microglia (Zhang H. et al., 2022). Nrf2 also inhibits the activation of the NLRP3 pathway in glial cells, thus leading to a reduction in neuroinflammation (Xie K. et al., 2020). In another animal experiment, little damage was observed to the BBB in rats with CLP after 24 h, suggesting that peripheral inflammatory cytokines such as IL-1β may not be able to enter the CNS at an early stage. The expression of IL-1β was upregulated in microglia and cerebral microvascular endothelial cells (CMECs), as detected by Western blot, ELISA and immunofluorescence co-localization techniques. The use of Fisetin to reduce IL-1 expression in CMECs limits its binding to IL-1R1 in microglia. This supports that IL-1β in microglia may originate from CMECs. IL-1R1/pNF-κB pathway further activates microglia, leading to neuroinflammation and cognitive impairment (Ding et al., 2022). Foxc1 is a transcription factor that can suppress oxidative stress and inflammation (Xia et al., 2019). In a study, it was discovered that the levels of Foxc1 and IκBα were reduced in the hippocampus of CLP mice. However, in mice with excessive Foxc1 expression, the overexpression of Foxc1 was found to hinder the migration of microglial cells, inflammation, and neuronal apoptosis in the hippocampus of CLP mice. This effect was achieved through the IκBα/NF-κB pathway. In addition, the expressions of Foxc1 and NF-κB inhibitor (IκBα) were significantly decreased, while the expressions of p65, IL-1β, and TNF-α were markedly elevated in microglia treated with LPS. After overexpressing Foxc1 through adenovirus transfection, the expression of IκBα was upregulated, while that of p65, IL-1β and TNF-α was downregulated. In addition, knockdown of IκBα resulted in a significant increase in IL-1β and TNF-α levels, as well as promotion of microglial migration. It was indicated that the diminished presence of Foxc1 within LPS-exposed microglia fosters neuroinflammation through the suppression of IκBα and the initiation of the NF-κB pathway (Wang et al., 2022b).

MicroRNAs (miRNAs) are a widely distributed group of small, non-coding RNA molecules that are single-stranded and have a crucial role in various pathological conditions (Fabbri et al., 2012). The study findings demonstrated a significant increase in RNA and miRNA levels in the plasma of CLP mice for seven consecutive days. This increase specifically included miR-146a, miR-145, miR-34a, and miR-122. *In vitro* experiments conducted on cultured microglia and astrocytes demonstrated a substantial and proportional reaction of IL-6 and CXCL2 upon exposure to synthetic miR-146a, miR-145, miR-34a, or miR-122. Furthermore, the absence of the TLR7 gene displayed a significant reduction in the expression of cytokines and activation of microglia. It was also observed that TLR7 was more prevalent in microglia in comparison to astrocytes. These studies propose that the activation of the miR-146a-5p stimulates the innate immune responses in the brain through the TLR7 pathway in microglia (Zou et al., 2022).

Autophagy is a process of catabolism that is evolutionarily conserved, aiming to recycle proteins and organelles that are damaged or become senescent. By increasing autophagy, there is a compensatory response that intends to restrict sepsis-driven harm to tissues (Tanida et al., 2008). Inhibiting mTOR can promote autophagy, which affects immune response and cytokine secretion processes (Weichhart, 2018). The study observed an increase in TNF-α, IL-6, HMGB1, and M1 type microglia in the hippocampus of CLP mice. The ratio of p-mTOR/mTOR and the expression of p62 were upregulated in LPS-treated microglia (BV-2 cells). Hydrogen-rich medium can regulate microglial polarization and reduce neuroinflammation through mTOR inhibition and autophagy (Zhuang et al., 2020). The findings from an another investigation in CLP mice models and primary microglia cultures indicated that sepsis elevated the expression of hippocampal CXCR5, leading to incomplete initiation of autophagy, polarization of microglia towards the M1 phenotype, generation of inflammatory cytokines, and manifestation of cognitive impairments. The downregulation of CXCR5 serves to reinstate autophagy, shift microglia towards an M2 phenotype, and suppress p38MAPK/NF-κB/STAT3 signaling, ultimately mitigating sepsis-induced neuroinflammation and cognitive deficits (Shen et al., 2021). Limited knowledge exists regarding the involvement of autophagy in the degradation of nuclear components in SAE. A study has identified the co-localization of the autophagy marker LC3B with the nuclear marker laminin B1 in the hippocampus of septic mice, suggesting the presence of nuclear autophagy (Xie et al., 2022).

2.2 Neuronal damage

Pyroptosis, which is also recognized as cellular inflammatory necrosis, manifests through the ongoing enlargement of cells until the point when the cell membrane ruptures, leading to the discharge of cellular components and initiation of an intense inflammatory reaction (Jorgensen and Miao, 2015). An overabundance of pyroptosis can pose a threat to the overall well-being of tissues and cells. In order to safeguard the host organism against harm, the regulation of pyroptosis is carried out with precision, with the involvement of inflammatory caspases like caspase-1, caspase-4, caspase-5, and caspase-11. It is generally considered to be induced by the cleavage of GSDMD by caspase-1 and other caspases. The study found that the downregulation of caspase-1 can inhibit GSDMD and its cleaved form GSDMD-NT expression, reduce brain pyroptosis and protect synaptic plasticity (Xu et al., 2019). Moreover, the investigation revealed a notable escalation in the quantity of NLRP3 and caspase-1 positive cells within the CA1 area of the murine cerebral cortex following a span of 7 days of CLP, as illustrated by means of immunohistochemical analyses. Conversely, Inhibition of NLRP3 (using MCC950) or caspase-1 (using Ac-YVAD-CMK) modulates pyroptosis by regulating GSDMD expression, and inflammatory responses by regulating IL-1β and IL-18 expression, in the hippocampus of CLP mice, thereby alleviating its impact. These findings solidify the proposition that sepsis can prompt the activation of the NLRP3 inflammasome pathway, consequently leading to the activation of caspase-1 and instigation of inflammatory cascade reactions and pyroptosis (Fu et al., 2019). NLRP3 has the capability to trigger apoptosis and pyroptosis via an intricate molecular mechanism. Maf1 serves as a conserved inhibitor of RNA polymerase (pol) III (RNAP III). Studies have shown that overexpression of Maf1 directly binds to the NLRP3 promoter, resulting in inhibition of NLRP3 inflammasome formation and pro-inflammatory protein release. Furthermore, Maf1 competitively inhibits the binding of NF-κB/p65 to the NLRP3 promoter. This inhibits the expression of inflammation-activated pyroprotein (GSDMD), upregulates the levels of antiapoptotic protein Bcl-2, and downregulates proapoptotic protein Bax, thereby

ameliorating NLRP3 inflammasome-induced apoptosis and pyrolysis (Chen S. et al., 2020).

Ferroptosis is a type of cell death and instead relies on the buildup of iron within cells, resulting in higher levels of harmful lipid peroxide ROS. Studies have found that SAE triggers hippocampal ferroptosis, which involves an increase in ROS, iron content, as well as malondialdehyde (MDA). Additionally, there is a decrease in glutathione (GSH) levels, and changes in the expression of ferroptosis-associated proteins (GPX4, ACSL4, and SLC7A11). Ferroptosis in hippocampal cells also triggers the recruitment of microglia, promoting an inflammatory microenvironment in SAE (Wang J. et al., 2022). Exosomes, which range in size from 30 to 150 nm, are small vesicles composed of a lipid membrane that carry a diverse array of complex molecules, including proteins and various forms of RNA, both coding and non-coding. NEAT1 binds to hsa-miR-9-5p, which targets the genes for transferrin receptor (TFRC) and glutamate-oxaloacetate transaminase 1 (GOT1). The transferrin receptor TFRC is responsible for transporting iron ions from outside the cell (Gammella et al., 2017; Xie et al., 2019). Recent research has revealed that sepsis can stimulate a substantial increase in exosome-associated lncRNA NEAT1. Moreover, these exosomes play a crucial role in transporting NEAT1 across the blood–brain barrier (BBB) and into the cerebral cortex. The implication of this mechanism is that it contributes to the exacerbation of severe acute encephalopathy (SAE) by inducing ferroptosis, a specific type of cell death, in brain microvascular endothelial cells. This damaging effect is achieved through the regulation of miR-9-5p, TFRC, and GOT1 axis (Wei et al., 2022).

During the initial stages of neural development in mice, the involvement of SOX2OT in the transcriptional regulation of embryonic neurogenesis processes can be observed (Tosetti et al., 2017). Research investigations conducted demonstrated that mice displayed a gradual elevation in the levels of both SOX2OT and SOX2 mRNA at distinct time points following CLP surgery, namely days 3, 7, and 14. Furthermore, this increase in expression was found to be associated with a decline in cognitive function. Analysis using immunofluorescence techniques indicated a reduction in neuronal markers (BrdU+/DCX+, BrdU+/NeuN+) within the dentate gyrus of the hippocampus, implying a decrease in the overall number of neurons. However, when the expression of SOX2OT was suppressed, it was observed that cell proliferation and survival in mature neurons were restored, resulting in an improvement in cognitive impairment (Yin et al., 2020). Tau protein has been investigated as a biomarker for brain injury. In a retrospective observational study, it was found that the average serum tau protein level in the group with SAE was significantly higher than in the non-SAE group. There was a strong correlation between serum tau protein levels in patients with severe sepsis and the occurrence of SAE (Zhao et al., 2019). In animal experiments, it was observed that the levels of ptau and ptau key kinases were elevated in the hippocampus of CLP mice. Studies have also found decreased dendritic spine density and the number of normal hippocampal neurons in CLP mice, indicating damage to neuronal synapses (Qi et al., 2023). Glutamate serves as the foremost excitatory neurotransmitter within the central nervous system, while the critical function of ionotropic glutamate receptors (NMDAR) involves the regulation of neuronal survival and synaptic plasticity. The abundance of synaptic glutamate can excessively activate NMDAR, thus resulting in excitotoxicity and subsequent harm to nerve cells (Asada et al., 2022). The research has shown that CLP rats exhibit elevated levels of glutamate in the hippocampus, in conjunction with an upregulated expression of the NMDAR1 glutamate receptor (Tang et al., 2023). Syntaxin1A primarily serves as an indicator for synaptic vesicles abundance in synaptically active regions (Ullrich et al., 2015). Munc18-1 is a crucial protein that is encoded by the STXBP1 gene. It plays a significant role in synaptic vesicle docking and fusion by interacting with Syntaxin1A. As a result, it has a direct impact on neurotransmitter transmission (Romaniello et al., 2015). Synapsin also plays a crucial function in synaptic vesicle trafficking, docking, and fusion, which holds significant significance (Evans and Cousin, 2005). The studies have found that the expressions of Munc18-1, Syntaxin1A and synapsin increased in the hippocampus of septic rats. Additionally, the expression levels of Syntaxin1A, synapsin and glutamate decreased after interference with Munc18-1 siRNA. These indicated that Munc18-1 may affect glutamate levels by regulating Syntaxin1A and synapsin, thereby participating in the process of hippocampal injury in septic rats (Tang et al., 2023).

2.3 Organelle dysfunction

A Retrospective cohort study found that Variations in mtDNA are associated with development of and protection from delirium during sepsis. This study highlights the role of mitochondrial dysfunction in sepsis as a crucial factor contributing to sepsis-related delirium (Samuels et al., 2019). A clinical study conducted on peripheral blood samples collected from 20 premature infants revealed a potential correlation between high expression of miRNA-1197 and low expression of miRNA-485-5p with the pathogenesis of oxidative respiratory chain and energy metabolism in premature infants with SAE. These findings suggest a possible association with mitochondrial dysfunction during SAE (Gong et al., 2022). Common neuroinflammation often affects mitochondrial health in SAE. Animal Studies have shown that the upregulation of NLRP3 and Nrf2 in microglia leads to mitochondrial dysfunction. Hydrogen can alleviate and improve mitochondrial function by inhibiting the Nrf2-mediated NLRP3 pathway (Xie K. et al., 2020). In experiments conducted on cell cultures, it has been observed that exposure to lipopolysaccharide (LPS) leads to a decrease in mitochondrial membrane potential (MMP) in hippocampal neuron cell lines (HT-22), which suggests the presence of mitochondrial dysfunction (Wang J. et al., 2022). Mitochondrial dysfunction plays a crucial role in sepsis-induced multiple organ failure, resulting in cytopathic hypoxia that impairs normal cellular function (Singer, 2014). After that, mitochondrial damages occur. The transmission electron microscope showed that the damaged mitochondria in the brain tissue were swollen and vacuolated. The damaged mitochondria also caused the accumulation of ROS, which further activated NLRP3 and aggravated the inflammatory response. Hence, alterations in the dynamics of mitochondria emerge as imperative for regulating the quality control of mitochondria amidst sepsis. Research has discovered that primary neurons treated with LPS undergo a transition from oxidative phosphorylation to glycolysis. This results in a reduction of both ATP production and MMP. Additionally, the expression of dynein-related protein 1 (Drp1) undergoes an elevation. While an inhibitor of the Drp1-Fis1 (P110) effectively improved mitochondrial health, suggesting that Drp1-Fis1 mediates mitochondrial dysfunction in

SAE (Haileselassie et al., 2020). Mitophagy acts as a crucial autophagic mechanism in preserving cellular homeostasis and disposing of impaired mitochondria (Doblado et al., 2021). However, mitophagy impairment may occur in SAE. Research has observed that the utilization of fisetin-induced medications has the ability to trigger mitophagy, which facilitates the elimination of impaired mitochondria and ROS during sepsis. Specifically, Fisetin enhances mitophagy in CMECs of CLP rats by increasing the expression of LC3-II, decreasing the levels of p62, and reducing ROS (Ding et al., 2022).

Excessive activation of endoplasmic reticulum (ER) stress is significantly associated with the cellular harm triggered by sepsis. ER stress occurs due to physiological or pathological events that disturb normal protein folding within the ER, giving rise to the unfolded protein response (UPR) (Sun et al., 2020). However, excessive UPR responses beyond cellular adaptation can lead to apoptosis (Lin et al., 2008). Therefore, maintaining normal levels of ER stress plays a crucial role in reducing sepsis-induced tissue damage (Gupta et al., 2010). Studies have found that the expression levels of UPR markers (GRP78, CHOP and PERK) significantly increased in two neuronal cell lines (PC12 and MES23.5) treated with LPS, decreased cell viability, and enhanced apoptosis. Furthermore, the expression levels of GRP78 increased with increasing doses of LPS treatment. Moreover, the overexpression of GRP78 led to decreased cell viability and increased apoptosis over time in both cell lines. These findings suggest that LPS-induced ER stress can promote apoptosis as a cellular adaptive response (Li et al., 2020).

2.4 Imbalance of neurotransmitters

Neurotransmitters are a group of chemical substances that transmit information between presynaptic and postsynaptic neurons. They can be divided into two types: excitatory neurotransmitters and inhibitory neurotransmitters. The imbalance of neurotransmitter may be associated with sepsis-associated encephalopathy (Tang et al., 2022). The main manifestations include increased levels of glutamate, decreased levels of acetylcholine, and reduced levels of γ-aminobutyric acid, etc. Restoring the balance of neurotransmitters could potentially be a target for treating SAE.

Glutamate, the primary excitatory neurotransmitter in the brain, can become excessively accumulated and result in neuronal excitotoxicity. A research study utilized a specialized imaging tool called glutamate-weighted chemical exchange saturation transfer (GluCEST) to identify alterations in glutamate signaling caused by neuroinflammation. The study revealed a substantial rise in glutamate levels in the hippocampus of rats with LPS-induced sepsis, which can potentially lead to disruptions in the neurotransmitter system. This observation sheds light on the detrimental effects of glutamate accumulation in the context of neuroinflammatory conditions (Lee et al., 2023). Another study found that inhibiting ferroptosis can alleviate cognitive disorders and neurological impairments in mice with sepsis-associated encephalopathy. Additionally, inhibiting ferroptosis can attenuate glutamate excitotoxicity induced by ferroptosis, thereby protecting the integrity of synapses and neurons (Xie et al., 2022). Its mechanism may also be related to the cystine/glutamate antiporter (System Xc-), which is a Na^+-independent reverse transport protein across the cell membrane. Its function is to uptake cystine and excrete glutamate. Redox imbalance occurs in cells with ferroptosis, which leads to the upregulation of System Xc- and excess accumulation of glutamate in the synaptic cleft, triggering excitotoxicity (Sato et al., 1999; Ogunrinu and Sontheimer, 2010). Furthermore, the prefrontal cortex (PFC)-hippocampus (HPC) pathway holds significant significance in cognitive functions, encompassing attention, decision-making, and both immediate and enduring memory (Jones and Wilson, 2005). One study found that cognitive dysfunction in CLP-induced septic mice was enhanced by chemical genetic activation of the HPC-PFC pathway, which could be blocked by glutamate receptor antagonists (Ge et al., 2023).

Acetylcholine is also an excitatory neurotransmitter, which differs from the excessive accumulation of glutamate. SAE exhibit a decrease in the expression of acetylcholine receptors within the hippocampus, leading to the inhibition of the cholinergic anti-inflammatory pathway in the vagus nerve. Consequently, this disruption triggers an unregulated inflammatory reaction and impairs neurological functionality (Hong et al., 2023). A study has found that defects in cholinergic nerve function and abnormal neuroinflammation have a synergistic effect on the pathogenesis of SAE. SAE rats exhibit defects in cholinergic neurological function, accompanied by overexpression of pro-inflammatory cytokines, increased neuronal apoptosis, and cognitive impairment in the brain. The acetylcholinesterase inhibitor huperzine (HupA) significantly improved cholinergic nerve function, attenuated abnormal neuroinflammation in SAE, and restored brain function (Zhu et al., 2016). In another investigation, murine p75-saporin immunotoxin (mu-p75-sap) was employed to induce specific harm to the cholinergic system within the basal forebrain of mice. It was discovered that animals with cholinergic defects exhibited acute and transient impairments in working memory when exposed to low-dose LPS, while control groups with impaired showed no effect. It has indicated that experiencing cholinergic depletion increases susceptibility to acute cognitive impairments that may arise after subsequent systemic inflammatory injuries (Field et al., 2012).

γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter. Parvalbumin (PV) interneurons are the most dominant subtype among GABAergic interneurons (Enwright et al., 2016). Most cortical PV interneurons are wrapped by a perineuronal net (PNN). PNN is a condensed form of extracellular matrix (Berretta et al., 2015) that is involved in the closure of developmental critical periods, regulates synaptic plasticity, and can be altered by oxidative stress. PNN plays a role in protecting and regulating PV interneurons (McRae and Porter, 2012; Cabungcal et al., 2013). One study found that mice treated by LPS exhibited significant cognitive disorder, which was associated with reduced densities of PNN and PV neuron. Active MMP-9-mediated PNN remodeling results in a decrease in inhibitory and excitatory inputs to PNN-wrapped PV interneurons, as well as a reduction in gamma oscillations in the hippocampal CA1 region (Zhang et al., 2023). This suggests that sepsis leads to decreased release of the inhibitory neurotransmitter GABA.

2.5 Blood–brain barrier disruption

The BBB, a dynamic "physical barricade," manages the movement of molecules between the brain and blood, thus preserving CNS stability (Sweeney et al., 2019). The components of the BBB encompass endothelial cells, astrocyte endfeet, and tight junctions (Abbott et al., 2010). Inflammation is the primary factor in BBB disruption in

SAE. We have previously highlighted glial cells in neuroinflammation. Astrocyte activation and brain-derived inflammatory factors appear to be among the causes of BBB disruption. Inflammation can also contribute to BBB disruption in sepsis by altering endothelial cell permeability. It has been reported that physiological doses of prostaglandin E2 (PGE2) are sufficient to induce brain endothelial cell permeability *in vitro* (Dalvi et al., 2015). After LPS treatment, the multifunctional protein-polymerase delta-interacting protein 2 (Poldip2) was significantly increased in the mouse cerebral cortex. The vascular permeability of Poldip2$^{+/+}$ mice was significantly increased, while that of Poldip2$^{+/-}$ mice was significantly reduced. In addition, the NF-κB/Cox2 signaling in the cerebral cortex of Poldip2$^{+/-}$ mice is inhibited. Cox-2 is a key mediator of arachidonic acid metabolism and can promote the synthesis of PGE2 (Minghetti, 2004). PGE2 is also down-regulated in the cerebral cortex. However, opposite results were observed in Poldip2$^{+/+}$ mice. We also observed that siPoldip2 significantly reduced LPS-induced endothelial cell permeability *in vitro*. This suggests that the Poldip2/COX-2/PGE2 signaling mediates changes in endothelial cell permeability in septic mice (Kikuchi et al., 2019). Inflammation also affects tight junctions in endothelial cells, which are maintained by important tight junction proteins, such as Claudin-5, Occludin, ZO-1, to preserve the integrity of the BBB (Sandoval and Witt, 2008). The study found that activation of NLRP3 after LPS treatment in the BBB *in vitro* model, which was constructed by co-culturing mouse CMECs and astrocytes, promoted inflammation, disrupted tight junctions, and reduced expression of tight junction-related proteins (Occludin, Claudin-5 and ZO-1) (Chen S. et al., 2020). In addition, the causes of nerve damage previously described, such as pyroptosis, mitochondrial dysfunction, and ferroptosis, also contribute to disruption of the BBB. We have summarized the pathogenesis of SAE in Figure 1 and Table 1.

3 Interaction between brain and peripheral organs in sepsis

3.1 Brain and gut microbiota

The disorders of gut microbiota mediated multiple organ damage associated with sepsis (Cryan et al., 2019). Microbial metabolites are increasingly recognized as key mediators in the functional effects of gut-brain communication. Due to various reasons, such as the use of broad-spectrum antibiotics, patients with sepsis may experience severe disruption in their intestinal flora distribution, resulting in dysbiosis that can have a negative impact on prognosis (Wei et al., 2021). A retrospective study revealed that the 28-day mortality rate of patients with SAE was significantly higher compared to those non-SAE. Additionally, the incidence of gastrointestinal infection was found to be significantly higher in the SAE group compared to the non-AE group (Chen J. et al., 2020). A clinical study conducted 16S rDNA sequencing on fecal samples obtained from 20 patients with SAE. The study revealed that these patients exhibited a decrease in both the diversity and number of their gut microbiota (Wang et al., 2022a). Acupuncture and Chinese herbal medicine can improve related intestinal symptoms by regulating the type of intestinal flora and the level of neurotransmitters such as 5-hydroxytryptamine (5-HT) (Ling et al., 2022; Sun et al., 2023). However, the role of gut microbiota in modulating sepsis-associated encephalopathy (SAE) is still not well understood. Previous research has shown that fecal microbiota transplantation (FMT) is a promising method to treat gut dysbiosis and enhance brain function in septic rats (Li et al., 2021). The study found considerable individual variation in the neurological reflexes of septic mice 36 h after CLP. Mice with a neurological score > 6 had lower mortality and were defined as SAE-resistant mice (SER), while those with a neurological score ≤ 6 were defined as SAE-susceptible mice (SES). There was a notable decrease in the abundance and variety of the gut microbiota in SES mice, whereas the gut microbiota in SER mice exhibited slight dysregulation with notable differences in composition among individuals. Additional FMT experiments exposed that the gut microbiota from SER mice conferred a survival benefit following CLP, leading to marked reductions in IL-1β and TNF-α levels in both the serum and cerebral cortex of the mice. These findings provide evidence that the gut microbiota can transfer neuroinflammation induced by CLP (Fang et al., 2022). Another study found that administering the probiotic *Clostridium butyricum* (Cb) by gavage after CLP treatment significantly reduced cognitive disorders and neuronal damage. Additionally, the excessive activation of microglia and the elevation of BDNF levels were significantly suppressed by Cb. Moreover, Cb effectively ameliorated gut dysbiosis in SAE mice (Liu et al., 2020).

Although it has been established that the dysfunction of gut microbiota may be one of the important causes of SAE, and the brain-gut axis (signaling between the gut microbiota and the brain through the neuroendocrine immune network) plays an important role in this process (Mayer et al., 2015), the exact mechanism remains to be elucidated. The gut-microbiota-brain axis could be influenced significantly by the vagus nerve. Previous research reveals that LPS mice treated with FMT showed improved spatial memory. Moreover, there was a notable decrease in the levels of IL-1β, IL-6, and TNF-α within the cerebral cortex, along with a decrease in the count of Iba-1-positive microglia. Nevertheless, the advantageous outcomes achieved through FMT were nullified once cervical vagotomy was performed (Li et al., 2018). Another study found that exposure to red light worsened learning disabilities and anxiety-like behaviors in aged septic mice, while also altering the diversity and composition of the fecal microbiota (increasing Bacteroidetes abundance while decreasing Firmicutes abundance). The same behavioral deficits were observed in pseudo germ-free mice transplanted with fecal suspensions from septic mice exposed to red light. However, it is noteworthy that subdiaphragmatic vagotomy reversed these behaviors deficits (Xie B. et al., 2020). This suggests that dysbiosis of the gut microbiota following sepsis signals through the cervical and subdiaphragmatic vagus nerves, leading to cognitive impairment and anxiety-like behavior.

Other studies have speculated that the gut microbiota may regulate the local immune response in mesenteric lymph nodes (MLN) through intestinal epithelial cells (IEC), thereby influencing the progression of SAE. Intestinal epithelial cells (IECs) have been found to possess the capability of releasing extracellular vesicles, specifically exosomes, that have immunological activity. These exosomes are able to transport crucial molecules like proteins, DNA, and various inflammatory cytokines. Ultimately, they play a significant role in shaping the immune environment (Xu et al., 2016). *In vitro*, exosomes from IECs of SAE rats induced M1 macrophages polarization and increased IL-1β levels, while exosomes secreted by FMT-treated CLP rats showed the opposite results. Exosome secretion inhibitor (GW4869) significantly suppressed M1-type macrophage polarization and IL-1β expression in MLN. More importantly, it

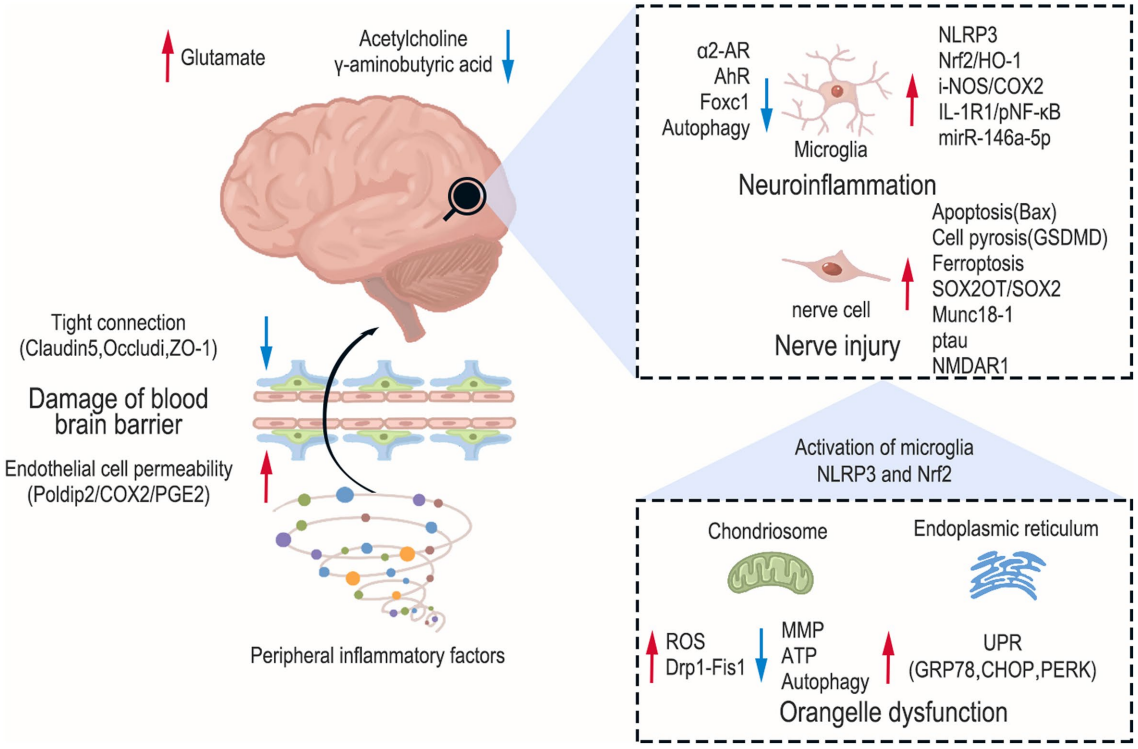

FIGURE 1
Pathogenesis of sepsis-associated encephalopathy. The red arrows indicate upward regulation and blue arrows indicate downward regulation. The brain sustains damage at various levels during SAE, encompassing brain neurotransmitter imbalance, activation of glial cells, mitochondrial dysfunction, and endoplasmic reticulum stress. Furthermore, peripheral inflammation can also compromise the permeability of the blood–brain barrier.

reduced serum and hippocampal IL-1β levels, and attenuated hippocampal damage, apoptosis and autophagy. The results are supported by the use of recombinant IL-1β and IL-1β antagonists. Furthermore, the supernatant of macrophages treated with IEC exosomes from SAE rats was co-cultured with neurons (H19-7), they found that microglia activation, apoptosis (Bcl-1, Bax, etc. protein), and autophagy (LC3II/I ratio) were present. However, IL-1β antagonists inhibited apoptosis and autophagy, which this was reactivated by rapamycin (RPA). These findings propose that the disruption of the gut microbiota might enhance the liberation of exosomes that originate from IEC, thereby having an impact on cognitive decline, inflammation, and damage to the hippocampus in SAE. This occurs through the polarization of M1 and the excretion of IL-1β in MLN, which leads to the impairment (Xi et al., 2021).

The metabolites of gut microbes into the bloodstream and subsequently into the brain, this is a pathway through which gut microbes establish a connection with the brain (Mayer et al., 2015). Several studies screened for differential metabolites in blood and brain of CLP mice, with l-gulono-1, 4-lactone levels decreasing in the blood and increasing in the brain (Han et al., 2023). Nonetheless, they possess the capability to transform into ascorbic acid (AA), which exhibits the potential to mitigate oxidative stress and endothelial dysfunction by augmenting endothelial NO synthesis. Therefore, their decrease in the blood is harmful (Kim et al., 2006), while the increase in the brain may be attributed to disruption of the BBB, leading to infiltration of macromolecules and entry into the brain. A prospective multicenter cohort study was conducted on 63 patients with sepsis to analyze their metabolic profile. The untargeted metabolomic analysis showed significant dysregulation of amino acid metabolism in sepsis patients. When compared to 43 normal controls, the sepsis multi-omics network exhibited key molecular changes associated with tryptophan biosynthesis (Chen et al., 2022). Tryptophan is crucial for maintaining immune homeostasis and optimizing gut barrier function. The metabolism of tryptophan, a precursor of 5-HT, can be modulated by the gut microbiota. Reduced levels of 5-HT in the brain can lead to abnormal behaviors such as anxiety, neurotic hallucinations, and insomnia (Roager and Licht, 2018). However, studies have found reduced levels of 5-HT in the blood and brain of CLP mice, which may be caused by disturbances in intestinal flora (Han et al., 2023). Another study focused on short-chain fatty acids (SCFAs), which are major fermentation metabolite of intestinal anaerobic bacteria. G protein-coupled receptor 43 (GPR43) interacts with SCFAs to exert anti-inflammatory effects in the central nervous system. The study revealed lower levels of acetate and propionate in the feces of SAE mice, as well as a decrease in the number of bacteria that produce SCFAs. After intragastric administration of SCFAsto SAE mice, it was found that SCFAs inhibited sepsis-induced cognitive disorders and neuroinflammation. However, the GPR43 antagonist (GLPG0974) counteracted the cognitive protective and anti-neuroinflammatory effects of SCFA (Liao et al., 2022).

3.2 Brain and liver metabolite

In addition to metabolites produced by gut microbes, the ketone body β-hydroxybutyrate (BHB), an intermediate metabolite in the

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TABLE 1 The pathological damage of SAE sepsis-associated encephalopathy.

	Ref.	Model	Test sites	Biochemical measurements
Neuroinflammation	Mei et al. (2021)	CLP	Hippocampus	TNF-α, IL-6, and IL-1β ↑ α2A-adrenoceptors (astrocytes) ↓
	Fang et al. (2022)	CLP	Cortex	NLRP3 and IL-1β ↑
	Shen et al. (2021)	CLP	Hippocampus	IL-1β, IL-6 ↑; microglial (M1 phenotype) ↑
	Saito et al. (2021)	CS	Cortex	IL-1β, IL-6 ↑; microglial, neutrophils, lymphocyte, CD 4^+ T cell, CD 8^+ T cell, Treg, Th2 ↑
	Ding et al. (2022)	CLP	Hippocampus	NLRP3, IL-1β (CMECs) ↑; TNF-α, IL-1R1, pNF-κB (microglial) ↑
	Wang et al. (2022b)	CLP, LPS	Hippocampus; microglial cells (BV-2)	IL-1β, TNF-α, p65 (microglial) ↑; Foxc1, IκBα (microglial, hippocampus) ↓
	Zou et al. (2022)	CLP	Cortex and hippocampus Primary microglia and astrocyte	miR-146a, TLR7 ↑;
Oxidative stress	Zhang H. et al. (2022)	CLP, LPS	Hippocampus; primary microglia	i-NOS, COX-2 and Nrf2/HO-1 (microglial) ↑
	Ding et al. (2022)	CLP	Hippocampus	i-NOS (microglial) ↑; ROS (CMECs) ↑
	Wang J. et al. (2022)	CLP	Hippocampus	ROS, MDA ↑; GSH ↓
	Wei et al. (2022)	CLP	Cortex; CMECs (bEnd.3)	ROS, MDA ↑; GSH ↓
	Haileselassie et al. (2020)	LPS	Brain CMECs, astrocyte and primary neuron	ROS, MitoSox, H_2O_2 ↑
	Xie et al. (2022)	CLP	Brain	MDA ↑
Autophagy	Zhuang et al. (2020)	CLP, LPS	Hippocampus microglial cells (BV-2)	TNF-α, IL-6, HMGB1 ↑; microglial (M1 phenotype) ↑; p-mTOR/mTOR, p62 (BV-2) ↑
	Shen et al. (2021)	CLP	Hippocampus	CXCR5 ↑
	Xie et al. (2022)	CLP	Hippocampus	LC3B, LaminB1 ↑
	Xi et al. (2021)	CLP	hippocampal neurons (H19-7)	The ratio of LC3II/I ↑
Neuronal damage	Xu et al. (2019)	CLP	Brain	Caspase-1, GSDMD, GSDMD-NT ↑; IL-1β, MCP-1, TNF-α ↑
	Fu et al. (2019)	CLP	Hippocampus	NLRP3, Caspase-1, GSDMD ↑; IL-1β, IL-18 ↑
	Chen S. et al. (2020)	LPS	Cortex; CMECs, astrocytes	Bax, GSDMD (*in vitro*) ↑; IL-1β, IL-18, NLRP3, p65 (*in vitro*) ↑
	Wang J. et al. (2022)	CLP	Hippocampus	GPX4, ACSL4, SLC7A11 ↑
	Wei et al. (2022)	CLP	Cortex; CMECs (bEnd.3)	Fe ion, Serum exosome-packaged NEAT1 ↑; TFRC, GOT1 ↑; miR-9-5p, GPX4 ↓
	Yin et al. (2020)	CLP	Hippocampus	SOX2OT, SOX2 ↑
	Qi et al. (2023)	CLP	Hippocampus	ptau, tau oligomer, TTBK1, ptau key kinase ↑
	Tang et al. (2023)	CLP	Hippocampus	Munc18-1, Syntaxin1A, Synaptophysin ↑
	Xie et al. (2022)	CLP	Brain	Transferrin, GPX4, GSDMD-NT ↑ pro-caspase-1, caspase-1 p20 ↑
	Xi et al. (2021)	CLP	Hippocampus	Bax, cleaved-caspase 3 ↑; Bcl-2 ↓

(Continued)

TABLE 1 (Continued)

	Ref.	Model	Test sites	Biochemical measurements
Organelle dysfunction	Wang J. et al. (2022)	LPS	Hippocampal neuronal cell line (HT-22)	MMP ↓
	Haileselassie et al. (2020)	LPS	Brain CMECs, astrocyte and primary neuron	p Drp1, p53, glycolysis ↑ MMP, ATP, oxidative phosphorylation ↓
	Ding et al. (2022)	CLP	CMECs	LC3-II, ROS ↑; p62, mitophagy ↓
	Li et al. (2020)	LPS	neuronal cell (PC12 and MES23.5)	UPR markers (GRP78, CHOP and PERK) ↑
Imbalance of neurotransmitters	Lee et al. (2023)	LPS	Hippocampus	Glutamate ↑
	Xie et al. (2022)	CLP	Brain	Glutamate, SXC, NR2B ↑
	Tang et al. (2023)	CLP	Hippocampus	Glutamate, NMDAR1 ↑
	Ge et al. (2023)	CLP	Prefrontal cortex	NMDAR, AMPAR, CaMKIIa、pCREB, BDNF ↑
	Zhu et al. (2016)	LPS	Hippocampus	AChE ↑; ChAT, CHRM1,ACh ↓
	Field et al. (2012)	LPS (mu-p75-sap)	Hippocampus	AchE, ChAT ↓
	Zhang et al. (2023)	LPS	Hippocampus	MMP-9 ↑; PNN, PV, gamma oscillations ↓
BBB disruption	Zou et al. (2022)	CLP	Brain	BBB permeability, TLR7 ↑
	Haileselassie et al. (2020)	LPS	Brain CMECs, astrocyte and primary neuron	BBB permeability, VCAM-1, ICAM-1 ↑ ZO-1, occludin ↓
	Kikuchi et al. (2019)	LPS	Cortex Primary CMECs	Poldip2, p65, Cox-2, PGE2 ↑
	Chen S. et al. (2020)	LPS	Cortex CMECs, astrocyte	Occludin、Claudin-5, ZO-1, Bcl-2 ↓ Maf1, p65, NLRP3, Bax, GSDMD ↑

↑, upregulated by intervention; ↓, downregulated by intervention. ACh, acetylcholine; AChE, acetylcholinesterase; BBB, Blood–brain-barrier; ChAT, choline acetyltransferase; CHRM1, muscarinic acetylcholine receptor-1; CLP, the cecal ligation and puncture; CMECs, cerebral microvascular endothelial cells; CS, cecal slurry; GSH, glutathione; LPS, lipopolysaccharide; MDA, malondialdehyde; MMP, mitochondrial membrane potential; mu-p75-sap, murine-p75-saporin; NR2B, N-methyl-D-asperate receptor subunit; PNN, perineuronal net; PV, parvalbumin; SXC, System xc-; UPR, unfolded protein response.

liver during fat oxidation metabolism, contributes to preventing cognitive impairment after sepsis. Studies have found that BHB levels in the hippocampus were significantly reduced and blood BHB levels were increased after CLP treatment, but other studies found that blood BHB levels were decreased during sepsis (Lanza-Jacoby et al., 1990; Umbarawan et al., 2017). After subcutaneous injection of BHB, it was found that the levels of BHB in both blood and hippocampus were significantly increased, indicating that subcutaneous injection of BHB can penetrate the bloodstream and brain. The following study found that subcutaneous injection of BHB improved neuroplasticity and reduced mRNA levels of hippocampal IL-1β and TNF-α, as well as the percentage of microglial activation in the CA1 region and dentate gyrus in mice that survived CLP. In addition, a significant decrease in peripheral blood leukocyte count and neutrophil percentage. Intracerebroventricular injection of BHB also resulted in decreased mRNA levels of hippocampal IL-1β and TNF-α. *In vitro*, it was further found that both HCA2 (BHB receptor) and MCT2 (BHB transporter) played a role in improving the BHB response to LPS-induced neuronal injury and inflammation. Additionally, the inflammatory response was more significantly impacted by HCA2 than by MCT2. Since BHB is considered as an alternative energy source for the brain during states of energy deficiency, the study also examined the ADP/ATP ratio in the hippocampus during sepsis. However, there was no significant change observed after administering BHB. These findings propose that the administration of BHB leads to a decrease in both neuroinflammation and peripheral inflammation in mice subjected to CLP. Importantly, it is suggested that this reduction occurs via the activation of HCA2 and MCT2 pathways (Wang et al., 2020). This also indicates that the immune system disorder in sepsis affects the level of liver metabolites in circulation, which further impacts the occurrence of neuroinflammation in SAE.

4 Interaction between brain and peripheral neuroimmune system in sepsis

4.1 Cholinergic pathways

We previously introduced the role of the vagus nerve in the brain-gut axis, and here we continue to discuss the regulatory mechanism of the vagus nerve and its associated neurotransmitters in

sepsis. The vagus nerve, which is the longest and most widely distributed of the 12 pairs of cranial nerves, is also the most important parasympathetic nerve. Furthermore, apart from its crucial involvement in controlling visceral function, the vagus nerve also possesses anti-inflammatory capacities, recognized as the cholinergic anti-inflammatory pathway (Borovikova et al., 2000). The involvement of cholinergic anti-inflammatory pathways in both CNS and peripheral inflammatory diseases has been extensively documented (Lee et al., 2010; Kolgazi et al., 2013). The CNS interacts bidirectionally with the immune system through the vagus nerve. The vagus nerve converts peripheral inflammation into nerve signals, which then enters the brain through afferent nerves. The brain immediately senses peripheral inflammatory stimuli and regulates peripheral immune responses through cholinergic efferent nerve fibers. Therefore, the vagus nerve acts as a "bridge" connecting peripheral inflammatory stimuli to the central nervous system (Olofsson et al., 2012).

A prospective, single-center study was conducted to enroll 45 patients with sepsis. The study observed that around one-third of sepsis patients with suspected SAE experienced a time-dependent increase in AChE activity in their blood samples. This increase in acetylcholinesterase activity results in the breakdown of acetylcholine, leading to cholinergic defects (Zujalovic et al., 2020). A research has investigated elements of cholinergic nerve activity in the hippocampus, such as acetylcholine transferase (ChAT), receptor-1 for muscarinic acetylcholine (CHRM1), acetylcholinesterase (AChE), and acetylcholine, in septic rats experiencing neuroinflammation in the hippocampus. It was found that LPS treatment resulted in the inhibition of ChAT and CHRM1 mRNA and protein expression in the hippocampal region of rats, decreased ACh concentration (measured by LC–MS/MS), enhanced AChE activity, increased TNF-α and IL-1β mRNA and protein expression, neuronal apoptosis (measured by TUNEL) and cognitive impairment. These results were reversed after administration of the acetylcholinesterase inhibitor huperzine (HupA). The effects of HupA include inhibiting the hydrolysis of AChE and promoting the expression of ChAT and CHRM1. It is suggested that the occurrence of inflammation in sepsis leads to the damage and dysfunction of cholinergic neurons, while HupA treatment improves SAE by promoting cholinergic nerve function and anti-inflammatory ability (Zhu et al., 2016). In another study examining electrophysiological changes in the hippocampus during sepsis, hippocampal function and long-term potentiation of excitatory synapses (LTP) in rat brain slices were studied using whole-cell patch-clamp single-cell electrophysiology techniques. The data showed that disruption of synaptic plasticity in the rat brain after LPS treatment was accompanied by an increase in after-hyperpolarization (AHP) mediated through small-conductance Ca^{2+}-activated potassium channels (SK). Inhibiting SK channels can partially restore sepsis-induced deficits in synaptic plasticity. This can be done by using an SK2 channel blocker like apamin, a highly selective muscarinic M1 receptor allosteric agonist such as TBPB, or by increasing the lifespan of endogenous acetylcholine through a cholinesterase inhibitor like physostigmine (Zivkovic et al., 2015). All of these studies have confirmed that sepsis impairs cholinergic nerve function in the hippocampus. Conceivably, damage to hippocampal cholinergic neurons could further affect the neural circuitry of the inflammatory reflex, impeding normal immunomodulatory function of peripheral immune cells that contain cholinergic receptors such as macrophages, neutrophils. Thereby exacerbating peripheral inflammation and causing multi-organ damage, thus creating a vicious cycle. However, further research is needed to understand the regulatory mechanisms.

In addition, numerous animal experiments have confirmed that the intervention of cholinergic anti-inflammatory pathways through peripheral injection of α7 nicotinic acetylcholine receptor agonists can improve heart, lung, and multiple organ dysfunction (Sallam et al., 2018; Shao et al., 2019; Wedn et al., 2019). The functioning of the heart significantly affects the perfusion of blood in the brain. Mean arterial pressure (MAP) is a crucial clinical factor that can predict the development of brain injury related to sepsis. While the brain has the ability to regulate blood flow on its own, if the mean arterial pressure falls below a certain threshold, it can lead to decreased perfusion of organs (Ge et al., 2022). According to a cohort study, the incidence of SAEs was found to increase when systolic blood pressure was less than 90 mmHg, diastolic blood pressure was less than 46 mmHg, mean arterial pressure was less than 65 mmHg, and lactate level was greater than 3.5 mmol/L (Zhao et al., 2022). According to the latest guidelines from the Surviving Sepsis Campaign, it is recommended to initiate fluid resuscitation and vasopressors as early as possible in order to achieve a MAP greater than 65 mm Hg and maintain organ perfusion (Singer et al., 2016). A prospective observational study was conducted to investigate the use of near-infrared spectroscopy (NIRS)-derived cerebral blood oxygenation index (COx) for monitoring autoregulation and determining optimal blood pressure in six selected SAE patients who were not sedated with pharmacological agents. The results indicate a strong correlation between brain autoregulation and neural status. It was observed that patients with lower GCS (Godzilla Coma Scale) had consistently higher hourly COx measurements, while patients with higher GCS had lower hourly COx measurements. This suggests that autoregulatory dysfunction may contribute to the pathophysiology of SAE (Rosenblatt et al., 2020). A prospective observational study conducted transcranial Doppler examination on 40 patients with sepsis. A positive correlation between pulsatile index (PI) and changes in peripheral vascular resistance. The results showed an increase in PI in delirium patients and a decrease in cerebral blood flow index (CBFi), suggesting that patients may have developed cerebral microcirculation disorders (Pierrakos et al., 2014). Therefore, impaired automatic regulation/cerebral perfusion is one of the more important pathological injuries in SAE patients.

4.2 Peripheral immune cells

Sepsis is triggered by infection and the disease develops rapidly, with an early feature being an uncontrolled inflammatory response (Singer et al., 2016). The activation of inflammatory signaling pathways, such as Toll-like receptors and NF-κB, stimulates immune cells to over-activate and release more inflammatory factors. This leads to cytokine cascade reactions and "cytokine storms," which amplify systemic inflammatory responses and damage systemic organs (Chousterman et al., 2017). Therefore, immune cells and their secreted cytokines play an irreplaceable role in the pathological process of sepsis.

A retrospective study was conducted on 86 patients diagnosed with severe sepsis. The study revealed that the percentage of cluster of differentiation $CD4^+$ T lymphocyte clusters in the blood samples of patients with severe SAE was lower, and $CD4^+$/cluster of differentiation $CD8^+$ ratio was also decreased. Additionally, the study found a higher

percentage of NK cells in these patients. This study provide evidence that immune imbalance plays a crucial role in the development of SAE (Lu et al., 2016). Following neuroinflammatory damage to the brain, peripheral secondary lymphoid organs have the potential to discharge abundant quantities of lymphocytes, encompassing T cells and B cells (Lewis et al., 2019). These immune cells are recruited from circulation to the brain along with many other peripheral immune cells. Brain-infiltrating T cells contribute to the recovery of depressive symptoms by addressing neuroinflammation (Ito et al., 2019). Nonetheless, limited knowledge persists regarding the mechanisms underlying T cell infiltration into the brain and their contribution to SAE progression. Investigating the impact of Cecal Serous (CS) treatment, the research revealed a substantial rise in both $CD4^+$ and $CD8^+$ T cell populations within the cerebral cortex of mice. In addition, the accumulation of T cells in the brains of CS mice was predominantly composed of naïve T cells, rather than effector memory T cells. Conversely, there was a noteworthy decrease in T cells in the peripheral blood and spleen of CS mice, which is a characteristic manifestation of sepsis-induced immunosuppression (Hotchkiss and Karl, 2003; Saito et al., 2021). They subsequently discovered that an increase in Tregs was only observed in cervical lymph nodes (CLN) among the numerous secondary lymphoid tissues in CS mice, suggesting that CLN may serve as an important source for T cell recovery following severe suppression caused by sepsis. FTY720, an antagonist of sphingosine-1-phosphate, inhibits the drainage of lymphocytes from lymph nodes, thereby preventing T cells from migrating into the brain. After intraperitoneal injection of FTY720, FTY720 administration was found to decrease the number of CD4+ and CD8+ T cells in the brains of CS mice. Moreover, a notable decline was observed in the concentrations of anti-inflammatory cytokines IL-4 and IL-10 expounded by T cells within the cerebral cortex. These changes lead to increased expression levels of IL-1β and TNF-α in the cerebral cortex and blood of CS mice, as well as an increase in microglia and a decrease in astrocytes in the cortex. This suggests that FTY720 delays the recovery of anxiety-like behavior and the expression of persistent neuroinflammation. Therefore, T cells derived from CLN enter the brain from circulation and contribute to attenuating neuroinflammation and recovering anxiety-like behavior (Saito et al., 2021).

The precursor of brain-derived neurotrophic factor (pro BDNF) is a distinct protein compared to mature BDNF. Signaling via pro BDNF-p75NTR promotes the apoptosis of neurons and axon pruning, while concurrently exerting negative control over learning and memory (Teng et al., 2005). Immunofluorescence detection revealed that proBDNF was upregulated in meningeal and peripheral blood immune cells ($CD3^+$ T cells, $CD4^+$ T cells, $CD19^+$ B cells) after LPS injection. Additionally, a significant decrease in the proportion of $CD4^+$ T cells was observed in splenocytes collected from septic mice that were treated with exogenous proBDNF protein. The subsequent intraperitoneal injection of proBDNF antagonist (McAb-proB) significantly restored the percentage of $CD4^+$ T cells in the meninges to normal levels and reversed the downregulation of anti-inflammatory cytokines IL-4, IFN-γ and IL-13 mRNA as well as the upregulation of pro-inflammatory cytokines IL-1β and IL-6 mRNA in the meninges. However, it is worth noting that the intracerebroventricular injection of McAb-proB did not play any role. The evidence presented indicates that the immune system's upregulation of proBDNF plays a role in the development of SAE. This occurs through the downregulation of circulating CD4+ T cells, thereby restricting their infiltration into the meninges. Additionally, it disrupts the balance between pro-inflammatory and anti-inflammatory factors within the meninges, leading to a disturbance in their homeostasis (Luo et al., 2020).

The SAE may also be affected by cytokines released from peripheral immune cells. The study found that the upregulation of IL-17R was accompanied by microglia activation (increased Iba-1 fluorescence intensity) in the brain tissue of CLP mice, as observed through an immunofluorescence assay. After administering of recombinant IL-17A through intraventricular injection, there was observed an elevation in the levels of pro-inflammatory cytokines (specifically, IL-1β and TNF-α) within the brain region. This led to a marked increase in the activation of microglia specifically within the hippocampus. Conversely, when both anti-IL-17A and anti-IL-17R antibodies were administered through intraventricular injection, there was a notable reduction in central nervous system (CNS) inflammation and a subsequent inhibition of microglial activation. These findings were further corroborated by *in vitro* experiments, thereby highlighting the influence of the IL-17A/IL-17R signaling pathway on microglial activation (Ye et al., 2019). Notably, earlier studies by the team have shown that IL-17A secreted by peritoneal γδ T cells rapidly enters the circulation in the early stages of sepsis. Intraperitoneal blockade of IL-17A reduces pro-inflammatory cytokines and neutrophil infiltration in alveolar lavage fluid, thereby ameliorating lung injury and improving survival (Li et al., 2012). Their two studies suggest that activation of peritoneal γδ T cells leads to the secretion of IL-17A, causing lung injury. In the presence of BBB injury, circulating IL-17A may also be transferred to the CNS and exacerbate SAE. However, further studies are still needed to establish it. We speculate that cytokines released by peripheral immune cells, such as macrophages and neutrophils, in addition to peritoneal γδ T cells, may worsen SAE by damaging the BBB.

In some studies, we have found that exosomes (Exo) are the most potential carriers for peripheral immune cells to act on the brain through the blood. Mesenchymal stem cells (MSC) have become clinically valuable therapeutic tools due to their high potential for differentiation, capacity for proliferative, and ability to modulate the immune system (Shetty et al., 2010). It was found that the injection of umbilical cord MSCs (UC-MSCs) via femoral vein significantly reduced the expression levels of TNF-α, IL-6 and HMGB1 as well as microglia activation (expression of Iba-1 markers) in brain tissue of LPS-treated mice. This improved cortical neuronal damage and cognitive impairment (Zhang Z. et al., 2022). Some studies suggested that MSCs can migrate into the brain through paracellular or transcellular pathways (Schmidt et al., 2006). It is possible that activation of endothelial cells and astrocytes in SAEs leads to decreased tight junctional integrity and reduced formation of the paracellular space, allowing cells to migrate via paracellular pathways (Liu et al., 2013). Nonetheless, the blood–brain barrier continues to be a significant physical barrier that stem cells need to overcome (Ballabh et al., 2004). In comparison, exosomes at the nanoscale (30-150 nm) can more easily penetrate the BBB while still exhibiting the same immunomodulatory and regenerative capabilities as their parent cells (Timmers et al., 2007). The role of exosomes is to enhance the communication between cells by transferring various components including cell surface receptors, cytokines, lipids, and RNA molecules from the donor cells to the recipient cells (van Niel et al., 2018). It has been demonstrated that intranasally administered MSC-Exo reach the brain and reduce microglia-mediated neuroinflammation in rats with

perinatal brain injury (Thomi et al., 2019). Another piece of evidence suggests that exosomes can participate in the pathological process of SAE through the BBB. In this study, exosomes were purified from CLP-treated rat cerebral cortex and exosome markers including TSG101, CD9, and CD63 were identified. To further investigate whether exosome packaging is the primary mode of delivery of lncRNA NEAT1, the expression of lncRNA NEAT1 in exosomes and cerebral cortex was examined by the researchers. Using qRT-PCR, it was found that the expression of lncRNA NEAT1 remained consistent in both exosomes and cerebral cortex. Moreover, an observable increase in lncRNA NEAT1 expression was observed in rats treated with CLP, and there was a positive correlation between their lncRNA NEAT1 expression levels in the cerebral cortex and exosomes. This suggests that exosomes carrying lncRNA NEAT1 can cross the blood–brain barrier and reach the cerebral cortex, promoting ferroptosis. In addition, it remains to be explored whether are the primary secretion of peripheral immune cells, serving as the main means of transporting lncRNA NEAT1 to the cerebral cortex (Wei et al., 2022). In conclusion, peripheral immune cells may be more involved in the pathological process of SAE through their derived exosomes. Finally, we summarized the interaction between the brain and peripheral system in sepsis in Figure 2 and Table 2.

5 Summary

In summary, the pathogenesis of SAE is broadly consistent with previous studies, mainly including microglia activation, programmed cell death (apoptosis, pyroptosis and ferroptosis), neuronal loss, mitochondrial damage and functional barrier, endoplasmic reticulum stress, neurotransmitters imbalance (glutamate, acetylcholine and γ-aminobutyric acid), as well as blood–brain barrier impairment due to altered endothelial permeability and disruption of tight junctions. With increasing attention to the multi-organ dysfunction caused by sepsis, there has been progressive demonstration of damage to organs such as the brain, intestinal tract, heart, and lungs. However, sepsis is a systemic inflammatory disease, and the interaction between organs during inflammation has received little attention. Therefore, we focus on the interplay between brain and peripheral system in sepsis. We discovered that several factors often exacerbate the condition of

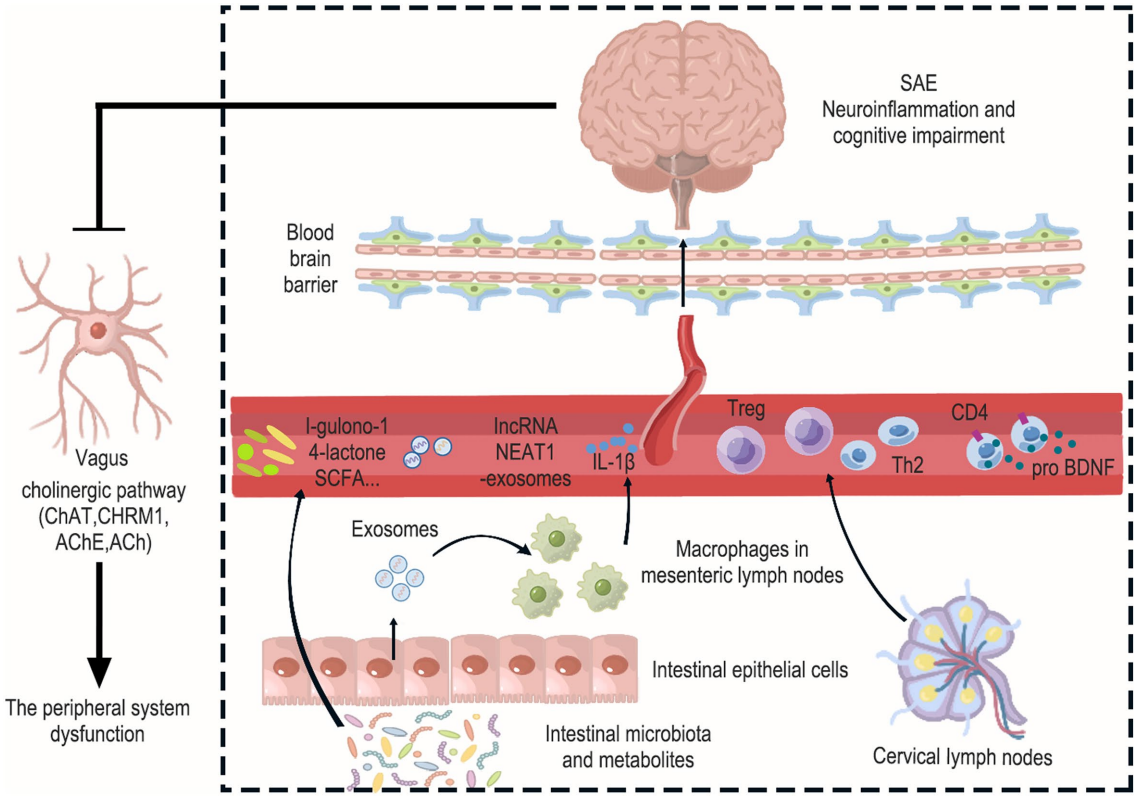

FIGURE 2
Interactions between the brain and peripheral immunity in sepsis. Peripheral system dysfunction is a crucial factor that should not be overlooked in the context of SAE. This encompasses disorders related to intestinal flora and their metabolism, the release of pathogenic exosomes from intestinal epithelial cells, the release of inflammatory factors from mesenteric immune cells, and alterations in the function of peripheral immune organs like cervical lymph nodes. Specifically, Levels of the gut microbiota metabolite l-gulonone-1,4-lactone were found to decrease in the blood and increase in the brain. Additionally, the number of bacteria producing short-chain fatty acids (SCFAs) in the gut microbiota decreased. This is significant because SCFAs have the ability to inhibit neuroinflammation. Furthermore, the expression of lncRNA NEAT1 increased in exosomes and the cerebral cortex, suggesting that exosomes carrying lncRNA NEAT1 may cross the blood–brain barrier and reach the cerebral cortex. And intestinal epithelial cell-derived exosomes promote M1 macrophage polarization and IL-1β production in mesenteric lymph nodes, leading to damage to hippocampal neurons. Interestingly, Treg cells and Th2 cells were found to increase in cervical lymph nodes (CLN), and these cells also showed an increase in the cerebral cortex. This suggests that T cells from the CLN may enter the brain from the circulation and have the ability to suppress neuroinflammation. Moreover, the upregulation of proBDNF in the immune system was found to suppress the number of circulating $CD4^+$ T cells and limit their infiltration into the meninges. Finally, the occurrence of neuroinflammation in the brain was observed to inhibit cholinergic anti-inflammatory pathways, which was evident through increased AChE and decreased ChAT, CHRM1, and ACh in the hippocampus. Collectively, these changes exacerbate peripheral system dysfunction.

TABLE 2 Interaction between peripheral system dysfunction and sepsis-associated encephalopathy.

	Ref.	Model	Test sites	Biochemical measurements
Gut microbiota	Fang et al. (2022)	CLP (FMT)	Cortex Serum	IL-1β, TNF-α, NLRP3 ↓; IPA ↑
	Liu et al. (2020)	CLP	Brain	Iba-1 ↑; BDNF ↓; gut microbiota dysbiosis
	Li et al. (2018)	LPS (FMT)	Cortex Hippocampus	Iba-1 (Cortex) ↓; TNF-α, IL-6, IL-1β (Hippocampus) ↓
	Xie B. et al. (2020)	LPS	Fecal microbial	Chao 1 index, Shannon index, ACE index ↓
	Xi et al. (2021)	CLP (FMT)	Hippocampus	IL-1β, IL-6, TNF-α, IBA-1 ↓; M1-type macrophage (MLN) ↓;
	Han et al. (2023)	CLP	Brain Fecal microbial	TNF-α, IL-6, HMGB1 ↑; Tryptophan ↓; Changes in the composition of gut microbiota
	Liao et al. (2022)	CLP	Hippocampus Fecal microbial	IL-1β, IL-6, TNF-α ↓; Acetate, propionate ↓; Relative abundance of SCFAs-producing bacteria ↓
Liver metabolite	Wang et al. (2020)	CLP	Hippocampus	BHB, neuroplasticity ↓; IL-1β, TNF-α, IBA-1 ↑;
Cholinergic pathways	Zhu et al. (2016)	LPS	Hippocampus	AChE ↑; ChAT, CHRM1,ACh ↓
	Zivkovic et al. (2015)	LPS	Hippocampus	AHP, SK channels ↑; LTP ↓
Peripheral immune cells	Saito et al. (2021)	CS	Cortex PB Spleen CLN	Neutrophils, lymphocyte, CD 4^+ T cell, CD 8^+ T cell, Treg, Th2 (Cortex) ↑; T cells (Spleen), CD 4^+ T cell, CD 8^+ T cell (PB) ↓ Treg, Th2 (CLN) ↑;
	Luo et al. (2020)	LPS	Meninges PB	IL-1β, IL-6 (meninges) ↑; IL-4, IFN-γ, IL-13 (meninges) ↓; $CD3^+$ T cell, $CD4^-CD8^+$ T cell, $CD11b^+$ monocytes/macrophages (meninges) ↑; $CD4^+CD8^-$ Th cells, $CD19^+$ B cells (PB) ↓; proBDNF (meninges, PB) ↑
	Ye et al. (2019)	CLP	Brain Hippocampus	IL-17A, IL-1β, TNF-α, IL-17R↑; CD11b, Iba-1 ↑
	Wei et al. (2022)	CLP	Cortex	lncRNA NEAT1, Exo- lncRNA NEAT1 ↑

↑, upregulated by intervention; ↓, downregulated by intervention. ACh, acetylcholine; AchE, acetylcholinesterase; AHP, after-hyperpolarization; BHB, the ketone body β-hydroxybutyrate; ChAT, choline acetyltransferase; CHRM1, muscarinic acetylcholine receptor-1; CLN, cervical lymph nodes; CLP, cecal ligation and puncture; FMT, fecal microbiota transplantation; HRV, heart rate variability; IPA, Indole-3-propionic acid; LPS, lipopolysaccharide; LTP, long-term potentiation; MLN, mesenteric lymph nodes; PB, peripheral blood; SK, small-conductance Ca^{2+}-activated potassium.

septic encephalopathy, including vagus nerve-mediated gut microbiota and its metabolites, exosomes secreted by intestinal epithelial cells, liver metabolites, cholinergic pathways, T cells and their cytokines in peripheral secondary lymphoid organs. Therefore, we suggest paying attention to the prevention and treatment of brain injury at all stages of sepsis to prevent a vicious cycle of uncontrolled systemic inflammation following CNS injury.

Author contributions

JZ: Writing – original draft. SC: Writing – original draft. XH: Writing – original draft. LH: Writing – review & editing. PL: Writing – review & editing. XY: Visualization, Writing – review & editing. ZL: Writing – review & editing. JL: Writing – review & editing. LG: Writing – review & editing. ZC: Supervision, Writing – review & editing. YG: Supervision, Writing – review & editing. BC: Supervision, Writing – review & editing.

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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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Glossary

5-HT	5-hydroxytryptamine
AA	ascorbic acid
AchE	acetylcholinesterase
AHP	after-hyperpolarization
AhR	aryl hydrocarbon receptor
BBB	blood–brain barrier
BHB	ketone body β-hydroxybutyrate
Cb	*Clostridium butyricum*
ChAT	acetylcholine transferase
CHRM1	muscarinic acetylcholine receptor-1
CLN	cervical lymph nodes
CLP	cecal ligation-punctured
CMECs	cerebral microvascular endothelial cells
CNS	central nervous system
COX-2	Cyclooxygenase-2
CS	Cecal Serous
ER	Excessive endoplasmic reticulum
FMT	fecal microbiota transplantation
GOT1	glutamate-oxaloacetate transaminase 1
GSH	glutathione
GPR43	G protein-coupled receptor 43
HupA	huperzine
IEC	intestinal epithelial cells
i-NOS	inducible nitric oxide synthase
IPA	indole-3-propionic acid
LPS	lipopolysaccharide
LTP	long-term potentiation
miRNA	microRNA
MDA	malondialdehyde
MLN	lymph nodes
MMP	mitochondrial membrane potential
NMDAR	Ionotropic glutamate receptors
PGE2	prostaglandin E2
pro BDNF	Brain-derived neurotrophic factor precursor
SAE	sepsis-associated encephalopathy
SER	SAE-resistant mice
SES	SAE-susceptible mice
SCFAs	short-chain fatty acids
SK	small-conductance Ca^{2+}-activated potassium channels
TFRC	transferrin receptor
Treg	regulatory T cells
UC-MSCs	umbilical cord MSCs
UPR	unfolded protein response

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Association between sleep-related phenotypes and gut microbiota: a two-sample bidirectional Mendelian randomization study

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Background: An increasing body of evidence suggests a profound interrelation between the microbiome and sleep-related concerns. Nevertheless, current observational studies can merely establish their correlation, leaving causality unexplored.

Study objectives: To ascertain whether specific gut microbiota are causally linked to seven sleep-related characteristics and propose potential strategies for insomnia prevention.

Methods: The study employed an extensive dataset of gut microbiota genetic variations from the MiBioGen alliance, encompassing 18,340 individuals. Taxonomic classification was conducted, identifying 131 genera and 196 bacterial taxa for analysis. Sleep-related phenotype (SRP) data were sourced from the IEU OpenGWAS project, covering traits such as insomnia, chronotype, and snoring. Instrumental variables (IVs) were selected based on specific criteria, including locus-wide significance, linkage disequilibrium calculations, and allele frequency thresholds. Statistical methods were employed to explore causal relationships, including inverse variance weighted (IVW), MR-Egger, weighted median, and weighted Mode. Sensitivity analyses, pleiotropy assessments, and Bonferroni corrections ensured result validity. Reverse causality analysis and adherence to STROBE-MR guidelines were conducted to bolster the study's rigor.

Results: Bidirectional Mendelian randomization (MR) analysis reveals a causative interplay between selected gut microbiota and sleep-related phenotypes. Notably, outcomes from the rigorously Bonferroni-corrected examination illuminate profound correlations amid precise compositions of the intestinal microbiome and slumber-associated parameters. Elevated abundance within the taxonomic ranks of class Negativicutes and order Selenomonadales was markedly associated with heightened susceptibility to severe insomnia (OR = 1.03, 95% CI: 1.02–1.05, $p = 0.0001$). Conversely, the augmented representation of the phylum Lentisphaerae stands in concord with protracted sleep duration (OR = 1.02, 95% CI: 1.01–1.04, $p = 0.0005$). Furthermore, heightened exposure to the genus *Senegalimassilia* exhibits the potential to ameliorate the manifestation of snoring symptoms (OR = 0.98, 95% CI: 0.96–0.99, $p = 0.0001$).

Conclusion: This study has unveiled the causal relationship between gut microbiota and SRPs, bestowing significant latent value upon future endeavors in both foundational research and clinical therapy.

KEYWORDS

sleep-related phenotypes, gut microbiota, causal effect, Mendelian randomization, insomnia

1 Introduction

In recent years, insomnia disorders have gradually emerged as a burden on public health (Glick et al., 2023). Recent studies indicate that the overall pooled prevalence of post-COVID sleep disturbances is 28.98% (Linh et al., 2023). These patients commonly experience challenges in initiating sleep, easy arousal, shortened sleep duration, early awakening, and disruptive snoring. Subsequently, daytime drowsiness becomes evident, presenting an air of lethargy. Sleep deprivation or inadequate sleep is correlated with elevated blood pressure (Makarem et al., 2021), weight augmentation (Nielsen et al., 2011; Ogilvie and Patel, 2017), heightened susceptibility to diabetes, cardiovascular ailments (He et al., 2017; Ogilvie and Patel, 2018), and compromised immune system functionality (Besedovsky et al., 2019). Managing sleep-related issues in a clinical setting remains inherently challenging. While the temporary alleviation of insomnia disorder symptoms can be achieved through the use of hypnotic medications, it fails to address the fundamental causes, resulting in patients excessively relying on such medications without resolving the underlying issues (Rocha et al., 2023). Sleep cognitive-behavioral therapy stands out as a primary therapeutic modality for ameliorating sleep problems. Unlike hypnotic drugs, it avoids side effects such as daytime somnolence, dizziness, weight gain, and addiction. Nevertheless, it is not without drawbacks, including discontinuation risks (Nam et al., 2023), relatively low accessibility (Koffel et al., 2018), and a time-consuming, intricate nature (Chung et al., 2023). The widespread occurrence of insomnia, coupled with negative clinical consequences and the lack of effective preventive and treatment strategies, emphasizes the need for comprehensive research to enhance understanding, investigate pathophysiology, analyze potential risk factors, and develop innovative approaches for prevention and therapy.

The pathophysiology of insomnia is exceedingly intricate, which is influenced by factors such as sleep environment, dietary habits, endocrine disruptions, psychological issues, and circadian rhythm disturbances, all of which can impact sleep. Among these, the gut microbiota (GM) engages in reciprocal interaction with brain function through the brain–gut microbiota axis (BGMA), constituting a complex and extensive bidirectional communication network. Previous research suggests that the maintenance of normal sleep physiology is inseparable from the assistance of GM, with the richness and diversity of GM fluctuating in accordance with alterations in sleep patterns, duration, and other related sleep features. Studies have identified *Lachnospira* and *Bacteroides* as signature bacteria distinguishing acute insomnia patients from healthy controls, while *Faecalibacterium* and Blautia serve as signature bacteria for distinguishing chronic insomnia patients from healthy controls (Li et al., 2020). This underscores the potential of gut microbiota as a vital indicator for the ancillary diagnosis of insomnia, offering potential novel therapeutic targets within the realm of insomnia disorders. Scholars have delved into specific genus differences between insomnia patients and healthy individuals, demonstrating the capability of addressing disrupted GM homeostasis and ameliorating insomnia, symptoms, as well as enhancing sleep quality through the administration of *Lactobacillus brevis* DL1-11 (Yu et al., 2020). Additionally, existing animal experiments validate that *Lactobacillus brevis*-fermented γ-aminobutyric acid improves sleep behaviors in fruit flies and rodent models (Jeong et al., 2021).

However, in observational studies, the association between intestinal microbiota and insomnia is easily affected by confounding factors such as age, environment, diet pattern, and lifestyle, which limits the confirmation of the causal relationship between intestinal microbiota and insomnia. Most antecedent investigations were configured as cross-sectional studies, posing challenges in ascertaining the duration of exposure and definitive outcomes. As an illustration, Grosicki et al. (2020) discerned that *Blautia* and *Ruminococcus* exhibited abundance in individuals attesting superior sleep quality, juxtaposed with *Prevotella*, which displayed diminished abundance, and a concomitant elevation in the α diversity of the gut microbiota. In addition, the results of some animal experiments are also inconsistent. For example, Poroyko et al. (2016) found that *Firmicutes* increased and *Bacteroidetes* decreased in mice with sleep fragmentation, while Maki et al. (2020) found that during the period of chronic sleep deprivation, *Bacteroidetes* decreased as compared with the control group. The abundance, F:B ratio, and α diversity of Firmicutes were decreased.

In this context, Mendelian randomization (MR) is a novel way to explore the causal relationship between the gut microbiota and SRPs. Mendelian randomization is an analytical technique in epidemiology that leverages genetic variation as an instrumental variable for exposure to explore a causal association between exposure factors and outcome events (Yavorska and Burgess, 2017). The advantage of MR is that because genetic variation is random, the analysis process is less influenced by environmental factors and lifestyle, which minimizes bias due to residual confounding (Haycock et al., 2016). Moreover, since the genetic variation is not altered by the disease state, this avoids the possibility of reverse causality in the analysis. MR has been widely used to explore the causal relationship between the gut microbiome and diseases, including metabolic diseases (Sanna et al., 2019), pregnancy-related diseases (Li P. et al., 2022; Li C. et al., 2022), autoimmune diseases (Xu et al., 2021), cardiovascular diseases (Zhang Y. et al., 2022), chronic kidney disease (Li N. et al., 2023), and rheumatoid arthritis (Inamo, 2021). In this study, we aimed to use pooled statistics from genome-wide association studies (GWAS) from MiBioGen and the UKB Consortium to elucidate potential causal

relationships between gut microbiota and SRPs using a two-sample MR approach. Identifying the relationship between them will help promote the prediction and prevention of related diseases and the further development of precision medicine. This will have a positive impact on population health and healthcare spending.

2 Methods

2.1 Data sources

The genetic variations within the gut microbiota were obtained through the most extensive genomic compilation of gut microbiota composition to date, which were carried out by the MiBioGen alliance (Kurilshikov et al., 2021). This study focused on the V4, V3–V4, and V1–V2 variable regions of the 16S rRNA gene, encompassing 18,340 individuals from 24 cohorts, with a predominant European ancestry ($n = 13{,}266$). A total of 211 gut microbiota profiles and 122,110 associated single nucleotide polymorphisms (SNPs) were recorded, and a taxonomic classification was conducted using direct taxonomic classification methods. Host genetic variations related to bacterial taxa abundance within the gut microbiota were identified through microbiota quantitative trait locus (mbQTL) mapping analysis. A total of 131 genera with an average abundance exceeding 1% were identified, including 12 unknown genera. Consequently, this study encompassed 119 genus-level taxonomic groups for analysis, comprising 196 bacterial taxa.

The summary statistics for GWAS of Sleep-Related Phenotypes (SRPs) were sourced from the IEU OpenGWAS project, encompassing traits such as sleeplessness/insomnia, chronotype (morning/evening preference), sleep duration, morning wakefulness, daytime napping, narcolepsy, and snoring. The GWAS ID, sample size, and SNP count for these traits are presented in Supplementary Table S1. Trait definitions utilized in this study are presented in Supplementary Table S2. Information pertaining to gut microbiota relevant to the study is presented in Supplementary Table S3.

2.2 The selection of instrumental variables

The following criteria guided IV selection as follows: (1) SNPs linked to each genus at a significance level of $p < 1.0 \times 10^{-5}$ were chosen as potential IVs (Li P. et al., 2022); (2) The linkage disequilibrium (LD) between SNPs was calculated using 1,000 Genomes Project European sample data as the reference panel, and among those with $R^2 < 0.001$ (using a clumping window size of 10,000 kb), only the SNPs with the most significant p-values were retained; (3) SNPs with a minor allele frequency (MAF) ≤ 0.01 were excluded; (4) In cases of palindromic SNPs, the forward strand alleles were determined based on allele frequency information.

2.3 Statistical analysis

In this study, we employed a range of statistical methods to investigate the causal relationship between exposure factors and the study's outcomes. Specifically, we used inverse variance weighted (IVW), MR-Egger, weighted median, and weighted mode methods. IVW, a classic technique, merged Wald ratio estimates from individual instrumental variables in a meta-analysis, essentially implementing a weighted linear regression relating instrumental variables to the outcome. IVW is advantageous as it provides unbiased estimates without horizontal pleiotropy. MR-Egger, based on the InSIDE assumption, primarily reflects the dose–response relationship between instrumental variables and outcomes, acknowledging some level of pleiotropy (Bowden et al., 2015). The weighted median method reduced class 1 errors and accommodated potentially invalid genetic variants. The weighted mode approach remained credible when most instrumental variables yielded consistent causal estimates, even if some did not meet MR method requirements. The primary method utilized is the IVW methods with a random effect assumption, assuming all SNPs to be valid instrumental variables or balanced horizontal pleiotropy, providing the most precise and unbiased estimates.

To ensure uniformity in effect alleles, we harmonized summary statistics and removed SNPs with unclear strands. We excluded palindromic SNPs to prevent allele effects on gut microbiota taxa and SRP causality. Horizontal pleiotropy and outliers were assessed using MR-Egger and MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) tests. Cochrane's Q test was used to test heterogeneity among instrumental variables. Leave-one-out sensitivity analysis tested outliers and result stability. To establish causality rigorously, we applied Bonferroni corrections based on the number of bacteria attributes (genera, families, orders, classes, and phyla).

A reverse causality analysis was conducted, and p-values within the range of the corrected values to 0.05 were considered nominally indicative of causation. The study was adhered to the STROBE-MR guidelines (Skrivankova et al., 2021), and statistical analyses were conducted using R software version 4.3.1, facilitated by the R package TwoSampleMR (version 0.5.6).

In summary, various statistical techniques were employed to comprehensively examine the causal relationship between exposure factors and outcomes, with IVW being the primary method. Sensitivity analyses and rigorous statistical corrections were performed to enhance the robustness and validity of the results. Additionally, reverse causality was considered.

3 Results

3.1 Selection of instrumental variables

We meticulously curated instrumental variables for 211 bacterial taxa. Those instrumental variables, which met the genome-wide significance threshold ($p < 1 \times 10^{-5}$) and had the confounding effects of specific taxonomic groups removed through linkage disequilibrium calculations, are presented in Supplementary Tables S4–S17. Upon rigorous examination, we observed an absence of weak instrumental variables, as all instrumental variables exhibited an F-statistic exceeding 10 (Supplementary Tables S32–S45).

3.2 Bidirectional two-sample MR analysis

3.2.1 Daytime dozing/sleeping (narcolepsy)

This study identified seven causal relationships between the gut microbiota and the risk of developing daytime dozing (Figure 1). A

FIGURE 1
IVW estimates from gut microbiota on SRPs. The color of each block represents the IVW-derived *p*-values of every MR analysis. *p*-values of <0.05 were shown in red and *p*-values of >0.05 were shown in yellow or green. *p*-value <0.05 is set as nominal significant, whereas ▲ represents significant [genera: 0.05/131 (3.81×10^{-4}), families: 0.05/35 (1.4×10^{-3}), orders: 0.05/20 (2.5×10^{-3}), classes: 0.05/16 (3.1×10^{-3}), and phyla: 0.05/9 (5.5×10^{-3})]. ↑ and ↓ represent, respectively, an increased and decreased risk of developing sleep-related traits in the gut microbiota.

higher genetically predicted class Gammaproteobacteria, phylum Bacteroidetes, genus *Butyricimonas*, genus *Clostridium sensustricto1*, genus *Coprococcus2*, and genus *Eubacterium eligens group* were associated with a higher risk of daytime dozing. Differently, genus *Intestinibacter* was associated with a lower risk.

In the reverse study, nine causal relationships were identified between daytime dozing and the risk of changes in gut microbiota. Daytime dozing may lead to a higher rate of *genus Flavonifractor*, *genus Anaerofilum*, *class Deltaproteobacteria*, and *genus Eubacterium eligens* group and a lower rate of *genus Fusicatenibacter*, *genus Oxalobacter*, *genus Slackia*, *genus Butyricicoccus*, and *genus Dorea*.

3.2.2 Getting up in the morning

This study identified seven causal relationships between the gut microbiota and the risk of getting up in the morning (Figure 1). A higher genetically predicted family Peptococcaceae was associated with a higher risk of getting up in the morning. Differently, genus *Intestinimonas*, order Bifidobacteriales, order Clostridiales, family Bifidobacteriaceae, genus *Anaerofilum*, and class Clostridia were associated with a lower risk.

In the reverse study, seven causal relationships were identified between getting up in the morning and the risk of changes in gut microbiota. Getting up in the morning may lead to a higher rate of

genus *Terrisporobacter,* order Bacteroidales, phylum Bacteroidetes, class Bacteroidia, and genus *Clostridium innocuum group* and a lower rate of genus *Intestinimonas and* genus *Slackia*.

3.2.3 Sleeplessness/insomnia

This study identified six causal relationships between the gut microbiota and the risk of developing insomnia (Figure 1). A higher genetically predicted genus *Oxalobacter,* class Negativicutes, order Selenomonadales, phylum Firmicutes, and genus *Eubacterium eligens group* were associated with a higher risk of insomnia. Differently, phylum Verrucomicrobia was associated with a lower risk.

In the reverse study, 12 causal relationships were identified between insomnia and the risk of changes in gut microbiota. Insomnia may lead to a higher rate of genus *Oxalobacter,* family Clostridiaceae1, and family Oxalobacteraceae and a lower rate of genus *Ruminococcaceae UCG013*, order Erysipelotrichales, order Rhodospirillales, class Alphaproteobacteria, family Erysipelotrichaceae, and family Lachnospiraceae.

3.2.4 Morning/evening person (chronotype)

This study identified seven causal relationships between the gut microbiota and the risk of developing being a morning/evening person (Figure 1). A higher genetically predicted genus *Parabacteroides*, genus *Prevotella7,* genus *Ruminococcus1,* and genus *Eubacterium coprostanoligenes group* were associated with a higher risk of being morning/evening person. Differently, family Peptococcaceae was associated with a lower risk.

In the reverse study, eight causal relationships were identified between being a morning/evening person and the risk of changes in gut microbiota. Being a morning/evening person may lead to a higher rate of *genus Ruminococcaceae UCG010*, family Porphyromonadaceae, and genus Butyricicoccus and a lower rate of *genus Streptococcus*, order Lactobacillales, class Bacilli, family Peptostreptococcaceae, and family Streptococcaceae.

3.2.5 Nap during day

This study identified six causal relationships between the gut microbiota and the risk of developing daytime napping (Figure 1). A higher genetically predicted genus *Oxalobacter*, order NB1n, genus *Butyricimonas,* and genus *Clostridium sensustricto1* were associated with a higher risk of daytime napping. Differently, genus *Holdemanella* and genus *Eubacterium fissicatena group* were associated with a lower risk.

In the reverse study, 10 causal relationships were identified between daytime napping and the risk of changes in gut microbiota (Figure 2). Daytime napping may lead to a higher rate of family Desulfovibrionaceae, class Deltaproteobacteria, genus *Desulfovibrio*, *genus Enterorhabdus,* and *genus Erysipelotrichaceae UCG003* and a lower rate of *genus Fusicatenibacter, genus Romboutsia,* order Desulfovibrionales, family Christensenellaceae, and *genus Butyricicoccus*.

3.2.6 Sleep duration

This study identified seven causal relationships between the gut microbiota and sleep duration (Figure 1). A higher genetically predicted genus *Odoribacter*, genus *Victivallis*, order Coriobacteriales, order Desulfovibrionales, phylum Lentisphaerae, family Coriobacteriaceae, genus *Anaerofilum*, class Coriobacteriia, and genus *Eubacterium fissicatena group* were associated with a higher risk of longer sleep duration. Differently, genus *Eubacterium hallii group* was associated with a higher risk of shorter sleep duration.

In the reverse study, seven causal relationships were identified between sleep duration and the risk of changes in gut microbiota. Longer sleep duration may lead to a higher rate of *o*rder Bacteroidales, family Bacteroidaceae, phylum Bacteroidetes, class Bacteroidia, genus *Alloprevotella,* and genus *Bacteroides* and a lower rate of genus *Lachnospiraceae UCG008,* genus *Senegalimassilia,* order Coriobacteriales, phylum Firmicutes, family Coriobacteriaceae and class Coriobacteriia.

3.2.7 Snoring

This study identified six causal relationships between the gut microbiota and the risk of developing snoring (Figure 1). A higher genetically predicted genus *Ruminococcaceae UCG010,* genus *Ruminococcus torques group,* genus *Terrisporobacter,* genus *Eubacterium ruminantium group* were associated with a higher risk of snoring. Differently, genus *Haemophilus* and genus *Senegalimassilia* were associated with a lower risk.

In the reverse study, four causal relationships were identified between snoring and the risk of changes in gut microbiota. Getting up in the morning may lead to a higher rate of genus *Anaerostipes* and genus *Erysipelotrichaceae UCG003* and a lower rate of genus *Lactobacillus* and genus *Peptococcus.*

3.3 Bonferroni-corrected test, sensitivity analysis, and reverse analysis

The results from the Bonferroni-corrected test revealed that a higher level of class Negativicutes retains a strong causal relationship with more severe insomnia (OR = 1.03, 95% CI: 1.02 to 1.05, $p = 0.0001$), whereas a higher level of order Selenomonadales retains a strong causal relationship with more severe insomnia (OR = 1.03, 95% CI: 1.02 to 1.05, p = 0.0001). At the same time, it turns out that a higher level of phylum Lentisphaerae leads to longer sleep duration (OR = 1.02, 95% CI: 1.01 to 1.04, $p = 0.0005$). In addition, more exposure to the *genus Senegalimassilia* has the potential to lead to improved symptoms of snoring (OR = 0.98, 95% CI: 0.96 to 0.99, $p = 0.0001$).

In Figure 1, we illustrate the causal links between intestinal flora and various outcomes. Notably, the F-statistics of our IVs ranged from 20.35 to 27.31, effectively mitigating any bias associated with weak IVs. Cochran's IVW Q test results revealed no significant heterogeneity among these IVs (Supplementary Tables S32–S38). Additionally, our MR-Egger regression intercept analysis showed no significant directional horizontal pleiotropy (Supplementary Tables S32–S38). Although potential IV outliers were observed upon visual inspection in scatter plots (Supplementary Figures 61–107), further analysis using MR-PRESSO did not identify any significant outliers. Consequently, there is insufficient evidence to support horizontal pleiotropy in the association between these bacteria and SRPs. Notably, our risk estimations for genetically predicted outcomes remained stable in the leave-one-out analysis, indicating that the causal relationship was not driven by specific SNPs (Supplementary Figures 61–107).

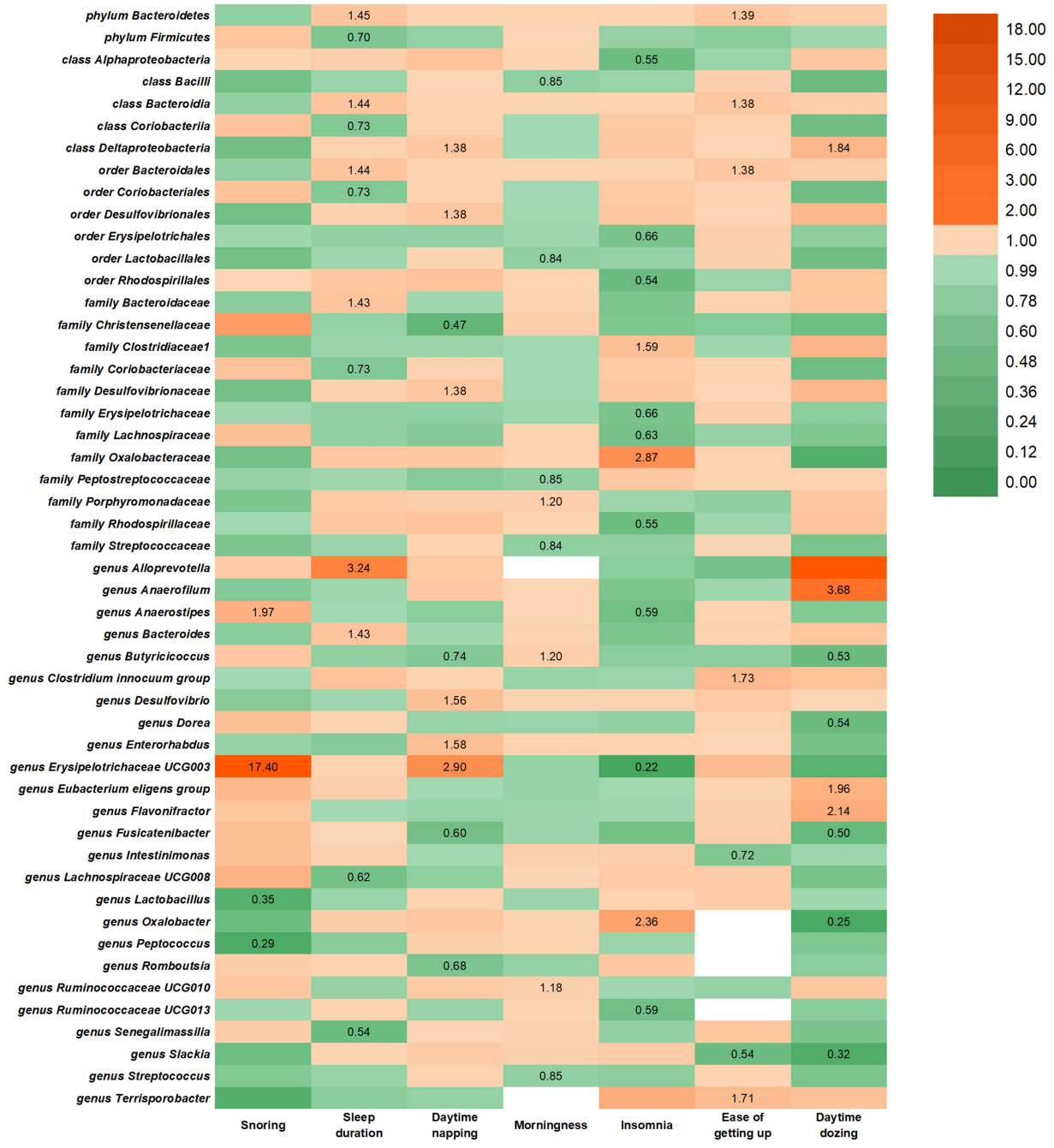

FIGURE 2
IVW estimates from SRPs on gut microbiota. The color of each block represents the IVW-derived odds ratio of every MR analysis. Odds ratio of <1.00 were shown in green and odds ratio of >1.00 were shown in yellow or orange.

Reverse MR analysis revealed a suggestive association between seven SRPs and 50 intestinal flora. However, Bonferroni-corrected testing indicated that only nominal significant associations were found between SRPs and other gut microbiota, as shown in Figure 2. Notably, Cochran's IVW Q test showed no significant heterogeneity among SRP IVs (Supplementary Tables S39–S45), and both MR-Egger regression intercept analysis and MR-PRESSO analysis failed to detect significant horizontal pleiotropy (Supplementary Tables S39–S45). Similar to forward analysis, our leave-one-out analysis (Supplementary Figures 1–60) demonstrated no significant changes in risk estimates for genetic predictions, confirming that causality was not driven by specific SNPs.

4 Discussion

4.1 Sleeplessness/insomnia

A multitude of preceding studies underscores a keen interest in unraveling the potential molecular mechanisms linking the intestinal

microbiota to the onset of insomnia. Disruption of the gut microbiome can precipitate intestinal inflammation and neural inflammation, thereby becoming embroiled in the pathogenesis of insomnia (de Theije et al., 2014; Sun and Shen, 2018). We have ascertained a positive correlation between the genus *Oxalobacter*, order Selenomonadales, phylum Firmicutes and the genus *Eubacterium eligens group*, with the propensity for insomnia. The order Selenomonadales, phylum Firmicutes, and the genus *Eubacterium eligens group* can generate butyrate (Louis and Flint, 2017; Gasaly et al., 2021), a metabolite intimately associated with inflammation. It is conceivable that butyrate may modulate inflammatory responses in the brain, employing microglial cells as target cells. An investigation suggests that butyrate, serving as a signaling molecule in the gut–brain axis, may ameliorate the cognitive impairments induced by sleep deprivation (Wang et al., 2023). Conversely, a lower association is established between insomnia and phylum Verrucomicrobia. An observational study corroborates our findings, demonstrating amelioration in sleep quality and a higher proportion of phylum Verrucomicrobia associated therewith. The assessment of sleep quality is conducted through the employment of the PSQI, underscoring that an augmentation in phylum Verrucomicrobia can engender an improvement in insomnia.

Insomnia exerts a multifaceted impact on the gut microbiota. Insomnia may lead to a reduction in the abundance of *genus Ruminococcaceae UCG013*, order Erysipelotrichales, order Rhodospirillales, and family Erysipelotrichaceae. In a study investigating sleep deprivation and inflammatory responses in mice (Zhang M. et al., 2023), a substantial increase in the abundance of *Rhodospirillales* and *Ruminococcaceae UCG013* was observed among sleep-deprived mice. Another study (Benedict et al., 2016), conducted on nine healthy adult males subjected to partial sleep deprivation, also noted a significant increase in the family Erysipelotrichaceae compared with baseline. These findings diverge from our research, potentially implying that experimentally induced sleep deprivation and subjective insomnia are not entirely congruent phenomena.

Furthermore, our investigation has unveiled that insomnia augments the abundance of the genus *Oxalobacter*, and the elevation of genus *Oxalobacter*, in turn, escalates the risk of insomnia. There exists a bidirectional causative relationship between these two factors, thus fostering a pernicious cycle of insomnia. However, it is worth noting that as of now, no study has definitively substantiated the causal link between these factors. Consequently, further research aimed at elucidating the causal relationship between them that may serve as a prospective avenue for future inquiries.

4.2 Sleep duration

In the investigation of the causal relationship between gut microbiota and sleep duration, we have discerned an association between the genus *Odoribacter* and an increased risk of prolonged sleep. A study concerning sleep disturbances in traumatic brain injury patients revealed a relative increase in the abundance of the genus *Odoribacter* within the intestinal microbiota of individuals who were suffering from post-traumatic sleep disturbances (Zhanfeng et al., 2022). This elevation may be correlated with excessively prolonged sleep following cranial trauma. Conversely, there exists a correlation between the genus *Eubacterium hallii group* and the risk of shortened sleep duration. Qiu et al. (2022) identified *Eubacterium hallii* as a microbial marker for sleep disturbances in individuals both before and after exercise interventions. The augmentation of *Eubacterium hallii* is associated with healthy sleep patterns. Based on the findings from MR analysis, it is conceivable that the genus *Eubacterium hallii group* may ameliorate sleep quality by modulating excessively extended sleep durations.

Regarding the study of the causal relationship between sleep duration and the risk of changes in the gut microbiota, it has been observed that longer sleep durations may lead to a diminished abundance of order Coriobacteriales, family Coriobacteriaceae, and class Coriobacteriia. In a cross-sectional investigation (Zhang et al., 2021), researchers found a negative correlation, which was statistically significant, between the relative abundance of *Coriobacteriia* and *Coriobacteriales* in individuals with type 1 narcolepsy and their total sleep duration, which aligns with the outcomes of our MR analysis.

Conversely, higher levels of order Coriobacteriales, family Coriobacteriaceae, and class Coriobacteriia may engender an increased risk of prolonged sleep duration. Lin et al. (2021) demonstrated that the probiotic *Lactobacillus PS150* can ameliorate sleep disturbances induced by the "first night effect" in mice, leading to an extension of sleep duration. They also noted a substantial increase in *Coriobacteriia* in the fecal samples of mice treated with *Lactobacillus PS150*. Therefore, in conjunction with the findings from our MR study, we have reasonable grounds to surmise that *Lactobacillus PS150* may indirectly contribute to the extension of sleep duration by reshaping the microbial composition, specifically with regard to *Coriobacteriia*.

Therefore, it is evident that *Coriobacteriia* plays a pivotal role in the bidirectional regulation of sleep duration. When considering therapeutic interventions for issues of excessive or insufficient sleep duration, it may be prudent to contemplate strategies that target this particular microbial community.

4.3 Easiness of getting up in the morning

This investigation unveils that the presence of the genus *Anaerofilum* mitigates the propensity for morning reluctance. A systematic review concerning the gut microbiota in individuals with depression has highlighted the heightened abundance of the genus *Anaerofilum* in this cohort. Previous studies (Kripke, 2016) have illustrated an inverse association between ease of early rising and depressive tendencies. Hence, the heightened abundance of the genus *Anaerofilum* in individuals with depression precipitates the challenge of early morning awakening.

Simultaneously, research has indicated that the use of sertraline, a commonly prescribed antidepressant, elevates the difficulty of morning arousal (Sun et al., 2017). This indirectly corroborates our findings. Furthermore, we have ascertained a bidirectional causal relationship between an increased abundance of the genus *Intestinimonas* and a reduction in the risk of morning reluctance. However, current evidence does not substantiate a definitive causative link between these factors. Thus, further investigations are imperative to ascertain the existence of a causal relationship between them.

4.4 Chronotype of morning/evening person

Previous investigations have elucidated diurnal fluctuations in the functionality and composition of the gut microbiota, which are intricately linked to circadian rhythms (Thaiss et al., 2014; Leone et al., 2015). Disruptions in the ecological equilibrium of the gut can lead to temporal misalignment (Wang et al., 2022). In our study, we have discerned that augmentation in the genus *Ruminococcus1* and the genus *Eubacterium coprostanoligenes group* escalates the risk of manifesting the chronotype of a morning or evening person. Carasso et al. (2021) have established a positive correlation between heightened fruit consumption and levels of the *genus Ruminococcus* within the microbiota, while increased intake of fruits and vegetables is associated with morning chronotypes. The gut microbiota plays a pivotal role in facilitating significant alterations in the expression patterns of circadian clock genes (Choi et al., 2021). For instance, in a study conducted on mice, it was observed that microbial populations, such as the genus *Eubacterium coprostanoligenes group*, generate butyrate, which modulates the expression of hepatic circadian clock genes (Leone et al., 2015). These findings align harmoniously with the outcomes of our investigation.

Similarly, perturbations in the host's circadian rhythms can impact the gut microbiota. Alterations in clock genes (Bmal1 or Per1/2) can influence the rhythmic oscillations of the gut microbiota. Long-term disruptions in circadian rhythms induced by changes in light exposure, jet lag, and shift work also exert an influence on the oscillatory patterns of the gut microbiota (Thaiss et al., 2014; Liang et al., 2015). However, the specific impact of circadian rhythm alterations on distinct microbial populations within the gut remains unclear. Currently, there is a growing interest in understanding how circadian rhythms can be harnessed for the prevention and treatment of psychiatric disorders. Future research endeavors may delve deeper into the intricate mechanisms through which circadian rhythms affect specific microbial communities within the gut, restore physiological circadian patterns, consider potential microbial transformations, and devise optimal intervention strategies (Zhang P. et al., 2023).

4.5 Daytime napping and daytime dozing

Our investigation reveals a bidirectional causal relationship between the gut microbiota and both daytime napping and daytime dozing, with considerable overlap in the specific microbial populations involved. For instance, an elevation in the levels of the genus *Butyricimonas* and genus *Clostridium sensustricto1* is associated with an increased susceptibility to daytime napping and daytime dozing. Conversely, engaging in daytime napping and daytime dozing results in an elevated proportion of class Deltaproteobacteria, accompanied by a decrease in the levels of the genus *Fusicatenibacter* and genus *Butyricicoccus*.

Previous research have uncovered a negative correlation between *genus Butyricicoccus* and the severity of rapid eye movement (REM) sleep, and in patients afflicted with Parkinson's disease (PD), a reduction in the abundance of *genus Butyricicoccus* is noted (Zhang Q. et al., 2023). This decrease may be linked to the heightened frequency of daytime dozing observed in PD patients. Furthermore, previous research (Zhang Q. et al., 2023) has indicated that individuals with Cushing's syndrome exhibit a significantly lower relative abundance of the genus *Eubacterium eligens group*, which correlates negatively with cortisol levels, systolic blood pressure (SBP), and diastolic blood pressure (DBP). Consequently, excess of the genus *Eubacterium eligens* group may lead to decreased daytime cortisol levels, thereby inducing daytime dozing, which, in turn, contributes to nocturnal difficulties in falling asleep. The underlying mechanisms driving the elevation in the abundance of the genus *Eubacterium eligens group* in response to daytime dozing remain a subject of exploration.

4.6 Snoring

Research concerning the interplay between the gut microbiota and snoring remains limited, while investigations into obstructive sleep apnea (OSA) have garnered considerable attention in recent years. Snoring is recognized as a hallmark of OSA (Campos et al., 2020). Further exploration of the relationship between snoring and the gut microbiota holds promise for future advancements in OSA treatments. An escalation in the levels of the genus *Ruminococcaceae UCG010* and genus *Ruminococcus torques group* is associated with an elevated risk of snoring. The study by Zhang C. et al. (2022) unveiled potential compensatory mechanisms in the alterations of the gut microbiota in the pathophysiology of OSA, proposing an association between OSA and the increased presence of *Ruminococcus_1* and *the Ruminococcus torques group* in patients. These findings are in remarkable congruence with our results.

Conversely, snoring also exerts an impact on the composition of the gut microbiota. We have observed that snoring leads to augmentation of the genus *Anaerostipes*. However, Li Q. et al. (2023) have reported a decrease in the genus Anaerostipes among individuals with severe OSA, presenting a contradiction with our findings. This incongruity may be attributed to differences between habitual non-apnoeic benign snorers and individuals with OSA. Additionally, snoring contributes to a reduction in the levels of the genus *Lactobacillus*. Studies (Liu et al., 2019) demonstrate that OSA can affect the gut microbiota of rodents by depleting lactobacilli. This observation may elucidate the decrease in *Lactobacillus*, resulting from snoring.

4.7 Clinical and research implications

The decline in sleep quality is considered a crucial catalyzing factor in the occurrence and exacerbation of various diseases, and is associated with an increase in healthcare expenditures. However, due to an incomplete understanding of potential mechanisms and a lack of early intervention measures, there has been a continuous quest for more effective, lower side-effect, and more convenient treatment methods. Our investigation supplements antecedent observational revelations and additionally scrutinizes the causal relationships between the gut microbiota and sleep-related attributes. These findings contribute to unveiling the impact of the gut microbiota on the development of sleep-related issues, paving novel avenues for etiological investigations into sleep-related concerns. We anticipate

that the aforementioned research findings can offer potential targets for intervening in insomnia through modulation of the gut microbiota. This, in turn, may serve as a reference for the prevention and treatment of sleep-related issues. For instance, by modulating the equilibrium of the gut microbiota, it may be plausible to mitigate the risk of developing insomnia or ameliorate the severity of these conditions. This could be accomplished through dietary alterations, probiotic supplementation, and analogous modalities. Nevertheless, the research landscape in this domain is still in its embryonic phase, necessitating further studies to corroborate these preliminary findings and ascertain the most effective intervention strategies. Furthermore, intricacy and individual variability of the gut microbiota pose a challenge, potentially requiring personalized therapeutic regimens.

4.8 Strengths and limitations

The strengths of this study encompass several facets. First, we have employed a multitude of sleep-related phenotypes, refining the scope of our sleep research. Second, the implementation of MR design has bolstered the credibility of causal inferences between the gut microbiota and sleep-related phenotypes by mitigating residual confounding and reverse causality. Third, the causal relationships unearthed in this investigation not only corroborate findings from previous studies but also pave the way for novel approaches in subsequent research, focusing on the targeted manipulation of specific gut bacterial populations to prevent and treat insomnia (Figure 3).

Nonetheless, certain limitations must be acknowledged. The stringent genome-wide statistical significance threshold (5×10^{-8}) for SNPs has compelled us to integrate SNPs meeting a genome-wide significance level of 1×10^{-5} into this study, given their limited availability. Second, a fraction of the gut microbiota data utilized in this study originates from populations of different ethnicities, potentially introducing bias into some of the research outcomes. Finally, we cannot discount the possibility of gene-diet or gene–environment interactions affecting the observed results, as these interactions may exert an influence on the ultimate outcomes under scrutiny.

5 Conclusion

This study has unveiled the causal nexus between the gut microbiome and SRPs, thereby endowing prospective clinical therapeutics and foundational inquiries with profound latent worth. On the clinical front, this revelation will substantiate the domain of personalized medicine and the genesis of novel therapeutic modalities, thereby mitigating pharmaceutical dependencies. In basic research, these findings will provide essential insights into the mechanisms behind insomnia disorders, enhancing our understanding of how the gut and brain interact and contribute to sleep difficulties. Furthermore, comprehension of these causal relationships is poised to galvanize the formulation of prophylactic measures, alleviating the burden of associated maladies and fostering interdisciplinary collaborations that burgeon innovative investigations. In summary, this discovery holds the promise of providing assistance to individuals troubled by insomnia disorders.

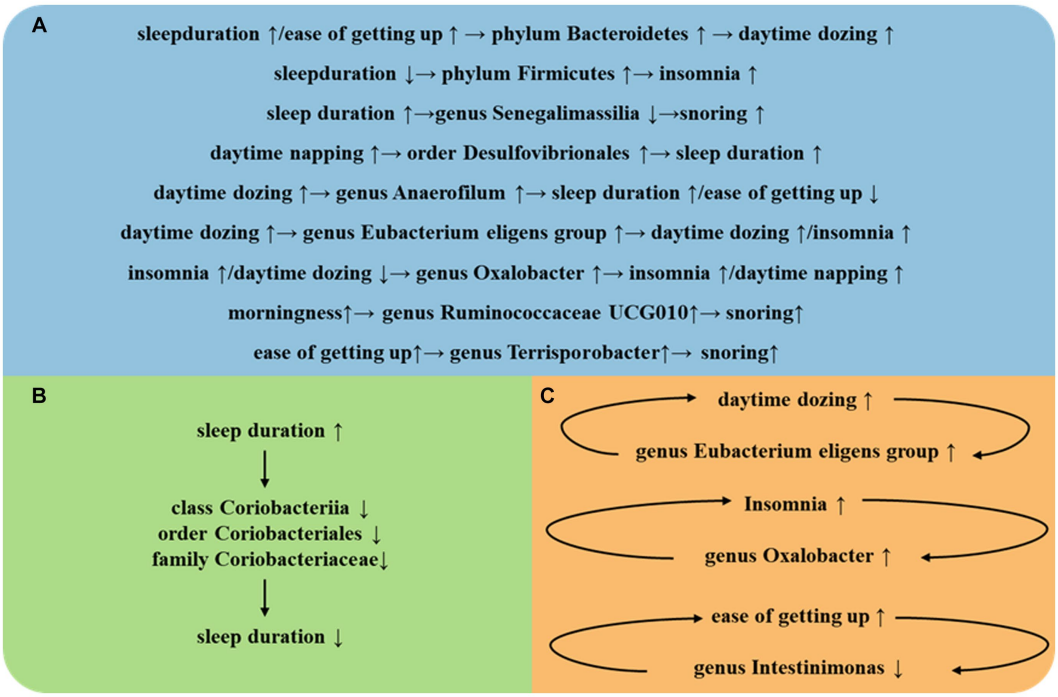

FIGURE 3
(A) Intestinal flora play an important role between different SRPs. **(B)** Intestinal flora play an important role in self-regulation between the same SRPs. **(C)** Intestinal flora play an important role in a vicious cycle between the same SRPs.

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

All the genes used in the research data are from the public database in the large-scale genome-wide association study (GWAS), so the study does not require ethical approval.

Author contributions

XW: Formal analysis, Methodology, Software, Writing – original draft. CW: Conceptualization, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. KL: Writing – original draft. QW: Writing – review & editing. WW: Writing – review & editing. CL: Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1341643/full#supplementary-material

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Fecal microbiota transplantation unveils sex-specific differences in a controlled cortical impact injury mouse model

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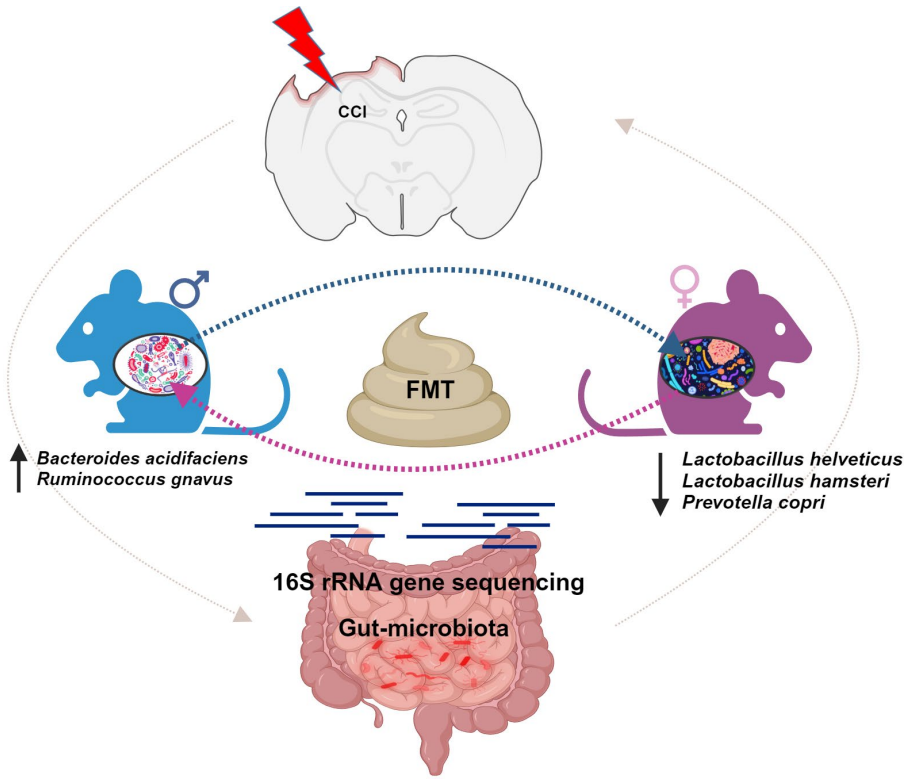
Introduction: Contusion type of traumatic brain injury (TBI) is a major cause of locomotor disability and mortality worldwide. While post-TBI deleterious consequences are influenced by gender and gut dysbiosis, the sex-specific importance of commensal gut microbiota is underexplored after TBI. In this study, we investigated the impact of controlled cortical impact (CCI) injury on gut microbiota signature in a sex-specific manner in mice.

Methods: We depleted the gut microflora of male and female C57BL/6 mice using antibiotic treatment. Thereafter, male mice were colonized by the gut microbiota of female mice and vice versa, employing the fecal microbiota transplantation (FMT) method. CCI surgery was executed using a stereotaxic impactor (Impact One™). For the 16S rRNA gene amplicon study, fecal boli of mice were collected at 3 days post-CCI (dpi).

Results and discussion: CCI-operated male and female mice exhibited a significant alteration in the genera of *Akkermansia*, *Alistipes, Bacteroides, Clostridium, Lactobacillus, Prevotella*, *and Ruminococcus*. At the species level, less abundance of *Lactobacillus helveticus* and *Lactobacillus hamsteri* was observed in female mice, implicating the importance of sex-specific bacteriotherapy in CCI-induced neurological deficits. FMT from female donor mice to male mice displayed an increase in genera of *Alistipes*, *Lactobacillus*, and *Ruminococcus* and species of *Bacteroides acidifaciens* and *Ruminococcus gnavus*. Female FMT-recipient mice from male donors showed an upsurge in the genus *Lactobacillus* and species of *Lactobacillus helveticus*, *Lactobacillus hamsteri*, and *Prevotella copri*. These results suggest that the post-CCI neurological complications may be influenced by the differential gut microbiota perturbation in male and female mice.

KEYWORDS

gut microbiome, fecal microbiota transplantation, traumatic brain injury, probiotics, controlled cortical impact



GRAPHICAL ABSTRACT

1 Introduction

Acute injuries linked with spinal cord and traumatic brain injury (TBI) as well as stroke pose several health problems, including neurological disabilities (Sun et al., 2018; Wang et al., 2018). TBI comprises a sudden mechanical injury to the brain by an external source (Coronado et al., 2005; Dewan et al., 2018). Each year, approximately 27–69 million people experience TBI incidence worldwide (Dewan et al., 2018; James et al., 2019). Experimental TBI in mice was reported to increase intestinal permeability and levels of endotoxins (Hang et al., 2003; Ma et al., 2017). Post-TBI gut microbiota perturbation was reported to negatively impact the recovery and lesion size (Nicholson et al., 2019). Indeed, growing literature documented the putative gut-brain crosstalk and the role of gut microbiota in the regulation of central nervous system (CNS) functions (Tan et al., 2011; Hazeldine et al., 2015; Treangen et al., 2018). The disturbed gut microbiota phenotypes have been evident in numerous neurological diseases such as autism, Alzheimer's disease, depression, TBI, and Parkinson's disease (Nicholson et al., 2019; Palepu and Dandekar, 2022; Rahman and Dandekar, 2023). Several clinical microbiome projects, investigating the connection of human gut microbiota with brain functionality and disease severity, are underway (Qin et al., 2010; The Human Microbiome Project Consortium, 2012a,b). It has also been suggested that the disruption of gut–brain crosstalk in the aftermath of TBI may affect the functioning of the central and enteric nervous system (Tan et al., 2011; Evans et al., 2015; Hazeldine et al., 2015; Sundman et al., 2017; Treangen et al., 2018; Ibrahim et al., 2022). The replenishment of healthy microbiota using probiotics confers positive impacts on several brain functions (Bienenstock et al., 2015; Sirisinha, 2016). For instance, the restoration of disrupted gut microbiota using probiotics was found useful in the management of dementia, depression, stroke, and inflammatory bowel diseases (Gao et al., 2017; Lye et al., 2018; Palepu and Dandekar, 2022; Rahman and Dandekar, 2023). Thus, perturbation of the gut microbiota may be directly linked to the progression of the disease (Louis, 2012; Sampson et al., 2016; Zheng et al., 2016; Jiang et al., 2017; Yuan et al., 2018).

In the acute CCI model, Treangen and colleagues reported a decreased abundance of the genus *Lactobacillus and Ruminococcus* and an elevation in *Eubacterium and Marvinbryantia* at 1 day post-injury (dpi) (Treangen et al., 2018). Nicholson et al. also showed that TBI may also results in gut microbiota dysbiosis, which was correlated with the lesion volume (Nicholson et al., 2019). Moreover, other TBI studies also reported changes in *Lactobacillus*, *Romboutsia,* and *Turicibacter* microbial genera following the injury (Kang et al., 2019; Koopman et al., 2019; Li et al., 2019; Du et al., 2021). Furthermore, probiotic treatment has shown potential benefits in animal models of spinal injuries, TBI, and stroke (Akhoundzadeh et al., 2018; Ma et al., 2019; Devi et al., 2021; Savigamin et al., 2022). In clinical settings, supplementation of *Lactobacillus*-rich probiotics showed improvement in TBI patients by modifying gut dysfunction (Tan et al., 2011). Additionally, replenishment of gut microbiota is also modified by fecal engraftment techniques improving neurological performance and shifting the pathobiome to healthy microbiota (Van Nood et al., 2013; Kelly et al., 2016; He et al., 2017). Taking into consideration the two-way connection between the gut microbiota and the brain, we intended to determine whether the gut microbiota is influenced by CCI.

Recent research has increasingly highlighted the sex-specific differences in TBI, encompassing variations in prevalence, disease severity, rates of emergency hospitalizations, and mortality rates (Faul

and Coronado, 2015; Cox et al., 2019). Furthermore, disparities in the constitution of the gut microbiome between male and female mice have been associated with the development of chronic diseases (Markle et al., 2013; Fransen et al., 2017). Notably, male mice tend to exhibit a higher abundance of genera like *Alistipes*, *Rikenella*, and *Porphyromonadaceae* in their gut microbiota, while female mice show increased levels of *Akkermansia* and *Lactobacillus* (Fransen et al., 2017). These distinctions in the gut microbiome composition potentially contribute to divergent responses to TBI severity and other associated comorbidities in male and female mice (Deitch et al., 2007; McGlade et al., 2015; Lavoie et al., 2017). Interestingly, there is an observed trend of greater resilience to post-traumatic consequences among women (Spychala et al., 2017). However, it is of crucial concern to note that the existing clinical evidence regarding sex-specific outcomes following TBI is still a subject of debate and controversy (Caplan et al., 2017).

This study aims to examine the sex-specific changes in gut microbiota following CCI surgery in both male and female mice. Moreover, the opposite-sex FMT approach was adopted, i.e., fecal slurry of male mice was transplanted into female mice and vice versa. The gut microbiota signature was evaluated from the samples collected at 3 dpi using the 16S rRNA v3 + v4 gene sequencing method. This study focused on the gut microbiome profiling, in particular, to identify the bacterial level abundance. Multiple studies have drawn observations stating that TBI-induced microbiome alterations often lead to a neuroinflammatory cascade ending up with secondary TBI (Ma et al., 2017; Nicholson et al., 2019).

2 Materials and methods

2.1 Animal housing, grouping, and CCI surgery

Adult, male and female, C57BL/6 mice (8–12 weeks) weighing 20–30 g were housed in polypropylene cages with free access to water and regular laboratory chow diet. The experimental and holding rooms were meticulously controlled to ensure a stable environment, with temperature maintained at 25 ± 2°C, humidity at 55 ± 5%, and an uninterrupted 12–12 h light–dark cycle. All experiments were conducted in compliance with the rules and guidelines of the Institutional Animal Ethics Committee of the NIPER, Hyderabad (Approval number: NIP/10/2020/PC/377).

Experimental mice were divided into six groups ($n = 4$): Group 1: Sham_M (Sham-operated male mice), Group 2: Sham_F (Sham-operated female mice), Group 3: CCI_M (male mice subjected to CCI surgery), Group 4: CCI_F (female mice subjected to CCI surgery), Group 5: CCI_M → F (female mice received FMT from male donor mice and then underwent CCI surgery), and Group 6: CCI_F → M (male mice received FMT from female donor mice and then underwent CCI surgery).

The surgical procedure of CCI has already been standardized in our laboratory (Rahman et al., 2022; Tentu et al., 2023). This procedure creates a highly reproducible brain injury that replicates the condition of a cortical contusion in humans. Briefly, mice were anesthetized with a mixture of isoflurane (4% for induction and 1–2% for maintenance) in 95% oxygen. The individual mouse was then placed on the stereotaxic apparatus (Stoelting) and secured with the use of a palate bar, nose clamp, and ear bars on both sides. CCI surgery utilizes a piston to apply controlled mechanical force through a surgical craniotomy directly to the exposed dura, typically 3–4 mm in size. Midway between bregma and lambda craniotomy was performed in the left parietal bone with the medial edge 0.5 mm lateral to the midline leaving the dura intact. The impact was delivered using a stereotaxic impactor (ImpactOneTM, Leica) at a velocity of 5 m/s with a 3 mm wide impactor, 0.1 s dwell time, and 1.5 mm deep followed by re-placing the skull flap on the exposed region to close the incision. A bead sterilizer (Steri 250) was used for the sterilization of surgicals across the animals during surgery. Sham mice of both sexes (Sham_M and Sham_F) also underwent the same surgical procedure, except impact was not delivered.

2.2 Fecal microbiota transplantation procedure

Fecal boli of male and female donor mice residing in different cages were collected at 3 dpi and immediately kept at −80°C for further use. Fecal samples of male and female donor groups were pooled and homogenized for 5 min using chilled phosphate buffer (120 mg feces/1 mL buffer) to make a paste-like consistency. Then, followed by centrifugation at 800x g for 3 min. Finally, the obtained supernatant was aliquoted in sterile tubes kept at −80°C for further use (Spychala et al., 2018).

Male and female mice were prepared for the FMT procedure by peroral administration of ampicillin sulfate (A), neomycin sulfate (N), metronidazole (M), and vancomycin hydrochloride (V) for 14 days (Morgun et al., 2015; Leclercq et al., 2020). Post-antibiotic treatment, the bowel cleansing (BC) was done using a solution of PEG3350, sodium bicarbonate, sodium sulfate, sodium chloride, and potassium chloride (Le Roy et al., 2019). Followed by the administration of prepared microbiota slurry by oral gavage (200 µL) to each recipient for 5 consecutive days once daily (Figure 1), FMT was carried out by administering a fecal slurry of male mice to the female recipient (CCI_M → F) and a fecal slurry of female mice to the male recipient (CCI_F → M). As described in Section 2.1, CCI surgery was adopted 30 days post-FMT.

2.3 Fecal collection for 16S rRNA gene amplicon study

Fecal samples were individually pooled from both male and female mice, which were housed temporarily in sterile cages without bedding on 3 dpi. These samples were then collected in sterile tubes and evenly distributed among all the experimental groups for further extraction steps.

2.4 DNA extraction

The genomic bacterial DNA was extracted from fecal samples using a QIAamp PowerFecal Pro DNA Kit (Qiagen catalog no: 51804). The procedure for the extraction of DNA from rodent fecal samples has been standardized and published in our earlier studies (Rahman et al., 2023). Briefly, 200–250 mg of fecal sample was added to the PowerBead Pro Tubes containing 800 µL of CD1 solution and then homogenized using a FastPrep-24™ bead grinder (MP Biomedicals, United States) followed by centrifugation at 15,000 × g for 1 min. Then, 200 µL of CD2 solution was added to the supernatant and centrifuged for 1 min. The supernatant was again transferred to a new tube containing 600 µL of CD3 solution, passed through the MB spin

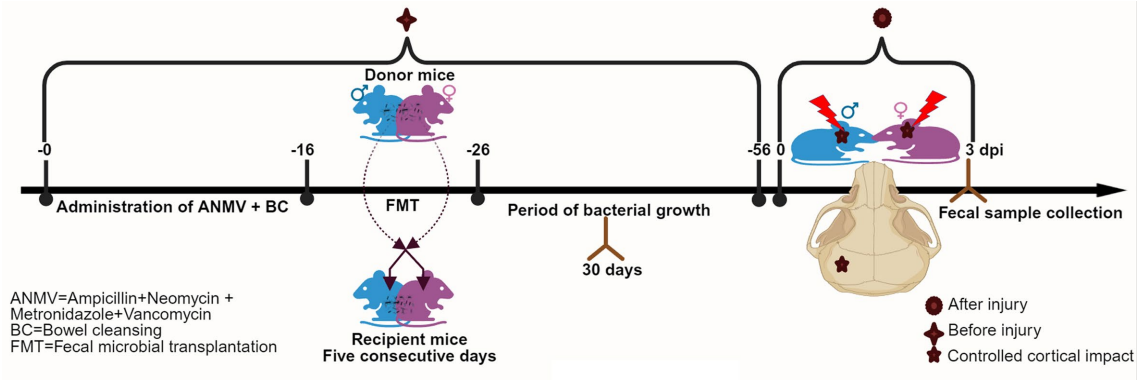


FIGURE 1
An *in-vivo* study design showing the timeline for antibiotic treatment, FMT, CCI, and fecal sample collection in mice.

column, and subsequently washed by 500 µL of EA and C5 solution. The column was then placed into a new tube, and 50–100 µL of solution C6 was added to the center of the white filter membrane. The DNA was collected after centrifugation at 15,000 × g for 1 min.

2.5 Library preparation

A total of 20 ng of DNA was used for the KAPA HiFi HotStart ReadyMix PCR Kit (Catalog: 0370, KAPA BIOSYSTEMS) and hypervariable regions (V3-V4) in the 16S gene amplicon assay (Amplicon et al., 2013). The process involves DNA (20 ng), primers of 100 nM concentration (341F and 785R), and KAPA HiFi HotStart ReadyMix (2X). PCR steps include denaturation for 5 min at 95°C, 20 cycles for 30 s at 95°C, accompanied by 45 s at 55°C, and for 30 s at 72°C, then followed by final extension for 7 min at 72°C and holding at 40°C. To confirm the amplification results, the PCR sample (3 µL) was loaded on agarose gel (2%) and the bands were notified at ~456 bp. To eliminate unused primers, cleansing of PCR entities was carried out (Catalog: A63881, Beckman Coulter) using XP beads 0.9X AMPure. Furthermore, eight cycles were performed for the purified PCR contents (4 µL) with a unique P7 and P5 barcoding process. The last set of PCR products underwent a secondary purification step with 0.9X AMPure XP beads, followed by elution of the final library in 15 µL (0.1X TE buffer) (Clevergene Biocorp Private Limited, Bangalore).

2.6 Library quantification

The DNA BR assay reagent stands out as an exceptionally sensitive detection dye for precisely quantifying DNA or library content in a solution. It offers a linear detection of fluorescence range from 100 pg./µL to 1,000 ng/µL. A ratio of 1:200 was utilized for diluting dye and buffer, followed by the addition of a library (1 µL). Furthermore, incubation was carried out for 2 min at room temperature, and subsequently, measurements were recorded by Qubit 3 Fluorometer (Life technologies/Catalog: Q33216). Prior to measurement, calibration was performed by the standards provided in the kit. The instrument was calibrated with the two standards supplied in the kit before measuring the sample.

2.7 Sequencing methodology

A total of 25 ng DNA was utilized for amplification of the (V3-V4) hypervariable region. The reaction involves modified primers (341F and 785R) of 100 nm and KAPA HiFi HotStart Ready Mix (Klindworth et al., 2013). PCR steps were similarly followed as described in Section 2.5. Ampure beads were used to remove unwanted primers. Furthermore, using Illumina barcoded adapters, 8 cycles of PCR were performed to prepare the sequencing libraries of 16S rRNA (V3–V4). Amplification of 16S bacterial rRNA gene was carried out using PCR targeting the V3-V4 regions using the following primers: (forward: 5′ CCTACGGGNGGCWGCAG; and reverse: GACTACHVGGGTATCTAATCC). A library was constructed by targeting the 16S V3–V4 regions, and sequencing was performed using the Illumina MiSeq platform with 300 bp paired-end libraries. The sequence data were produced using the Illumina MiSeq platform, and its quality was assessed using FastQC (Edgar et al., 2011) and MultiQC (DeSantis et al., 2006) software.

3 Bioinformatic analysis

3.1 Operational taxonomic units and statistical analyses

The sequences obtained from PCR were amplified and processed for quality inspection. Furthermore, reads were filtered using Trim Galore[1] via the Cutadapt tool. UCHIME algorithm (Edgar et al., 2011) was applied to flag contigs containing chimeric regions. Using the GREENGENES v.13.8-99 database, the filtered contigs were processed and classified into taxonomical categories (DeSantis et al., 2006). Operational taxonomic units (OTUs) were obtained after clustering the contigs followed by estimation of OTU abundance. Mothur pipeline (Schloss et al., 2009) was used to determine the alpha and beta diversity indices. Phylum, genus, and species level taxonomical analysis was plotted using the PAST 4.03 tool and GraphPad Prism statistical software. A p-value of < 0.05

1 http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

was regarded as statistically significant using one-way ANOVA with *post-hoc* Dunnett's multiple comparisons test.

3.2 Metagenomic data analysis

For the removal of the degenerate primers, reads (20 bp) were processed by trimming from the 5′ end, followed by the elimination of low-quality bases and adapter sequences using Trim Galore. Reads that passed the quality control test were streamlined into Mothur (Schloss et al., 2009), making the aligned pairs form contigs. The contigs whose size falls within the range of 300–532 bp were reserved after the process of error screening. Furthermore, high-quality contigs were quantified for similar sequences while any ambiguous base calls were rejected. The contigs were then aligned to a standard database for 16S rRNA. Despite the use of pre-designed 16S bacterial primers, chances of non-specific amplification exist. Most of the contigs were aligned to their specific target databases. Based on the amplification of variable regions, any ambiguous contigs aligned to non-specific sites on the database were removed. Then followed by the removal of overhangs and gaps at their end sites from the contigs to be processed for the removal of chimera, which are raised due to PCR errors. Later, the UCHIME algorithm (Edgar et al., 2011) was availed to flag contigs with chimeric regions. Standard references for all the chimeric sequences were utilized to recognize and eliminate possible chimeric sequences. GREENGENES v.13.8–99 database was additionally used to classify OTUs after processing the filtered contigs (DeSantis et al., 2006). Finally, for the final estimation of abundance, contigs were clustered into OTUs (Supplementary file S1).

4 Results

4.1 Gut microbiota richness and diversity analysis

The alpha diversity indices of gut microbiota were evaluated at 3 dpi using a 16S rRNA gene amplicon (Figures 2A–L; Supplementary file S2). One-way ANOVA revealed a reduced microbiota richness in both male and female mice after CCI (CCI_M and CCI_F) compared with sham-operated mice, as indicated by observed OTUs, ACE, and Chao 1 index. Moreover, reduced abundance and evenness (Shannon, Simpson, and Fisher indices) of gut microbes were observed in CCI_M and CCI_F mice. These findings were also evident in a few earlier studies (Du et al., 2021; Hou et al., 2021).

In the FMT module, we noticed that female mice receiving microbiota from male donors (CCI_M → F) had a higher OTU score than male mice who received microbiota from female donors (CCI_F → M). Assessing the gut microbiota richness using the ACE and Chao 1 indices revealed a notable increase in richness in CCI_M → F mice compared with CCI_F → M mice. Furthermore, CCI_M → F mice also demonstrated the greater evenness and abundance, as indicated by Shannon, Simpson, and Fisher indices, than both male and female sham-operated mice.

Gut microbiota beta diversity (Supplementary file S3) was assessed by analyzing OTU-level differences across the different groups. The dissimilarities index was calculated on a variance and co-variance matrix using a 4-point eigenvalue scale and represented in a principal component analysis (PCA) plot using PAST 4.03 (Figure 3). The plot showed minimal dissimilarities between Sham_F vs. CCI_F and Sham_M vs. CCI_M groups. Similarly, no major beta diversity was seen in male and female mice after CCI at 3 dpi. In fecal-engrafted groups also, we did not observe major sex-specific differences in OTU diversity.

4.2 Phylum level changes in gut microbiota

The phylum-level changes in the gut microbiota phenotypes are depicted in a stacked bar chart (Figure 4, Supplementary file S4). The most abundant phyla in Sham_F constitute *Bacteroidetes* (47%), *Firmicutes* (43.65%), *Proteobacteria* (7.2%), and others (1.44%). The compositional distribution of Sham_M mice was *Bacteroidetes* (50.75%), *Firmicutes* (39.70%), *Proteobacteria* (7.5%), and others (1.96%). At 3 dpi, CCI_F and CCI_M mice displayed a decline in the abundance of *Bacteroidetes*. Furthermore, changes in the phylum abundance were also noticed in FMT-recipient mice. Specifically, among CCI_M → F mice, there was an increase in *Bacteroidetes* (46.8%) and a decrease in *Firmicutes* (39.9%) composition. Conversely, CCI_F → M mice exhibited the opposite pattern with a decline in *Bacteroidetes* (37.4%) and a rise in *Firmicutes* (54.7%). In the *Firmicutes/Bacteroidetes* (F/B) ratio (Figure 5), the increased F/B ratio was evident in CCI_M and CCI_F mice, signifying gut dysbiosis; additionally, in fecal-engrafted mice, a ratio of 1.1 (CCI_M → F mice) and 1.6 (CCI_F → M) was noticed.

4.3 Genus level changes in gut microbiota

Analysis of fecal gut microbiome samples through polar plots in PAST 4.03 unveiled a rapid shift in the abundance of the genera at 3 dpi such as *Akkermansia*, *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, *Prevotella*, and *Ruminococcus* in CCI_M and CCI_F mice, as well as in FMT groups. Unlike CCI_M mice, a notable decline in the levels of *Alistipes*, *Lactobacillus*, and *Prevotella* genera was observed in the CCI_F group compared with sham. Furthermore, a noticeable increase in the relative prevalence of *Akkermansia* and *Ruminococcus* genera was also seen in CCI_M and CCI_F mice of both sexes. Considering the significant shifts in gut microbial communities, it is worth mentioning that the CCI_F group exhibited a marked reduction in the *Lactobacillus* genus compared with the sham-operated female mice.

The examination of sex-specific changes in gut microbiota using the FMT approach has revealed a notable change in *Lactobacillus* genus in CCI_M → F group as compared to that in CCI_F → M (Figure 6; Supplementary file S5). Marked elevation of *prevotella* was noticed in the CCI_M → F group, while the increase in *Alistipes* and *Ruminococcus* was seen in CCI_F → M engrafted mice. Furthermore, when examining the abundance in sham mice of both sexes, no notable changes have been observed. However, sham_F mice exhibited a higher percentage of *Lactobacillus* abundance than sham_M mice.

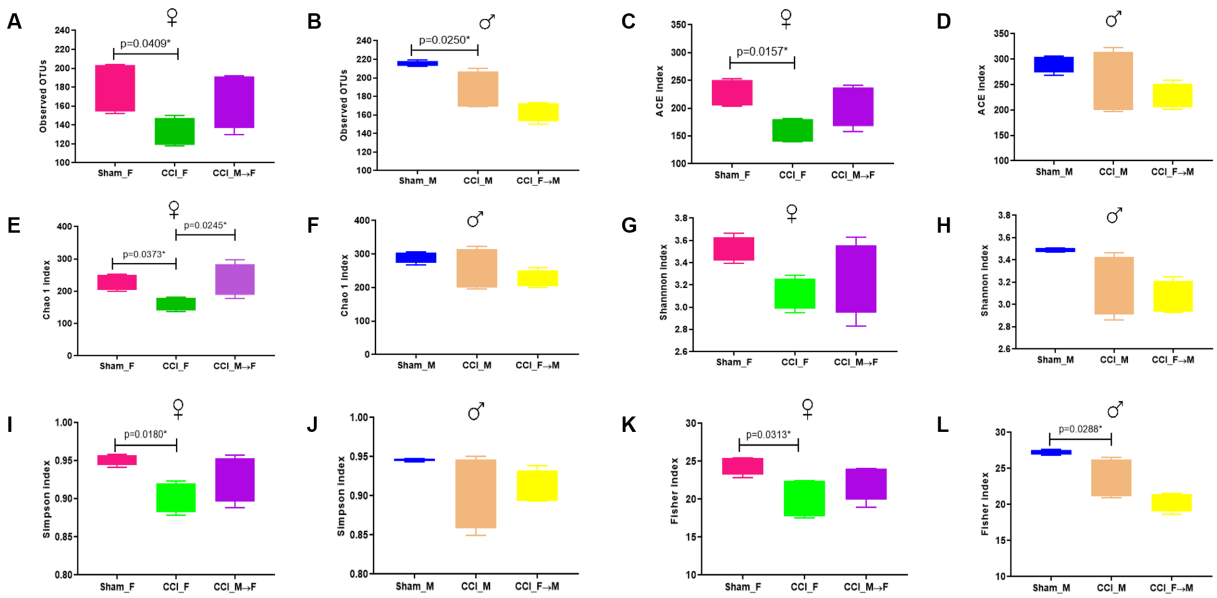


FIGURE 2
Graphs showing the richness and diversity index. Observed OTUs were **(A)** $p = 0.0409$ vs. CCI_F and **(B)** $p = 0.0250$ vs. CCI_M mice. ACE index showing $p = 0.0157$ vs. CCI_F mice **(C,D)**. No significant difference compared with CCI_M mice. Chao 1 index **(E)** $p = 0.0373$ vs. CCI_F, and no significant difference was seen in CCI_M mice **(F)**. Shannon index **(G,H)** represents a greater decline in CCI mice of both sexes compared with Sham and FMT groups. Simpson index showing $p = 0.0180$ vs. CCI_F **(I)**, and no significant difference is seen in CCI_M **(J)**. Fisher index **(K,L)** represents $p = 0.0313$ vs. CCI_F and $p = 0.0288$ vs. CCI_M mice, respectively.

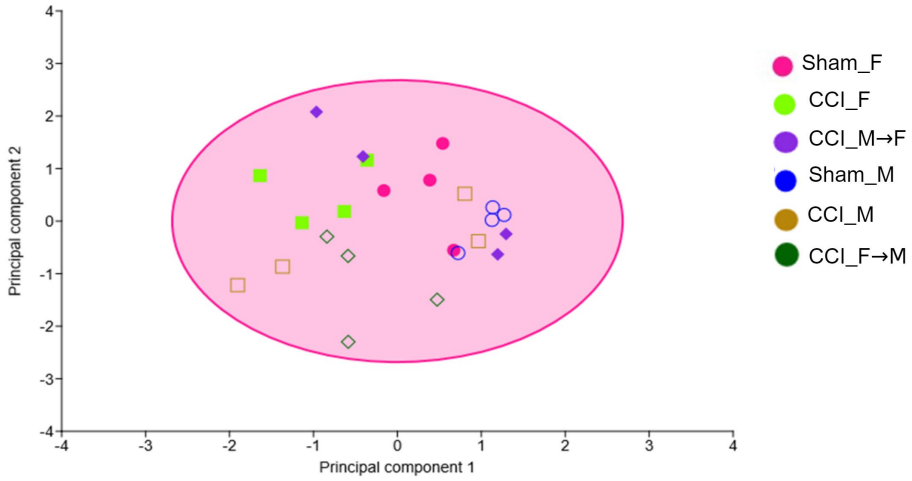


FIGURE 3
The graph showing the principal component analysis (PCA) of the fecal samples at 3 dpi. Symbols with different colors and shapes represent different groups (six).

4.4 Species-level changes in gut microbiota

Sham_F mice displayed a greater abundance of specific species (*Bacteroides* and *Lactobacillus*) in contrast to sham_M mice, as depicted in the stacked plot (Figure 7). At 3 dpi, a rapid shift in the abundance of *Akkermansia muciniphila*, *Alistipes finegoldii*, *Bacteroides acidifaciens*, *L. hamsteri*, *L. helveticus*, *Prevotella copri*, and *Ruminococcus gnavus* was observed at 3 dpi in both sexes as well as in FMT groups. In contrast to CCI_M mice, CCI_F mice displayed a marked decline in the abundance of *L. helveticus* and *L. hamsteri*. A significantly decreased abundance of *L. helveticus* was observed in CCI_F mice ($p = 0.0095$ vs. sham_F) as compared to CCI_M mice (Figure 8A). Furthermore, *L. hamsteri* showed the reduced abundance in CCI_F mice compared to CCI_M mice, but this change was not significant statistically when compared to sham_F mice (Figure 8B). One-way ANOVA followed by *post-hoc* Tukey's multiple comparisons test revealed a decline in the abundance of *Alistipes finegoldii* and *Bacteroides acidifaciens* (Figures 8C,D), with no major statistical significance.

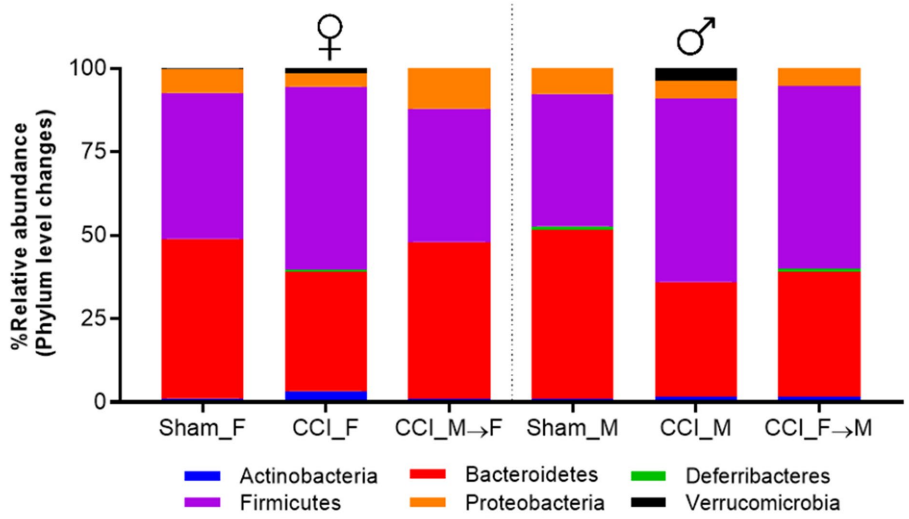


FIGURE 4
Graph representing the percentage relative abundance at top 6 phyla across the Sham male and female mice, CCI-induced female (CCI_F) and male (CCI_M) mice and fecal microbial recipient groups, i.e., CCI_M → F (male mice as a donor to female mice as the recipient) and CCI_F → M (Female mice as a donor to male mice as the recipient) using GraphPad Prism software.

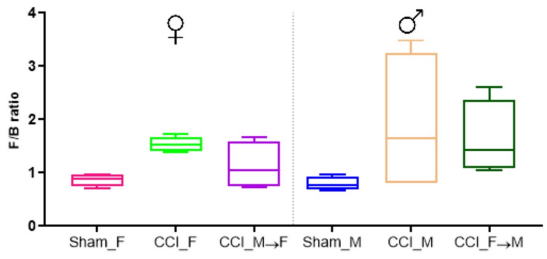


FIGURE 5
Graph representing the *Firmicutes* to *Bacteroidetes* (F/B) ratio across the sham mice of female and male (Sham_F and Sham_M), CCI-induced female (CCI_F), and male (CCI_M) mice and fecal microbial recipient groups, i.e., CCI_M → F (male mice as donor to female mice as recipient) and CCI_F → M (Female mice as a donor to male mice as recipient) using GraphPad Prism software.

Radar plots were employed to depict the sex-specific changes at the species level (Figure 9). These plots represented a greater richness and abundance in *L. helveticus* and *L. hamsteri*. Notably, a greater richness of these *Lactobacillus* species was noticed in CCI_M → F group as compared to CCI_F → M. These findings underscore the significance of *Lactobacillus* in potential therapeutic interventions (Supplementary file S6).

A Venn diagram (Figures 10A,B) illustrated the occupancy and distribution of the core microbiome of 568 OTUs among six groups. The Venn plot (Supplementary file S7) also revealed the combined OTUs among groups such as sham_F vs. CCI_F vs. CCI_M → F (Ma et al., 2017) and sham_M vs. CCI_M vs. CCI_F → M (Evans et al., 2015). The UpSet plot (Supplementary file S8) displayed the interaction size of genera and species among six groups (Figure 11). It was observed that 104, 97, 113, 104, 83, and 73 OTUs were unique to sham_F, CCI_F, CCI_M → F, sham_M, CCI_M, and CCI_F → M groups, respectively, and five OTUs were commonly shared across all the groups.

5 Discussion

A key impact of gut microbiota has been demonstrated in the regulation of inflammation, immune responses, anxiety-related outcomes, and post-injury consequences (Kelly et al., 2016; Robertson et al., 2017; Davis et al., 2022; Medel-Matus et al., 2022). Numerous studies also emphasized the importance of gut microbiota in post-TBI complications (Hang et al., 2003; Ma et al., 2017; Nicholson et al., 2019; Du et al., 2021). The CCI surgery in rodents mimics several clinical symptoms of the contusion type of TBI and also displays a wide change in gut microbiota phenotypes (Evans et al., 2015; Hazeldine et al., 2015; Treangen et al., 2018; Zhu et al., 2018). In this study, we chose a 3-dpi time point for the investigation of phylum, genus, and species level changes in fecal samples, as it is a critical time point with respect to hippocampal neurogenesis and histopathological changes in the brain after CCI (Sun et al., 2007; Kernie and Parent, 2010; Pabón et al., 2016). We observed a substantial decrease in bacterial richness (ACE and Chao 1 index) after CCI in both male and female mice. Moreover, reduced evenness and abundance of gut microbiota were reflected in Shannon, Simpson, and Fisher indices in both male and female mice. These alpha diversity-related changes in gut microbiota following CCI correspond with the earlier research findings (Nicholson et al., 2019; Davis et al., 2021, 2022; Du et al., 2021; Frankot et al., 2023). The bidirectional crosstalk of the brain and gut microbiota has also been evident in the management of brain injuries such as stroke, spinal cord injuries, and TBI (Singh et al., 2016; Akhoundzadeh et al., 2018; Sun et al., 2018; Ma et al., 2019; Devi et al., 2021; Frankot et al., 2023). Thus, as suggested in earlier studies (Benakis et al., 2016; Ma et al., 2019), rebuilding gut microflora biodiversity may be essential for recovery from brain injury.

In this study, CCI-operated male and female mice showed noticeable changes in the genera of *Akkermansia*, *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, *Prevotella*, and *Ruminococcus* at 3 dpi. The *Alistipes* genus is abundantly found in human gut

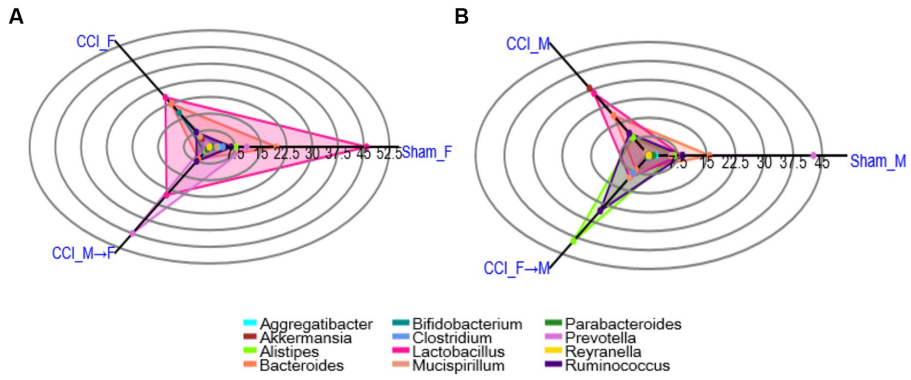


FIGURE 6
Polar plots showing the abundance of top 12 genus level across the Female **(A)** and Male **(B)** mice. Groups representing: sham mice of female and male (Sham_F and Sham_M), CCI induced female (CCI_F) and male (CCI_M) mice and fecal microbial recipient groups i.e., CCI_M→F (male mice as donor to female mice as recipient) and CCI_F→M (Female mice as a donor to male mice as recipient) using PAST 4.03 software.

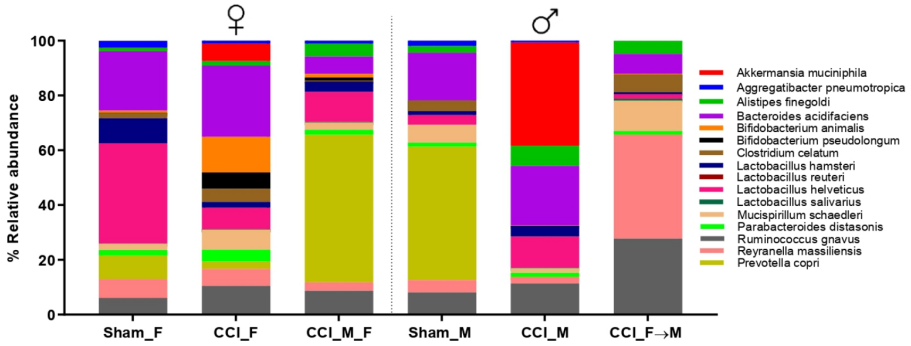


FIGURE 7
Stacked bar plot showing the abundance of top 16 species level across the sham mice of female and male (Sham_F and Sham_M), CCI-induced female (CCI_F) and male (CCI_M) mice, and fecal microbial recipient groups, i.e., CCI_M → F (male mice as donor to female mice as recipient) and CCI_F → M (Female mice as a donor to male mice as recipient) using GraphPad Prism software.

microbiota (Hou et al., 2021), which offers dimorphic effects, i.e., protection against colitis and autism spectrum disorder, and also potentially contributes to anxiety, myalgic encephalomyelitis/chronic fatigue syndrome, and depression (Koren et al., 2013; Morgan et al., 2013; Manrique et al., 2016; Parker et al., 2020). *Ruminococcus* bacterium plays a key role in stress management in wild and domestic animals (Rowin et al., 2017). In line with earlier TBI studies, an increase in *Ruminococcus* species in both male and female CCI mice was noticed (Celorrio et al., 2021). Recent investigations on *Lactobacillus* and *Bifidobacterium* containing probiotics illustrated the role of these bacteria in the reversal of anxiety, chronic psychological stress, neuronal death, and cognitive dysfunction in mice (Messaoudi et al., 2011a,b; Zakostelska et al., 2011).

We noted a greater abundance of *Akkermansia* at 3 dpi in male mice than in female mice. The increased abundance of *Akkermansia* has already been indicated in TBI patients and brain injury rat models (Hou et al., 2021). The variable levels of *Clostridium* species were seen in both male and female mice after CCI, which influences inflammatory bowel disease (Kabeerdoss et al., 2013; Lopetuso et al., 2013). Moreover, we noted differences in *P. copri* abundance, which are connected with elevated serum amino acids, especially branched-chain amino acids (Pedersen et al., 2016). The relevance of *P. copri* has been correlated with bacterial vaginosis, rheumatoid arthritis, periodontitis, and other chronic inflammatory states (Pianta et al., 2017; Accetto and Avguštin, 2021). We also noted the increased abundance of *P. copri* in the CCI_M → F group. In this way, sex-specific characterization of gut microbiota phenotypes may be useful for predicting post-TBI neurological recovery and crafting better therapeutic approaches (Luczynski et al., 2016; Sundman et al., 2017; Davis et al., 2021; Du et al., 2021).

TBI reduces gut microbial diversity and disrupts the ratio of healthy to opportunistic bacteria. Our 16S rRNA gene amplicon results revealed a substantial decline in microbial diversity. We found a notable change in the diversity of *Lactobacillus* genera, indicating the therapeutic importance of *Lactobacillus*. The *Lactobacillus* genus was decreased in CCI_F compared with CCI_M mice. Moreover, the abundance of *L. helveticus* and *L. hamsteri* has indicated that specific beneficial gut microbiota may play a pivotal lead in the recovery process following a brain injury. Furthermore, the potential neuroprotective benefits of probiotics have already been reported for neurotraumatic events (Fransen et al., 2017). For instance, *L. acidophilus* and *L. reuteri* improve post-TBI neuronal complications and brain edema by increasing the expression of occludin and neuronal survival (Deitch et al., 2007; Lavoie et al., 2017). These findings provide

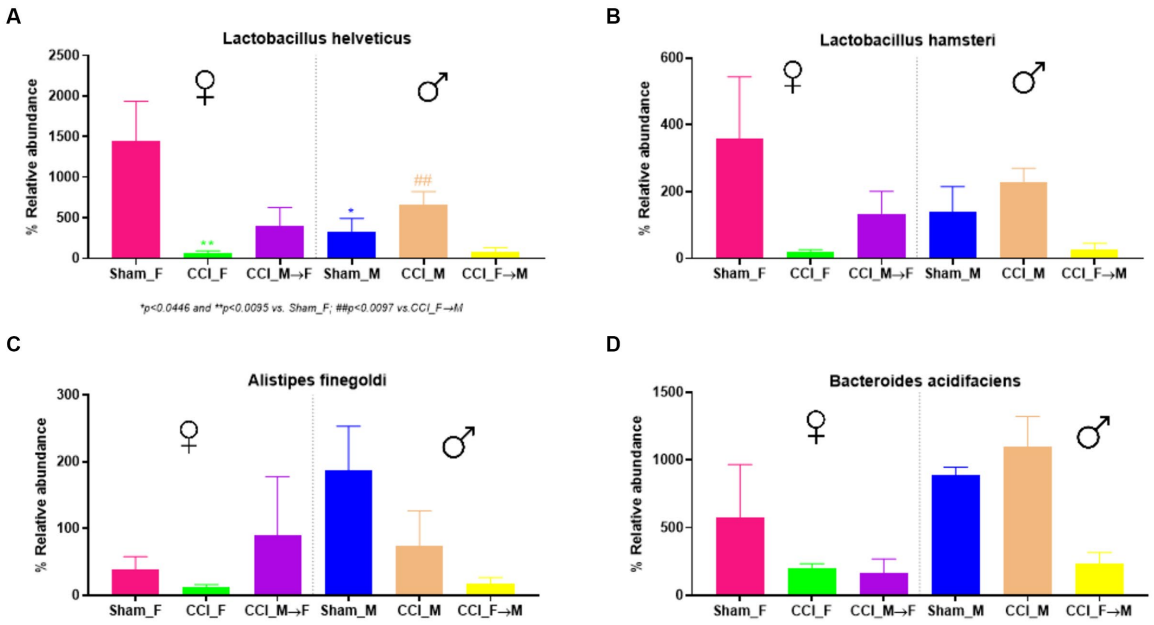


FIGURE 8
Graphs showing the abundance of *Lactobacillus helveticus* **(A)**, *Lactobacillus hamsteri* **(B)**, *Alistipes finegoldii* **(C)**, and *Bacteroides acidifaciens* **(D)** using GraphPad Prism software. The significance of $p = 0.0095$ vs. CCI_F was noticed in female mice.

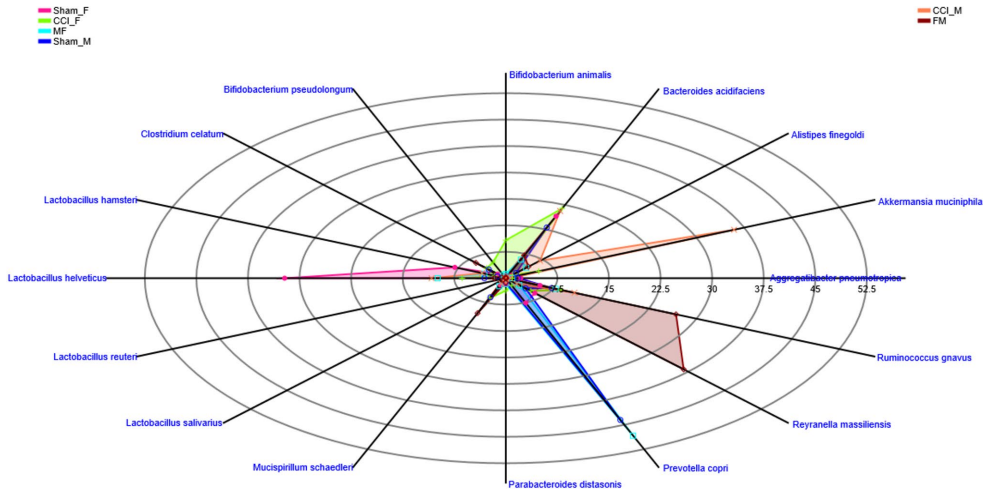


FIGURE 9
Radar plot representing the sex-specific percentage distribution of species among all the groups showing the greater richness and abundance across 16 represented species using PAST 4.03 software.

promising avenues for better therapeutic strategies in treating neurological illnesses (Messaoudi et al., 2011a,b; Zakostelska et al., 2011). As probiotics impose a positive impact on brain functions, they also possess a role in influencing the diversity of the gut microbiota, which is crucial for overall wellbeing (Bienenstock et al., 2015; Sirisinha, 2016; Akhoundzadeh et al., 2018; Ma et al., 2019; Devi et al., 2021; Savigamin et al., 2022). These preliminary results indicate that the sex-specific restoration of gut microbiota may reverse the CCI-generated neurological complications.

6 Conclusion

In this study, we observed an increase in the *Alistipes* and *Bacteroidetes* genera following CCI surgery in both male and female mice. The sex-specific changes were identified in the *P. copri*, *L. helveticus*, and *L. hamsteri* species at 3 dpi. We suggest that the modulation of these microbial strains may hold promise for developing sex-specific gut microbiota-based interventions in cases of brain injury.

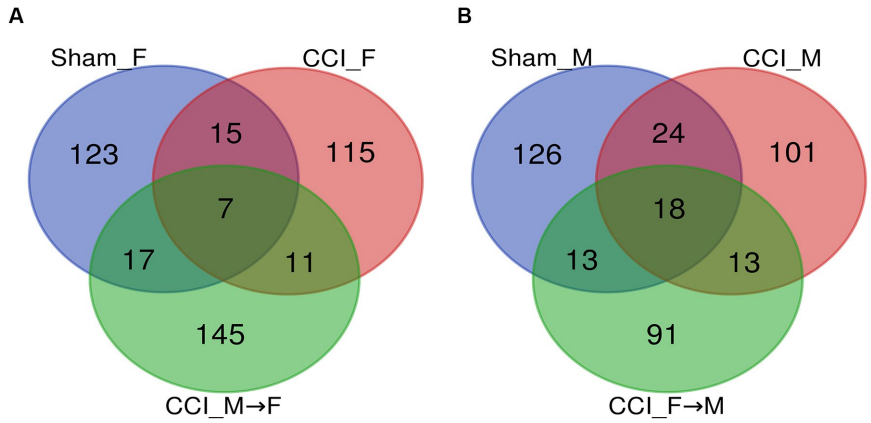


FIGURE 10
Venn diagram representing bacterial community OTUs (genus and species) distribution, and intersection among the female **(A)** and male mice **(B)**.

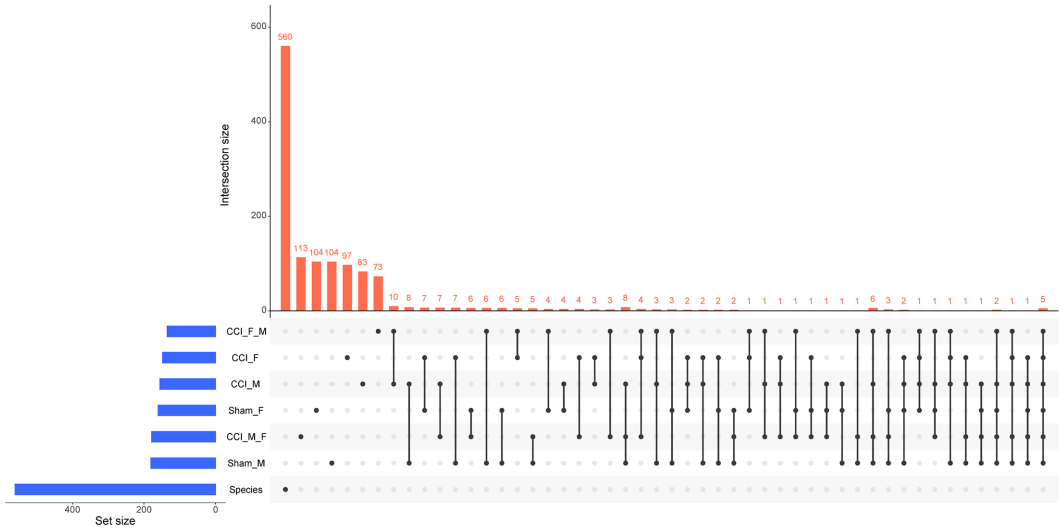


FIGURE 11
UpSet plot eliciting the intersection of species among different groups. The total size of each set is represented on the left bar plot. Every connection is represented by the bottom plot, and their occurrence is shown on the top bar plot.

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) SRA (Sequence Read Archive) files were submitted to NCBI with accession number BioSample accession: SAMN38196246 and Bioproject ID: PRJNA1037720) can be found in the article/Supplementary material.

Ethics statement

The animal studies were approved by Institutional Animal Ethics Committee of the NIPER, Hyderabad (Approval number: NIP/10/2020/PC/377). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

TP: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. MD: Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1336537/full#supplementary-material

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Alteration of the gut microbiota profile in children with autism spectrum disorder in China

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Background: Autism spectrum disorder (ASD) is associated with alterations in the gut microbiome. However, there are few studies on gut microbiota of children with ASD in China, and there is a lack of consensus on the changes of bacterial species.

Purpose: Autism spectrum disorder (ASD) is associated with alterations in the gut microbiome. However, there are few studies on gut microbiota of children with ASD in China, and there is a lack of consensus on the changes of bacterial species.

Methods: We used 16S rRNA sequencing to analyze ASD children (2 to 12 years), HC (2 to 12 years).

Results: Our findings showed that the α-diversity, composition, and relative abundance of gut microbiota in the ASD group were significantly different from those in the HC groups. Compared with the HC group, the α-diversity in the ASD group was significantly decreased. At the genus level, the relative abundance of g_Faecalibacterium, g_Blautia, g_Eubacterium_eligens_group, g_Parasutterella, g_Lachnospiraceae_NK4A136_group and g_Veillonella in ASD group was significantly increased than that in HC groups, while the relative abundance of g_Prevotella 9 and g_Agathobacter was significantly decreased than that in HC groups. In addition, KEGG pathway analysis showed that the microbial functional abnormalities in ASD patients were mainly concentrated in metabolic pathways related to fatty acid, amino acid metabolism and aromatic compound metabolism, and were partially involved in neurotransmitter metabolism.

Conclusion: This study revealed the characteristics of gut microbiota of Chinese children with ASD and provided further evidence of gut microbial dysbiosis in ASD.

KEYWORDS

gut microbiota, autism spectrum disorder, Chinese children, metabolic pathways, gut-brain axis

Introduction

Autism spectrum disorder (ASD) refers to a group of complex neurodevelopmental disorders that typically present in early childhood and are characterized by stereotyped behavior, language, and social interaction disorders (Valentino et al., 2021). The prevalence of ASD has increased significantly in recent decades, affecting 1 in 59 children in the United States (Maenner et al., 2020) and approximately 39.23/10000 Chinese children aged 1.6–8 years (Wang et al., 2018). Patients with ASD require lifelong treatment and intervention, making it a serious public

health concern (Joon et al., 2021). Both genetic and environmental factors are believed to be potential triggers for ASD (Wang et al., 2016; Modabbernia et al., 2017; Lord et al., 2018), with abnormal gut microbiota being an important environmental factor. Gastrointestinal problems are common in children with ASD, affecting 9–91% of cases (Ferguson et al., 2019) and often correlating with the severity of ASD (Adams et al., 2011).

Research has shown that the human gut microbiota plays a crucial role in influencing the brain through a communication pathway, called the gut-brain axis (Fowlie et al., 2018; Iannone et al., 2019; Lacorte et al., 2019; Sharon et al., 2019; Srikantha and Mohajeri, 2019). Changes in the gut microbiota can regulate gastrointestinal physiology, immune function, and behavior through the gut-microbiota-brain axis (Li et al., 2019; Sharon et al., 2019; Tomova et al., 2020; Nogay and Nahikian-Nelms, 2021). Germ-free animal studies have demonstrated that the gut microbiota shapes the brain by influencing synaptic signaling and gene transmission (Sanlier and Kocabas, 2021). *Lactobacillus reuteri* treatment has been shown to selectively reverse social deficits in mouse models of ASD, further highlighting the involvement of gut microbiota in the pathogenesis of ASD (Sgritta et al., 2019). While several human studies have shown alterations in the gut microflora in ASD, there is little consensus on the specific bacterial species involved. Conflicting results have been reported in different population studies, with some showing increased bacterial diversity (Wan et al., 2022), others showing no change (Kandeel et al., 2020), and still others showing a decrease (Dan et al., 2020) compared with healthy controls. Some studies have found that potentially harmful microbiota, such as Clostridium (Wang et al., 2019), Sutterellaceae (de Angelis et al., 2013), and Enterobacteriaceae (Ding et al., 2020), were more abundant in children with ASD, while Firmicutes (Liu et al., 2019) and Prevotellaceae (Pulikkan et al., 2018) were decreased in the ASD group. However, other studies have shown the opposite results (Rose et al., 2018; Zou et al., 2020; Ye et al., 2021). Studies have also found that Clostridium and Bacteroidetes/Firmicutes ratios were increased in ASD children with functional gastrointestinal disease (Luna et al., 2017), while a study in Slovakia showed increased numbers of Lactobacillus and a significant decrease in the Bacteroidetes/Firmicutes ratio (Tomova et al., 2015). Another study suggested that decreased Bifdobacteria abundance might lead to reduced folate production in individuals with ASD, so abnormal folate metabolism might be associated with ASD (Frye et al., 2017). In conclusion, while the results on gut microbiota and ASD are still inconsistent and mainly focused on Western populations, there is evidence to suggest that altered gut microbiota is associated with ASD. Further research is needed to better understand the role of gut microbiota in ASD and to identify non-invasive biomarkers for early diagnosis.

Given the inconsistent findings on the gut microbiota in ASD and the limited research on Chinese populations, it is important to investigate the changes in gut microbiota in Chinese children with ASD. Based on fecal 16S ribosomal DNA (rDNA) sequencing data from 957 children with ASD and 161 healthy controls in China, we aimed to determine the taxonomic composition of gut microbiota of children with ASD and the changes in the gut microbiota compared with healthy controls. Additionally, we identified potential biomarkers of bacterial species that could serve as non-invasive tools for early diagnosis of ASD.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of Second Affiliated Hospital, Army Medical University (Ethics NO. 2023–001-01). Written informed consents were signed by all the children's legal guardians prior to the study. The clinical trial number: ChiCTR2300074832.

Study subject recruitment and fecal sample collection

In total, 1,118 participants from 12 provinces of China including 957 participants with clinical definition as ASD (aged between 2 and 12 years, average age 4.6) and 161 health children (HC) (aged between 2 and 12 years, average age 4.8) were recruited (Supplementary Table S1).

All children with ASD participating in this study were enrolled from social groups of unrelated families with autism (Participants had been diagnosed with ASD according to DSM-5 (Diagnostic and Statistical Manual of Mental Disorders - 5th Edition) before enrollment). The exclusion criteria included diseases such as depressive disorder, cerebral palsy, schizophrenia, bipolar disorder, significant sensory impairment, and clinically significant inflammatory conditions.

The control group of children were recruited from kindergartens and primary school. Children with psychiatric conditions (such as depressive disorder, schizophrenia, and bipolar disorder) were excluded on the basis of entrance examinations and parent interviews.

All subjects had not taken antibiotics, antipsychotics, probiotics, and prebiotics in the month prior to the sample collection. Feces samples were used to analyze the genes associated with microbiota. Supplementary Figure S1 presents the study flowchart.

Feces were collected by parents in hospitals or at home. The feces were collected according to the instructions and delivered immediately at a low temperature. The frozen feces were transported overnight with dry ice to CNNC Yili (Tianjin) Medical Laboratory Co., Ltd., where they were frozen at −80°C until DNA was extracted.

16S rRNA sequencing and analysis

DNA from intestinal contents was prepared from subjects in the ASD and HC groups using a fecal DNA extraction kit (DP712, Tiangen Company, Beijing, China). The analysis was repeated with three biological replicates in each group. DNA quality was monitored on 1% agarose gels. The hypervariable V3-V4 region of the 16S-rDNA gene was amplified by PCR using the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), where the barcode was an eight-base sequence unique to each sample. PCR was performed in 30-μL reactions composed of 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM forward and reverse primers, and 10 ng of template DNA. The thermal cycle consisted of an initial denaturation step at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and

elongation at 72°C for 30 s. Finally, the reactions were incubated at 72°C for 5 min. PCR products were detected on 2% agarose gels by electrophoresis and then purified using the GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using the Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, United States) according to the manufacturer's recommendations, and index codes were added. Sequencing was performed using the Illumina NovaSeq MiSeq PE300 platform (Genecloud Co. Ltd., Chongqing, China). The sequence analysis was performed using the QIIME software package 2 (version 2020.2). The raw data of 16S rRNA sequencing underwent decomposition and quality filtering using version 0.20.0 of fastp, and merged using FLASH version 1.2.7 with the following merging conditions: (i) Any reads with an average quality score < 20 in a 50 bp sliding window were truncated at any position. Reads shorter than 50 bp were discarded, as well as reads containing ambiguous characters. (ii) Assembly was performed only based on overlapping sequence lengths greater than 10 bp, with a maximum mismatch ratio of 0.2 in the overlapping region. Reads that could not be assembled were discarded. (iii) Samples were differentiated based on barcodes and primers, and the sequence orientation was adjusted. Barcodes were matched exactly, while primer matching allowed for 2 nucleotide mismatches. Using the MOTHUR workflow, cluster effective tags with a similarity of ≥97% into operational taxonomic units (OTUs). The representative sequence within each cluster was selected as the tag sequence with the highest abundance. Bacterial alpha diversity was determined based on OTU analysis of samples and expressed as Chao index, Shannon index and Simpson index, which were calculated using the R program package "vegan." Principal coordinate analysis (pCoA) was performed using the R package[1] to represent the β diversity of the microbiome. Using the phyloseq package to calculate Bray-Curtis metric distances, UniFrac distances, and weighted UniFrac distances. The wilcoxon rank sum test was used to compare the differences of bacterial phyla, classes, orders, families and genera between the two groups. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to analyze the bacterial community dominance between groups.[2] LEfSe and Kruskal–Wallis rank sum test ($p < 0.05$) to identify features with significant differences in abundance levels between specified taxa, and to evaluate the effect size of each feature using LDA (LDA score (log10) =3.5 as the cutoff value). The R language mixOmics package was used to calculate Pearson correlation coefficient, and the correlation coefficient r^2 and p value of differential gut microbiota and differential metabolites were calculated. The metabolic pathways of intestinal microorganisms were analyzed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[3] We evaluated the metabolic pathway enrichment of the two groups. The metabolic pathway was considered enriched, while when the p value of the metabolic pathway was <0.05. Correlation analysis was performed by calculating Pearson's correlation coefficient using the R language mixOmics package to calculate the correlation coefficient r^2 and the p value of differentially bacterial and differentially metabolites.

The raw data of 16S rRNA sequence of this paper have been deposited in the Genome sequence Archive (Genomics, Proteomics & Bioinformatics 2021) of the National Genomics Data Center (Nucleic Acids Res 2022) and the National Center for Biological Information of China/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA - human: HRA004410), and can be in open access at https://ngdc.cncb.ac.cn/gsa-human.

Co-occurrence network analysis

To understand the correlations among different genera, we constructed co-occurrence network based on the 16S rRNA data (Frye et al., 2017; Wang et al., 2018). The bacterial correlations in the ASD and HC samples were analyzed, respectively, according to the relative abundance of each genus using Spearman's correlation coefficient to construct the co-occurrence network. The significant correlated genus ($p < 0.05$, $r \geq 0.7$) were visualized by Gephi 0.9.7 (www.netbeans.org). Then, the similarity between the two network structures was measured by node closeness and shared correlations. Closeness of the nodes was analyzed by Gephi to predicate node centralities in each network. The shared correlations between two groups were defined the edges with the same nodes in two co-occurrence networks. Only genera existed in at least 10% sample were included in the network analysis.

Statistical analysis

The data was presented as mean ± standard error (SEM). SPSS 26.0 software (SPSS, Inc., Chicago, IL, United States) was used to perform independent sample t test for the two groups of data. A two-tailed Wilcoxon rank-sum test was performed for the analysis of gut microbiota sequencing data by R Project. $p < 0.05$ was considered statistically significant.

Results

Differences of microbial diversity between ASD and healthy controls

The alpha diversity of gut microbiota was measured using various indices, including the Shannon index, Chao1 index, and Simpson index. The alpha diversity of gut microbiota in the ASD group showed a significant difference when compared with those of the HC groups (Figure 1). Specifically, these diversity indices showed a similar trend, that is, the alpha diversity of gut microbiota was higher in the HC group than in the ASD group (Figures 1A–C, online Supplementary Figure S2). The Anosim-analysis also showed that the differences between HC and ASD groups were significantly greater than those within the group, indicating meaningful grouping (Figure 1D). Figure 1E illustrated that the samples obtained from the ASD group had 1,152 features, while only 490 features were detected in the samples from the HC group, both groups shared a total of 432 features. These findings suggested that a significant number of features were present exclusively in the ASD group, indicating a higher relative abundance of certain species in the gut microbiota of children with ASD compared to healthy children. The pCoA principal component analysis based on bray-curtis clearly showed

1 http://www.R-project.org/

2 http://huttenhower.sph.harvard.edu/lefse/

3 https://www.genome.jp/kegg/pathway.html

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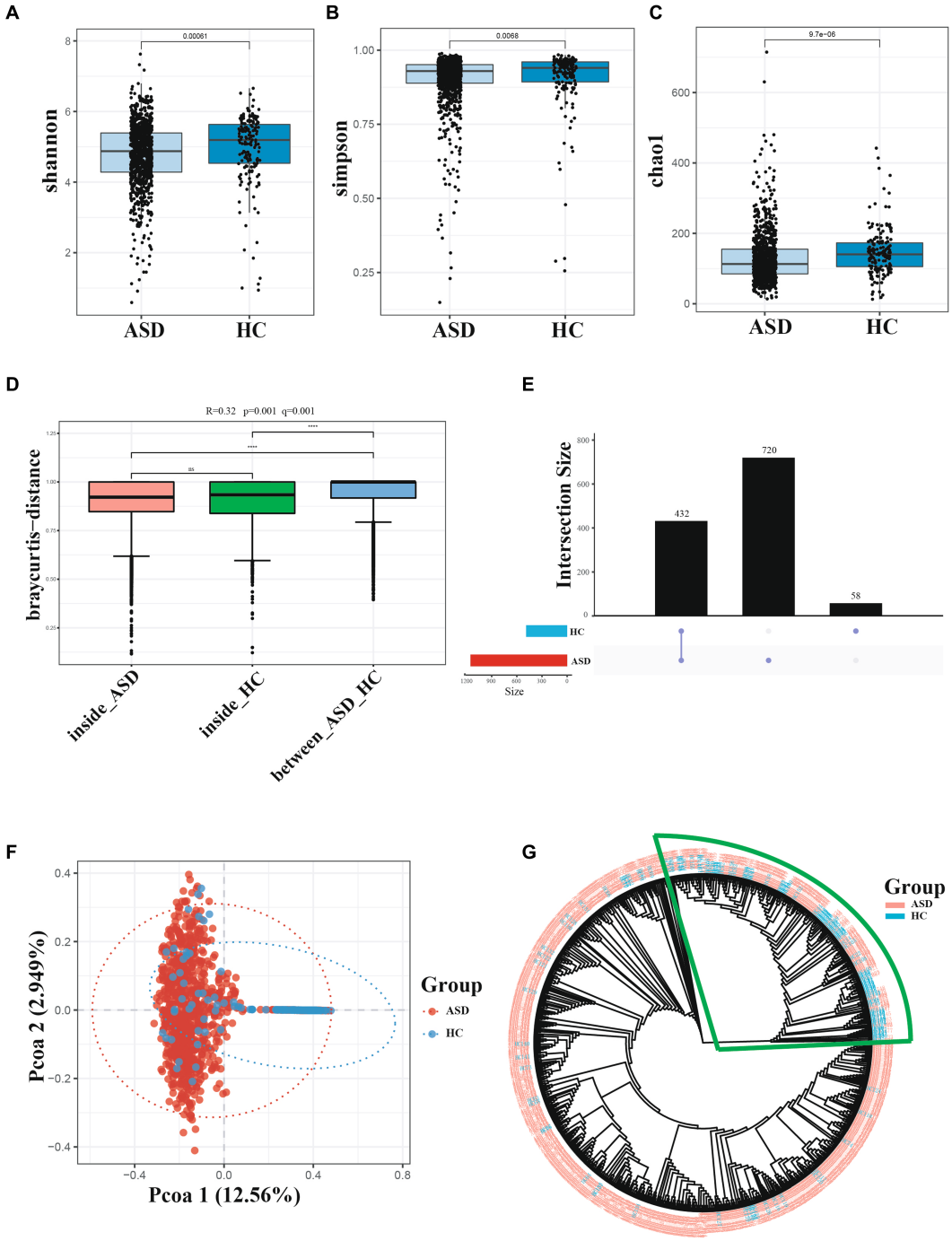

FIGURE 1
Diversity of gut microbiota are different across ASD and HC. **(A–C)** Index of Alpha diversity (the Shannon, simpson, and Chao1). **(D)** Anosim-analysis results, between represents the difference between groups; others are within groups; the greater the distance is, the greater the difference is; and the thickness is the sample size. **(E)** UpSet plot of intersections and unique among different group. **(F)** pCoA of beta diversity. **(G)** Cluster evolutionary tree analysis. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

the microbiota differences between the ASD and HC groups. There were significant differences along the PCO1 axis, accounting for 12.56% of the total variation (Figure 1F). Sample cluster evolutionary tree analysis also effectively clustered the ASD and HC group (Figure 1G). In conclusion, the species richness and diversity of gut microbiota in children with ASD were significantly different from those in HC group.

Differences of microbial comparison between ASD and healthy controls

Next, the composition of gut microbiota in ASD, and HC group was further analyzed. At the phylum level, Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria were found to be dominant in the different groupings (Figure 2A). In comparison to the HC group, the

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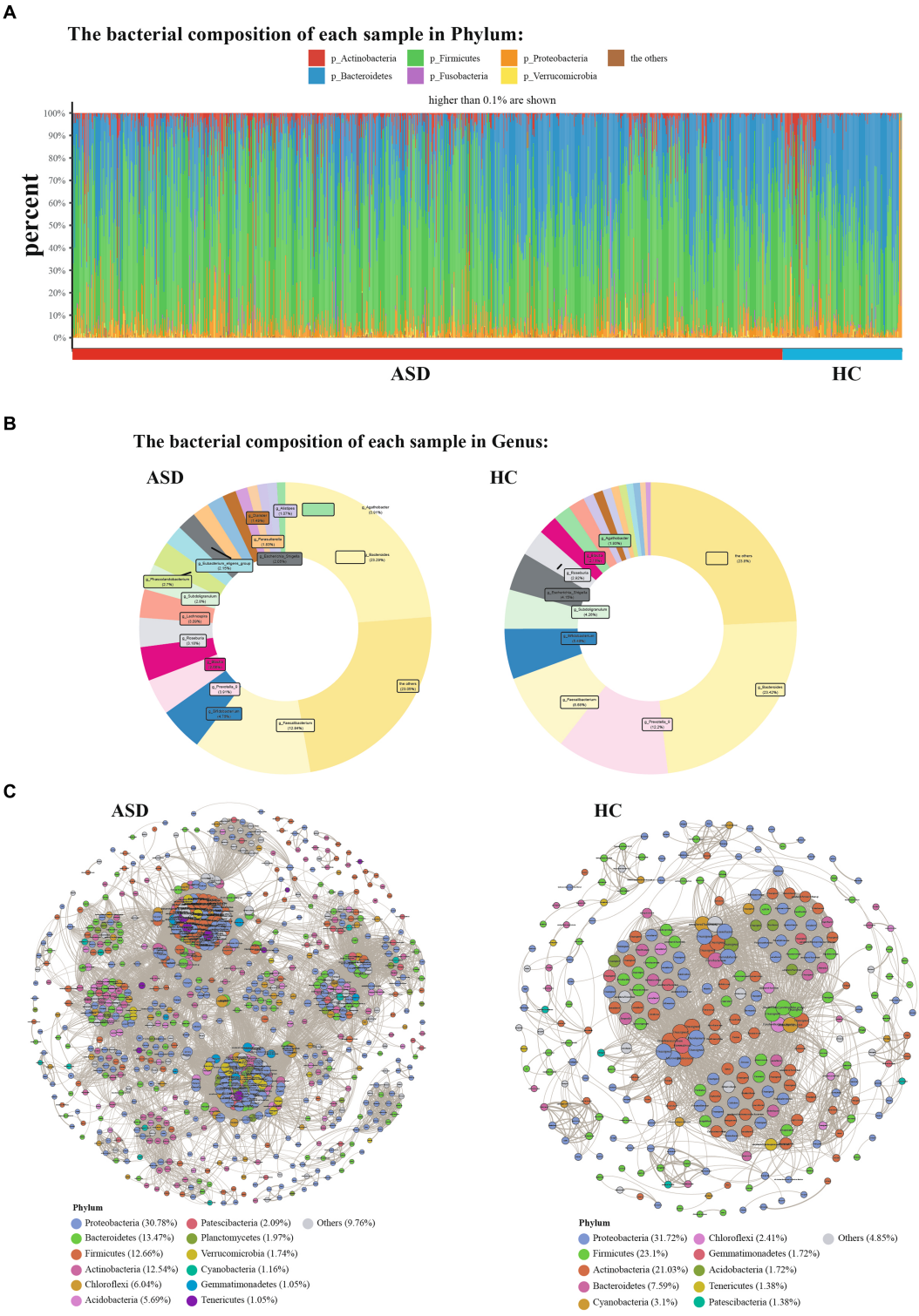

FIGURE 2
Phylum and Genera of gut microbiota are strikingly different across ASD and HC. **(A)** Relative abundance of the indicated phylum. **(B)** Relative abundance of the indicated genus. **(C)** Genera co-occurrence network between ASD and HC group based on the Spearman correlation algorithms. Each node presents a bacterial genus. The node size indicates the weighted value of each genus per group, and the thickness of the line represents the Spearman coefficient ($p < 0.05$, $r \geq 0.7$).

ASD group exhibited higher levels of Firmicutes and a significantly lower Bacteroidetes/Firmicutes ratio (ASD = 0.54, HC = 0.89). At the genus level, g_Bacteroides, g_Faecalibacterium, g_Prevotella_9, g_Bifidobacterium, g_Phascolarctobacterium and g_Blautia were found to be dominant in the different groupings (Figure 2B). At the phylum level, the ASD group showed a significant decrease in

p_Lentisphaerae, p_Bacteroidetes, p_Euryarchaeota, p_Patescibacteria, while p_Fusobacteria, p_Firmicutes, and p_Verrucomicrobia were significantly increased compared with the HC group (Figure 3A). At the genus level, we observed 316 significantly different species between ASD and HC groups (Figure 3B). Among them, the top 20 genera that showed a significant increase in the ASD group compared with the HC group included g_Blautia, g_Lachnoclostridium, g_Lachnospira, g_Lachnospiraceae_NK4A136_group, g_Eubacterium_eligens_group, g_Faecalibacterium, g_Dialister, g_Parasutterella, the significantly reduced bacterial flora included g_Prevotella_9, g_Alistipes, g_Agathobacter (Figure 3C). Then analysis at the species level was carried out to find out the bacteria with significant differences, among which the s_Bacteroides_fragilis in the ASD group was significantly increased compared with the HC group, while s_Bacteroides_coprocola_DSM_17136, s_Bacteroides_plebeius, s_Bacteroides_stercoris_ATCC_43183, and the s_Parabacteroides_merdae was significantly reduced (Figure 3D). It showed that indicating that these differential flora may be involved in the gastrointestinal symptomsof ASD.

According to the former study, it demonstrated that the complex microbial ecosystem of the human intestinal tract is unevenly influenced by individualtaxa within different microbial communities (Swiss IBD Cohort Investigators et al., 2019). To characterize potential relationships among bacteria in gut microbial communities, we further constructed co-occurrence networks for each taxon based on significant Spearman correlations. The HC taxa were mainly composed of six co-occurrence networks consisting of dispersed genera from four primary phyla: Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, while the ASD taxa were mainly composed of 15 co-occurrence networks consisting of dispersed genera from seven primary phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Chloroflexi, Acidobacteria, and Patescibacteria (Figure 2C). Two group displayed a co-occurrence network with strong positive correlation among genera. As shown in Figure 2C, the microbial community of the ASD group featured more complicated network. The correlation between the microbiota in the ASD group was distinctly increased compared to that of HC group. To quantify such differences, the average degree of edges (connections) and nodes (genera) were counted in the three microbial networks [ASD (49.78), HC (26.71)]. Taken together, the above analyses further indicating that there may be a disorder in the intestinal microecology of children with ASD.

The linear discriminant analysis (LDA) distribution diagram analysis (LAD score > 3.5) showed a clear alteration of the microbiota characterized by higher p_Frimicutes and p_Actinobacteria levels in ASD individuals (Figure 3E). However, p_Bacteroidetes levels were significantly decreased in ASD group (Figure 3E). The g_Faecalibacterium, g_Blautia, g_Eubacterium_eligens_group, g_Parasutterella, g_Lachnospiraceae_NK4A136_group and g_Veillonella were more abundant in ASD group, while the g_Prevotella 9, g_Agathobacter were more abundant in HC group (Figure 3E). The f_Ruminoccaceae, f_Lachnospiraceae was more abundant in ASD group, while f_Prevotellaceae and f_Enterobacteriaeae was more abundant in HC group (online Supplementary Figure S3). These apparently distinct species could be used as non-invasive biomarkers for the early diagnosis of autism. Taken together, compared with the HC group, the microbial composition of the ASD group showed alterations, further indicating that gut microecology dysbiosis existed in children with ASD.

Significant differences in metabolic pathways between the ASD group and the HC group

Ninety-seven Kegg pathways were different between the ASD and HC groups By KEGG pathway analysis (Figure 4A). In particular, among the Top 20 KEGG pathways, One carbon pool By folate pathways, carbon fixation In photosynthetic organisms pathways were significantly less enriched In The ASD group compared with The HC group, while The biosynthesis of ansamycins pathways, valine, leucine and isoleucine biosynthesis pathways, C5—branched dibasic acid metabolism pathways, pentose phosphate pathway pathways, fatty acid biosynthesis pathways, lysine biosynthesis pathways were significantly more enriched (Figure 4B).

To explore the potential relationships between the gut microbiome changes and metabolic products, a correlation matrix was generated using Spearman correlation (Figure 4C). The abundance of most species such as g_Blautia, g_Prevotella_9, g_Agathobacter, and g_Faecalibacterium, was positively correlated with the level of Penicillin_and_cephalosporin_biosynthesis, Carbon_fixation_in_photosynthetic_organisms, Arachidonic_acid_metabolism, Staphylococcus_aureus_infection. In summary, the abnormal gut microbiota in children with ASD leads to abnormal gut metabolites, which may be the potential reason for the effect of gut microbiota on ASD, although further research was needed to validate the results.

Discussion

Based on our study, we found significant differences in the diversity, structure, and relative abundance of intestinal microflora between ASD children and healthy children. Therefore, it is crucial to detect the gut microbiota in ASD children for intervention treatment.

Our results showed a significant decrease in gut microbiota diversity in ASD children compared with the HC group (Dan et al., 2020; Wan et al., 2022). These results were consistent with some previous studies (Carissimi et al., 2019; Wang et al., 2019; Dan et al., 2020; Ye et al., 2021). In addition, the Bacteroidetes/Firmicutes ratio of gut microbiota in ASD children was significantly reduced, which was consistent with the observation of gut microbiota in other autistic patients (Tomova et al., 2015; Strati et al., 2017). We found that different bacterial genera, such as g_Blautia, g_Faecalibacterium, g_Lachnospiraceae_NK4A136_group, g_Parasutterella, g_Eubacterium_eligens_group, g_Parasutterella, g_Akkermansia, and g_Anaerostipes significantly increased in the ASD group, while g_Prevotella_9, g_Alistipes, and g_Agathobacter were significantly reduced. These differential gut microbiotas can be used as biomarkers for early screening of ASD. In fact, the research results about ASD gut microbiota are often contradictory (Ho et al., 2020; Iglesias-Vázquez et al., 2020). At the genus level, more controversial findings have been reported, with g_Akkermansia reported to be more abundant in children with ASD (de Angelis et al., 2013; Li et al., 2021), but some studies have shown that the relative abundance of g_Akkermansia is significantly reduced in the gut microbiota of children with ASD

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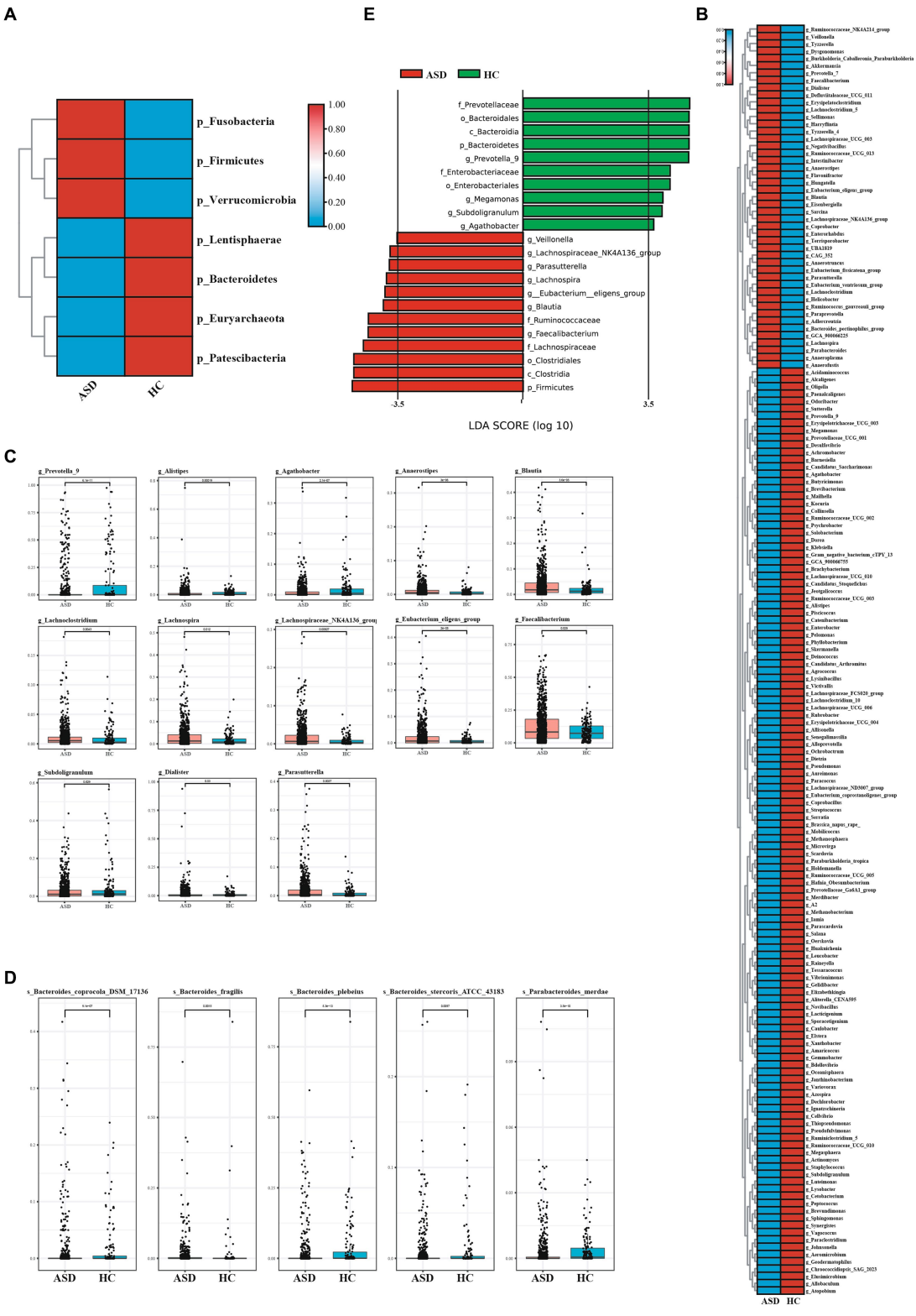

FIGURE 3
The shift of gut microbiota in ASD and HC. **(A)**Heatmap of selected differentially abundant phylum in ASD and HC ($p < 0.05$). **(B)** Heatmap of selected differentially abundant genus in ASD and HC ($p < 0.05$). **(C,D)** The top 20 of differentially abundant analysis between ASD and HC at the level of genus **(C)** and species **(D)**. **(E)** LDA scores for the bacterial taxa showed different abundant between ASD and HC (LAD score > 3.5) ($p < 0.05$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

(Wang et al., 2011; Zhang et al., 2018). Our data found that the relative abundance of g_Akkermansia in autistic patients was significantly increased in the ASD group. In addition, studies have shown that the relative abundance of g_faecalibacterium in the ASD sleep disorder group was significantly lower compared to healthy controls (Hua et al., 2020), while a meta-analysis reported that g_Faecalibacterium was

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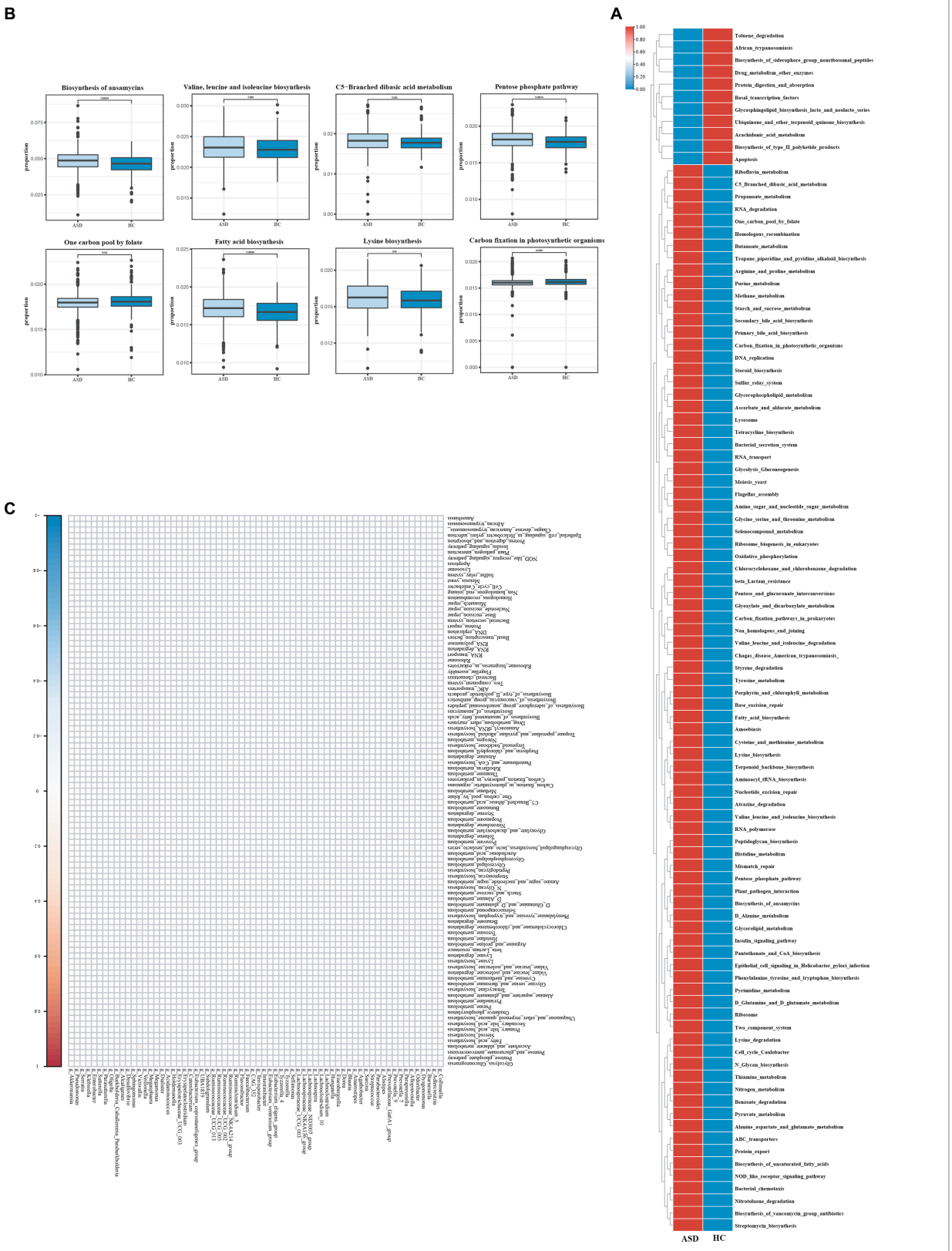

FIGURE 4
Different metabolic patterns in ASD and HC. **(A)** The average abundance of KEGG pathway differentially enriched in ASD and HC ($p < 0.05$). **(B)** The top 20 of KEGG pathway differentially enriched in ASD and HC ($p < 0.05$). **(C)** The heatmap of correlation between differential gut microbiota and differential metabolites ($p < 0.05$). Red color represents positive correlation, blue color represents negative correlation, and darker color corresponds to a stronger correlation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

more abundant in ASD children (Iglesias-Vázquez et al., 2020), consistent with our current research results. Some studies have shown that the Ruminococcaceae family, including Faecalibacterium, Subdoligranulum varia, Clostridium, and Eubacterium, is positively correlated with caproic acid levels (Dan et al., 2020), and ASD is associated with higher levels of caproic acid in the blood (Wang et al., 2012). The acetic acid produced by these intestinal bacteria is a ligand for GPR84, which can enhance the production of pro-inflammatory cytokine IL-12 and its subunit IL-12 P40 induced by lipopolysaccharide (Zhao et al., 2012). It has been reported that IL-12 or IL-12 P40 significantly increased in ASD children (Ashwood et al., 2011; Saghazadeh et al., 2019).

In addition, it was noted that g_Prevotella_9 was depleted in the gut microbiota of ASD children, while g_Blautia was enriched. The results of our g_Blautiade study are contrary to those of previous studies (Xie et al., 2022). However, the decline in Prevotella levels in ASD children was consistent with the results of previous studies (Dan et al., 2020; Zhang et al., 2021). Prevotella was the main bacterial species in the human gut, which could use polysaccharide to produce succinic acid (Kovatcheva-Datchary et al., 2015; Strati et al., 2017). Succinic acid could bind to the succinic acid receptor GPR91 on the surface of dendritic cells to enhance the immune response of antigen-specific T cells and protect the health of the host (Rubic et al., 2008). There is growing evidence that many ASD children have immune dysfunction (Ashwood et al., 2011). Therefore, it was speculated that the immune dysfunction of ASD might be related to the decrease of Prevatella bacteria in the gut, which further affected the physiological and behavioral characteristics of children with ASD. Why do different studies reach different conclusions, which we suspect that it may be due to differences in genetic background, varying manifestations of autism symptoms, as well as differences in age and living environment. Anyhow, these results again indicated that the gut microbiota of children with ASD was significantly different from that of non-ASD individuals.

According to the former study, it showed that Ecological networks of gut microbiota are considered critical for host health and well-being, because it shows that beneficial symbionts and their associated functions are maintained over time (Lozupone et al., 2012; Relman, 2012). In our study, the gut microbial community of children with ASD showed a more complex ecological network where more interactions between bacterial species were observed. It has been shown that highly cooperative microbial communities are perceived as less stable (Coyte and Rakoff-Nahoum, 2019). As previous studies have shown, only gradually increasing the proportion of cooperative interactions within a community almost always reduces the overall rate of return and the likelihood of stability (Coyte et al., 2015). As for key taxa in the network in ASD, Bacteroidetes produce propionic acid and other short-chain fatty acids (SCFA). Rat models administered with propionic acid show increased restrictive/repetitive behavior as well as impaired social behavior (MacFabe et al., 2011). In our study, Bacteroidetes showed the most interactions and identified as key species in the network in children with ASD. The associations between these bacteria and ASD requires further exploration. Besides this, Clostridium genus associated with neurological disorders (Averina et al., 2020) were connected closely with each other under the same genera. Interactions between harmful bacteria may promote and accelerate disease progression (Singer, 2010). Taken together, we observed an unstable and unfavorable ecosystem in the gut microbiome of ASD.

Numerous studies have shown that gut microbiota plays a key role in the biological and physiological characteristics of neurodevelopment, and the establishment of early community relationships among microorganisms may affect the neurodevelopment of children (Dinan and Cryan, 2017; Srikantha and Mohajeri, 2019; Liu et al., 2022). The results showed that, compared with HC group, abnormal microbial function in ASD patients was mainly concentrated in metabolic pathways related to fatty acid, amino acid and aromatic compound metabolism, and partly involved in the metabolism of neurotransmitters. Previous studies have shown that these functions were related to individual nervous system development, neurotransmitter biosynthesis and neuronal response regulation (Needham et al., 2020). For example, abnormalities in the bacterial pathway of tryptophan metabolism have been observed in patients with ASD, and its production of neuroprotective canine and neurotoxic quinolinic acid has been shown to be significantly associated with the severity of ASD (de Angelis et al., 2013; Lou et al., 2022). Our functional analysis of the gut microbiome provided some clues to these potential mechanisms that need to be further investigated in the future.

The differential microbiota and abnormal metabolic pathways identified in our study may have important clinical implications in the early diagnosis and treatment of autism. A large number of previous studies have shown that gut microbiota can be used as a new method for early screening and treatment of autism. Xinyan Xie et al. conducted a case–control study on 101 ASD children and 103 healthy controls in China, and found that Actinobacteria, Proteobacteria, Enterobacteriaceae, and Escherichia were significantly higher in ASD children. However, the numbers of monospheraceae, Blautia and unclassified lachnospiraceae were significantly reduced in ASD children. The gut microbiota of ASD children may have disorders in functional pathways such as amino acid metabolism, coenzyme and vitamin metabolism, and AMPK signaling pathway (Xie et al., 2022). Zhou Dan et al. performed 16S sequencing and metagenomic sequencing analysis on 143 children with clinically diagnosed ASD and 143 age- and sex-matched typically developing (TD) individuals. They found that ASD was associated with alterations in gut microbial profiles and abnormal metabolic activity. ASD patients showed gut ecological imbalance at the phylum, genus and species levels. The findings provide the possibility for future ASD interventions targeting specific microbiota related to neurotransmitter metabolism (Dan et al., 2020). Yingxin Zhao et al. analyzed and compared the gut microbiota of 36 ASD children with gastrointestinal symptoms and 40 TD children. Compared with TD, the gut microbiota of ASD patients with GI symptoms showed a decrease in alpha diversity and butyrate-producing bacteria (such as Faecalibacterium and Coprococcus). The results support the use of gut microbiota as a potential biomarker for early identification of ASD and intervention targeting specific gut microbiota (Zhao et al., 2023).

A strength of this study was the relatively large sample size compared with previous studies on the gut microbiota of ASD children (Supplementary Table S1). However, there were several limitations that need to be addressed in future studies. First, this study was observational only, and longitudinal studies were needed to explore how a disturbed gut microbiome contributes to the

development of ASD symptoms. Secondly, due to the availability of data, we were only able to obtain and analyze gut microbiota data, and lacked other data (such as diet, clinical indications, physiology and biochemistry, metabolomics, etc.), which limited the ability of in-depth causal inference. These problems were expected to be solved in the following research.

In summary, our study showed that children with ASD exhibited intestinal microecological disorders at the phylum, genus, and species levels compared with healthy children. These findings supported previous studies on gut microbiota changes in children with ASD and contributed to the development of new approaches for early screening and treatment of ASD based on gut microbiota. However, due to the heterogeneity of ASD phenotypes, further studies in independent populations with larger sample sizes are still needed.

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 studies involving humans were approved by his study was approved by the Ethics Committee of Second Affiliated Hospital, Army Medical University (Ethics No. 2023–001-01). Written informed consents were signed by all the children's legal guardians prior to the study. The clinical trial number: ChiCTR2300074832. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

HL: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. WG: Investigation, Resources, Writing – review & editing. SL: Investigation, Resources, Writing – review & editing. BS: Writing – review & editing. NL: Writing – review & editing. DX: Investigation, Writing – review & editing. ZD: Writing – review & editing. DL: Investigation, Writing – review & editing. WC: Writing – review & editing. WF: Writing – review & editing. JZhe: Writing – review & editing. JZhu: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1326870/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Study fowchart.

SUPPLEMENTARY FIGURE S2
Alpha diversity across microbiome samples of ASD and HC.

SUPPLEMENTARY FIGURE S3
Cladograms generated by LEfSe indicating differences in the bacterial taxa between ASD and HC.

SUPPLEMENTARY TABLE S1
Characteristics of study participants.

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Electroacupuncture at ST25 corrected gut microbial dysbiosis and SNpc lipid peroxidation in Parkinson's disease rats

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Introduction: Parkinson's disease (PD) remains one kind of a complex, progressive neurodegenerative disease. Levodopa and dopamine agonists as widely utilized PD therapeutics have not shown significant positive long-term outcomes. Emerging evidences indicate that electroacupuncture (EA) have potential effects on the therapy of nervous system disorders, particularly PD, but its specific underlying mechanism(s) remains poorly understood, leading to the great challenge of clinical application and management. Previous study has shown that acupuncture ameliorates PD motor symptoms and dopaminergic neuron damage by modulating intestinal dysbiosis, but its intermediate pathway has not been sufficiently investigated.

Methods: A rat model of PD was induced using rotenone. The therapeutic effect of EA on PD was assessed using the pole and rotarod tests and immunohistostaining for tyrosine hydroxylase (TH) in the substantia nigra (SN) of brain. The role of gut microbiota was explored using 16S rRNA gene sequencing and metabonomic analysis. PICRUSt2 analysis, lipidomic analysis, LPS and inflammatory factor assays were used for subsequent exploration and validation. Correlation analysis was used to identify the key bacteria that EA regulates lipid metabolism to improve PD.

Results: The present study firstly reappeared the effects of EA on protecting motor function and dopaminergic neurons and modulation of gut microbial dysbiosis in rotenone-induced PD rat model. EA improved motor dysfunction (via the pole and rotarod tests) and protected TH+ neurons in PD rats. EA increased the abundance of beneficial bacteria such as Lactobacillus, *Dubosiella* and *Bifidobacterium* and decreased the abundance of Escherichia-Shigella and Morganella belonging to *Pseudomonadota*, suggesting that the modulation of gut microbiota by EA improving the symptoms of PD motility via alleviating LPS-induced inflammatory response and oxidative stress, which was also validated by various aspects such as microbial gene functional analysis, fecal metabolomics analysis, LPS and inflammatory factor assays and SNpc lipidomics analysis. Moreover, correlation analyses also verified strong correlations of *Escherichia-Shigella* and *Morganella* with motor symptoms and SNpc lipid peroxidation, explicating targets and intermediate pathways through which EA improve PD exercise symptom.

Conclusion: Our results indicate that the improvement of motor function in PD model by EA may be mediated in part by restoring the gut microbiota, which intermediate processes involve circulating endotoxins and inflammatory mediators, SNpc oxidative stress and lipid peroxidation. The gut-microbiome - brain axis may be a potential mechanism of EA treatment for the PD.

KEYWORDS

Parkinson's disease, electroacupuncture, gut microbiota, microbial metabolites, lipidomics, lipid peroxidation

Introduction

Parkinson's disease (PD), the second most common neurodegenerative disease, characterized by the accumulation of α-synuclein (α-syn) and the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), with consequent motor features, such as tremor, bradykinesia postural instability, and non-motor manifestations, including constipation, sleep disorders, dementia and other symptoms involved in various functions of the gastrointestinal, central nervous system (CNS) and autonomic nervous system (ANS) (Goedert, 2015). The pathogenesis of PD relates complex polygenic factors interacting with environmental exposures. As the gastrointestinal tract represents a primary site for exposure to pathogens and gastrointestinal motility disorders is one of the main prodromal dysfunctions, the role of which the microbiome-gut-brain axis in PD has become a subject of great interest to the field (Travagli et al., 2020). Lipids in the gut microbiome play important roles in many metabolic and inflammatory diseases, and provide important signals for cell trafficking, neuronal function in the brain, and lipid raft protein function. Microbiome lipid metabolism is important for regulating brain function and may affect chronic inflammation in the brain, leading to many diseases particularly starting in the gut, such as PD.

Although the etiology of PD is generally unknown, the formation of α-syn aggregates seems to be closely associated with an altered lipid metabolism (Sarchione et al., 2021). Abnormal accumulation of lipid droplets in neurons can induce a conformational change of α-syn, which causes α-syn accumulation in human neurons (Girard et al., 2021). Phosphatidic acid (PA), especially PA (18:1/18:1), demonstrated a substantially enhancement of generation of α-helical, multimeric and PK-resistant α-syn protein (Mizuno et al., 2017). Recent data from animal models suggest that PE deficiency disrupts the homeostasis of α-syn and induces its aggregation (Wang et al., 2016), suggesting that PE may delay the pathologic progression of PD. However, the specific role of gut microbiota and lipid metabolism for PD and the factors modulating such processes along the microbiome-gut-brain axis are still largely unknown.

So far, levodopa and dopamine agonists are widely used as treatment options for PD. Nevertheless, these drugs are not showing significantly positive long-term outcomes (Raza et al., 2019). It is crucial to widening the research scope to find alternatives that can improve symptoms for PD patients in a long run. With the wide distribution of PD cases globally, more and more complementary and alternative treatments are being used for the care and control of PD. Acupuncture has promising clinical effects when used to treat dyskinesia and other related neurological disorders (Wen et al., 2021). Due to the advantages in improving the symptoms of neurological disorders and few side effects, acupuncture is considered to be a safe and useful treatment for PD (Huang et al., 2020). Although an increasing number of studies revealed acupuncture's therapeutic effects both in clinical and animal experiments research for PD, the mechanisms of the effectiveness of acupuncture for the treatment of PD remain largely unknown (Li et al., 2022). Previous studies showed that acupuncture has neuroprotective, anti-neuroinflammatory and antiapoptotic effects in PD mice models (Park et al., 2003, 2015, 2017; Jeon et al., 2008; Kim et al., 2011a,b). It has also been shown that acupuncture enhances motor function and protects dopaminergic neurons by regulating gut microbial dysbiosis (Jang et al., 2020). However, the specific mechanisms by which acupuncture ameliorates dyskinesia and dopaminergic neurons damage in PD through gut microbiota have not been sufficiently investigated.

EA at ST25 is a commonly used clinical experience in the treatment of constipation and its efficacy has been fully confirmed (Liu et al., 2016; Wang et al., 2019). The regulating effect of EA at ST25 on colonic motility has also been well demonstrated by our previous work (Sun et al., 2018a; Xu et al., 2019). PD has the characteristics of encephalo-intestinal disease and constipation is a precursor and one of its characteristic symptoms (Yu et al., 2018). The efficacy of EA at ST25 on PD motor function and constipation symptoms was confirmed by a randomized controlled multicentre trial (Li et al., 2023). In our previous study on the mechanism of EA at ST25 on PD constipation symptoms, its effects on motor symptoms were also observed (Song et al., 2022, 2023), and this paper further explored the therapeutic mechanism of motor symptoms on the basis of it. In the previous study, we also found that EA at ST25 changed the characteristics of gut microbiome in PD rats (Song et al., 2023). According to the current cutting-edge research progress, the changes of gut microbiome are closely related to the onset and treatment of PD (Travagli et al., 2020), which aroused our interest and prompted us to conduct related research. The present study firstly reappeared the effects of electroacupuncture (EA) on protecting motor function and dopaminergic neurons and modulation of gut microbial dysbiosis in rotenone-induced PD rat model. Then, possible pathways by which EA at ST25 influence PD via gut microbiome were explored through microbiota function prediction and fecal metabolomics changes.

Materials and methods

Establishment of the experimental animal model

In this study, eight-week-old Sprague Dawley (SD) rats were supplied by the Beijing Vital River Laboratory Animal Technology Co., Ltd. [No.110011220101889264, under grant SCXK(JING)2021–0011]. The experimental rats were kept in a barrier environment with stable parameters (conditions: 12/12 h light/dark cycle; temperature, 22 ± 2°C; relative humidity 60% ± 5%). The animals were randomly numbered and divided into three groups: The control group, the model group, and the EA group, with six animals in each group. They were kept in cages of the same size in groups with free access to food and water.

PD was induced by giving a low dose of rotenone (Cannon et al., 2009). Selection of the appropriate route of administration and concentration is critical for model stabilization and appropriate plasma concentrations (to avoid systemic toxicity) (Innos and Hickey, 2021). We formulated the following program based on previous research (Liu et al., 2015) and our prior investigations. The model and EA groups were

injected subcutaneously with rotenone solvent on the back of the neck at a dose of 1 mL/kg once a day for 5 days a week. The solvent was prepared by dissolving 200 mg of rotenone (M6209; Abmole Bioscience Inc., Houston, TX, USA) in 3 ml of dimethyl sulfoxide (DMSO, D8370; Beijing Solarbio Science & Technology, Tongzhou, Beijing, China), and then fixed to 100 ml with sunflower oil to make up 2 mg/ml of rotenone sunflower oil solvent. The control group was injected with an equal volume of solvent mixture (3% DMSO sunflower oil solvent). Motor function was assessed at the end of each weekly intervention (refer to the "Motor coordination assessment" below for specific methods). The modeling was considered successful when there was a statistically significant difference in motor scores between the modeled rats and the control rats. This animal study was reviewed and approved by the Scientific Investigation Board of the Nanjing University of Traditional Chinese Medicine, Nanjing, China (permission no. 202112A047).

Motor coordination assessment

The pole test and rotarod test (Park et al., 2017; Yan et al., 2022) were used to evaluate Motor mediation and balance impairment. The homemade climbing pole (100 cm high, 1 cm in diameter) was wrapped with medical gauze to ensure sufficient friction. The rats were placed on the top of the pole with their heads facing upward, and the time required to turn from vertical upward to vertical downward and to climb from the top of the pole to the bottom of the pole was recorded. The rotarod test used the KW-6D rat rod instrument (Nanjing Calvin Biotechnology Co., Ltd., Nanjing, Jiangsu, China). The total experiment time was 180 s. The first 90 s from 0 to 30 RPM uniform acceleration, the next 90 s maintain a uniform speed of 30 s per minute. Before the test, each rat was guided to perform two times of acclimatization training. Each rat was tested three times, with each interval of not less than 10 min, and the average value was taken.

EA intervention

The rats in the EA group were received EA treatment on bilateral ST25 (Tianshu, located 5 mm lateral to the intersection between the upper 2/3rd and the lower 1/3rd in the line joining the xiphoid process and the upper border of the pubic symphysis) after gas anesthesia with isoflurane (2–5%; 9020000522; Shenzhen Ruiwode Lift Technology). Meanwhile, the same anesthesia was administered to rats in the Model group but without performing EA. For the EA group, two stainless steel acupuncture needles (20162270970; Suzhou HUATUO Medical Instruments, Suzhou, Jiangsu, China) of 0.2 mm in diameter were inserted at a depth of 5 mm into the bilateral ST25 acupoint (Figure 1A). EA at ST25 was conducted with the HANS-100A (HAN ACUTENS WQ1002F; Beijing Anlong Photoelectric Technology, Haidian, Beijing, China) apparatus set to a current of 2 mA and a frequency of 2/15 Hz. EA treatment starts from the fifth week: 20 min a day for 5 days a week, 1 week a course, over four continuous courses of treatment.

Samples collection

Samples were collected after 4 weeks of intervention. Stool samples were obtained using sterile instruments and containers and stored at −80°C. Blood samples were obtained by orbital blood sampling after anesthesia (Ethyl carbamate, 1,000 mg/kg, I.p), centrifuged (4°C, 3000 rpm, 15 min) within 1 h and the supernatant was stored at −80°C. After animals sacrificed under an overdose of anesthesia, fresh intact brains were pre-frozen in liquid nitrogen after removal, and subsequent treatments were carried out at low temperatures to ensure fixed tissue morphology and facilitate accurate sampling.

DNA isolation and 16S rRNA gene sequencing

Total genome DNA from samples was extracted using the CTAB method. DNA concentration and purity was monitored on 1% agarose gels. 16S rRNA genes of distinct regions (16S V3-V4) were amplified used specific primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) with the barcode. PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2,100 system.

The library was sequenced on an Illumina NovaSeq platform, generating 250 bp paired-end reads. First, the raw data were screened and sequences were removed if they were shorter than 200 bp, had a low-quality score (≤20), contained ambiguous bases or did not match primer sequences and barcode tags. Qualified reads were separated based on the sample-specific barcode sequences and trimmed with Illumina Analysis Pipeline Version 2.6. Then, the dataset was analyzed using VSEARCH. The sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97%, to generate rarefaction curves and calculate the richness and diversity indices. The Ribosomal Database Project (RDP) Classifier tool was applied to classify all sequences into different taxonomic groups. Clustering analyses and principal component analysis (PCA) were used based on OTU information from each sample using R-Studio to examine the similarity between different samples.

linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was used to find species that differed significantly in abundance between groups (i.e., biomarkers). Species with significant differences in abundance between groups were first detected using the parametric test ANOVA test in multiple samples, with a threshold set at 0.05. Then the significantly different species obtained in the previous step were analyzed for between-group differences using the Wilcoxon rank sum test in groups, with a threshold set at 0.05. Finally, LDA was used to downscale the data to assess the significant differences in the influence of species (i.e., LDA score) was assessed using LDA, with a threshold set of 3/3.5. The obtained differential species were used for the next analysis.

Metabonomic analysis based on liquid chromatography-mass spectrometry

The sample stored at-80°C refrigerator was thawed on ice. A 400 µl solution (Methanol: Water = 7:3, V/V) containing internal standard was added into 20 mg sample, and vortexed for 3 min. The sample was sonicated in an ice bath for 10 min and vortexed for 1 min and then placed at −20°C for 30 min. The sample was then centrifuged

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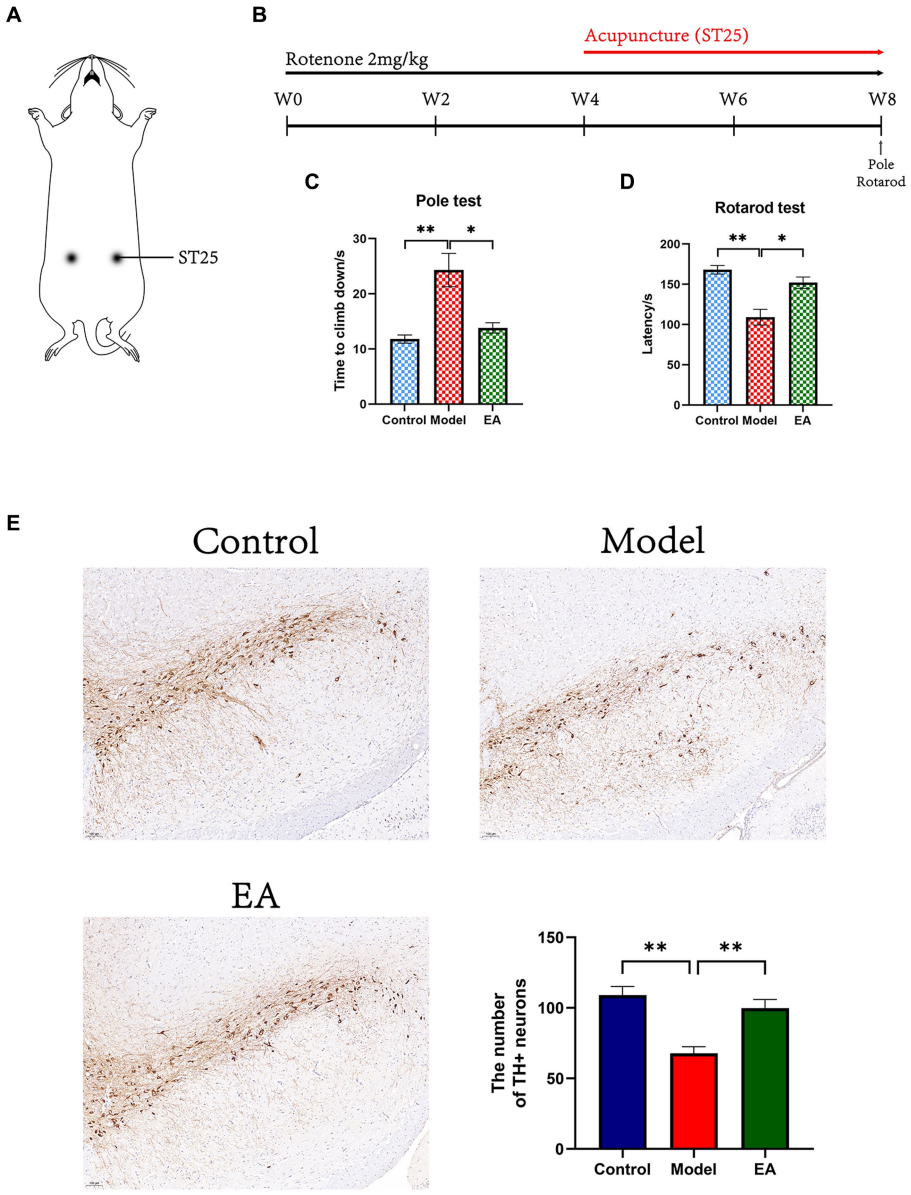

FIGURE 1
Effects of EA on motor functions in rotenone-induced PD rat. **(A)** EA on ST25 of PD rat treated with rotenone for 4 weeks (EA group). **(B)** Experimental schedule of the EA treatment in the rotenone-induced PD rats. All motor behavioral tests including **(C)** pole, and **(D)** rotarod tests were performed the ending day of the EA intervention ($n = 6$, Student t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). **(E)** Immunohistostaining for tyrosine hydroxylase (TH) in the SN of brain extracted from Control, Model, and EA group and counts of TH^{+} neurons ($n = 4$. Scale bar: 100 μm). W, week.

at 12,000 rpm for 10 min (4°C). And the sediment was removed, then centrifuged the supernatant was at 12,000 rpm for 3 min (4°C). A 200 μl aliquots of supernatant were transferred for LC–MS analysis.

All samples were acquired by the LC–MS system following machine orders. The analytical conditions were as follows, UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm*100 mm); column temperature, 40°C; flow rate, 0.4 ml/min; injection volume, 2 μl; solvent system, water (0.1% formic acid): acetonitrile (0.1% formic acid); The column was eluted with 5% mobile phase B (0.1% formic acid in acetonitrile) at 0 min followed by a linear gradient to 90% mobile phase B (0.1% formic acid in acetonitrile) over 11 min, held for 1 min, and then come back to 5% mobile phase B within 0.1 min, held for 1.9 min.

The original data file acquired by LC–MS was converted into mzML format by ProteoWizard software. Peak extraction, peak alignment, and retention time correction were, respectively, performed by the XCMS program. The "SVR" method was used to correct the peak area. The peaks with a detection rate lower than 50% in each group of samples were discarded. After that, metabolic identification information was obtained by searching the laboratory's self-built database, integrated public database, AI database, and metDNA.

Unsupervised PCA was performed by statistics function prcomp within R. The data was unit variance scaled before unsupervised PCA. For two-group analysis, differential metabolites were determined by VIP (VIP > 1) and p-value (p-value <0.05, Student's t-test). VIP values were extracted from OPLS-DA results and were generated

using the R package MetaboAnalystR. The data was log transform (log2) and mean centering before orthogonal partial least squares discriminant analysis (OPLS-DA). In order to avoid overfitting, a permutation test (200 permutations) was performed. Identified metabolites were annotated using the KEGG Compound database,[1] and annotated metabolites were then mapped to the KEGG Pathway database.[2] Significantly enriched pathways are identified with a hypergeometric test's *p*-value for a given list of metabolites.

Lipidomics analysis of serum and brain samples

The lipids of individual serum or brain SNPC samples were extracted by using a modified protocol of Bligh and Dyer (1959) in the presence of internal standards as previously described (Han and Gross, 2005). Each lipid extract was resuspended with 200 µl chloroform/methanol (1:1, v/v)/mg protein, and stored at −20°C for lipid analysis. Derivatization of the primary amine in phosphoethanolamine-containing species (such as PE and lysoPE) with fluorenyl methoxycarbonyl chloride was conducted according to the previously reported method (Han, 2005; Hu et al., 2020). Individual lipid species including FA isomers and regioisomers were identified using multidimensional MS analysis (Yang et al., 2009; Hu et al., 2022).

Lipidomics analysis of serum and brain samples was conducted with a triple-quadrupole mass spectrometer (Thermo TSQ Quantiva) equipped with an automated nanospray ion source (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY, USA) as previously reported (Han and Gross, 2005). To prevent possible lipid aggregation, the solutions of lipid extracts were diluted in $CHCl_3$/MeOH/isopropyl alcohol (1:2:4, v/v/v) prior to direct infusion. All mass spectral data were acquired by different customized sequence subroutines operated under Xcalibur software (Xcalibur 3.0, Thermo Fisher Scientific Inc., San Jose, CA, USA). Data processing was performed based on the previous method (Yang et al., 2009).

Analysis of serum cytokines and endotoxin

The serum concentrations of interleukin-6 (IL-6), IL-10, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and lipopolysaccharide (LPS) were determined using ELISA assay kits (Quanzhou Ruixin Biological Technology Co., Ltd., Meile Street East Section, Dacheng Town, Sanyuan County, Shaanxi. China) according to the manufacturer's protocol.

Data analysis

All data analyses were performed using GraphPad Prism 9.4 (GraphPad Inc., La Holla, CA, USA) and R. $p < 0.05$ was considered to indicate statistical significance. As a variety of statistical methods are used, specific methods will be marked in detail in the Methods and Results section and figure note.

Results

EA improves motor dysfunctions in rotenone-induced PD rats

Experimental schedule of the EA treatment in the rotenone-induced PD rats was shown in Figure 1B. At the end of the 4-week rotenone intervention, the modeled rats showed a significant decrease in motor function (Supplementary Figure S1). EA intervention was started from week 5 and after 4 weeks of intervention, the motor coordination ability of rats in each group was compared. In the pole test, the time climbing down of rats in the Model group were significantly extended ($p < 0.01$ vs. Control group), from about 10s in the Control group to more than the 20s in the Model group, and the change factor was more than two times (Figure 1C). The time climbing down of the EA group was significantly reduced compared to the Model group ($p < 0.05$). In the rotarod test, the reduction of latency time induced by rotenone injection ($p < 0.001$ vs. Control group) was restored by the EA treatment ($p < 0.001$ vs. Model group; Figure 1D).

EA counteracts rotenone-induced deficits in dopaminergic neurons

We confirmed the neuroprotective effects of EA on dopaminergic neurons in the SNpc of rotenone-induced rats. In the SN, a significant difference was observed among the three groups in the number of TH-positive neurons. There were decreased in the Model group ($p < 0.01$ vs. Control group) and were increased in the EA group ($p < 0.01$ vs. Model group; Figure 1E).

EA alters dysbiosis of the gut microbiome in rotenone-induced PD rats

The dilution curve (Rarefaction curve), Shannon-Wiener curve, species accumulation curve, and goods coverage index generated by OTUs indicate that all samples achieved high sampling coverage (≥99%) (Supplementary Figure S2; Supplementary Table S1). This indicates that the sequencing depth is sufficient for studies of the gut microbiota. The Venn diagram shows 1,000 unique OTU in the control group,1,575 unique OUTs in the model group, and 1,012 unique OUTs in the EA group. There was 1,118 OTU in the EA group but not in the Model group (Supplementary Figure S3A).

It was found by α-diversity analysis, rotenone treatment increased the community richness (Figure 2A; Supplementary Figures S3B,C) and diversity (Figures 2B,C) of intestinal microbiota, suggesting that the model group had gut microbiota disorder, and EA intervention played a positive role in this situation. β-diversity analysis showed that there were significant differences among the three groups (Figures 2D,E; Supplementary Figures S3D,E), the model group and control group separated along the longitudinal axis, the EA group and model group separated along the horizontal axis, partial least squares discriminant analysis (PLS-DA) showed the most obvious difference (Figure 2D). The

1 http://www.kegg.jp/kegg/compound/

2 http://www.kegg.jp/kegg/pathway.html

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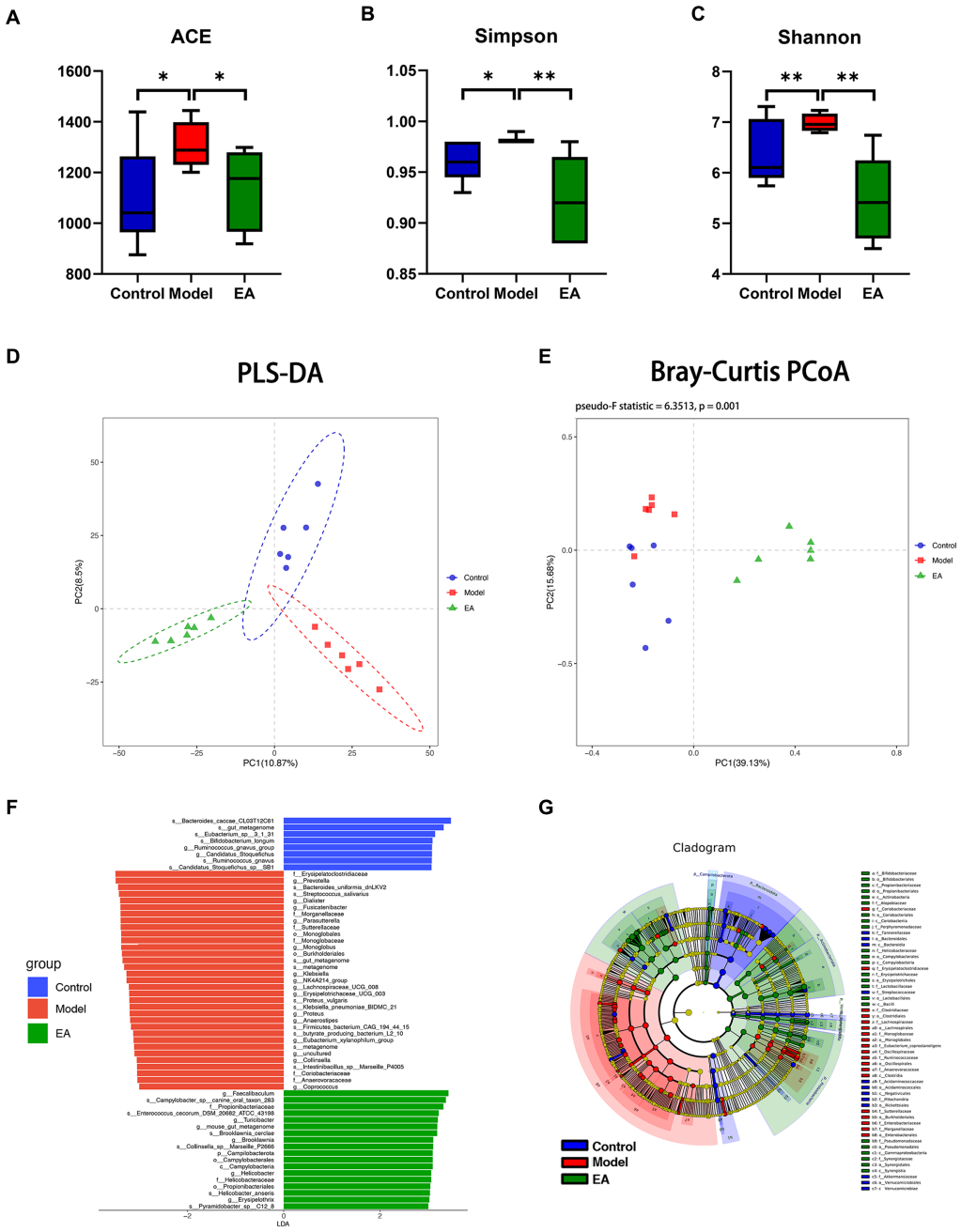

FIGURE 2
Diversity analysis of gut microbiota. Rotenone treatment increased ACE **(A)**, Simpson index **(B),** and Shannon **(C)** of the model group (n = 6, Student t-test, * $p < 0.05$, ** $p < 0.01$). Compared with the control group, EA decreased ACE, Simpson index and Shannon index **(A–C)**. PLS-DA and PCoA (PERMANOVA: Pseudo-F Statistic = 6.3513, p = 0.001) analysis showed that the bacterial communities in each group were far apart and clearly demarcated **(D,E)** (Amova: $p < 0.001$; Anosim: R = 0. 9,593, p < 0. 01). **(F)** LDA scores for the bacterial taxa differential abundant (LDA > 3.5). **(G)** Cladograms generated by LEfSe indicate differences in the bacterial taxa (LDA > 3.0).

LDA distribution diagram analysis (LAD score > 3.5) showed the differential microbiota in model group rats, including 2 orders, 6 families, 15 genera, and 10 species (Figure 2F). After the EA intervention, 1 phylum, 1 class, 2 orders, 2 families, 6 genera, and 6 species were significantly altered (Figure 2F). The cladogram shows the differential microbiota after the LAD score threshold was relaxed to 3.0 (Figure 2G).

Genus-level analyses showed a significant distinction between the gut microbiota of the EA and Model groups. A total of 68 genera were identified and differed significantly among the three groups (Supplementary Table S2; FDR $p < 0.05$, Kruskal–Wallis one-way ANOVA). Among these genera, 15 were significantly different between the Control and Model groups (Supplementary Table S2; $p < 0.05$, Metastats); 14 genera of them in the Model group were enriched compared to the Control group. The EA treatment significantly reversed the changes in the relative abundance of 13 genera (Figure 3B), of which *Escherichia-Shigella* and *Morganella* belonged to *Pseudomonadota* (Figure 3C). Among these 13 genera, the relative abundance of *Escherichia–Shigella* was far ahead. In addition, compared to the Model group, 13 genera were significantly elevated in the EA group (Figure 3D), especially *Lactobacillus*, *Dubosiella*, and *Bifidobacterium* (Figure 3E).

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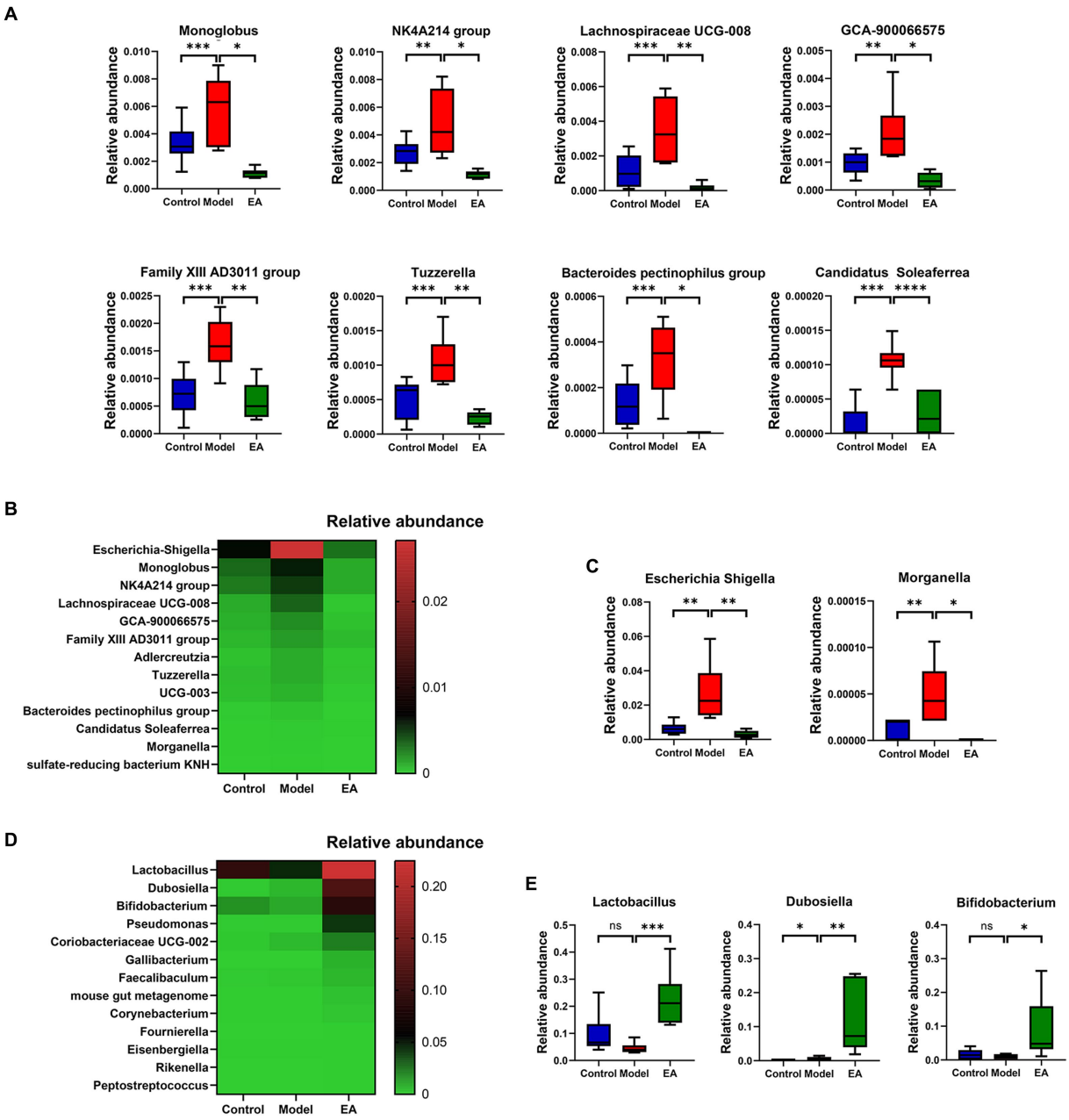

FIGURE 3
Analysis of species differing at the genus level. **(A)** Differential microbiota at genus level belong to *Bacillota*. **(B)** Genera whose relative abundance was significantly increased in the Model group and decreased in the EA group, **(C)** of which *Escherichia-Shigella* and *Morganella* belong to *Pseudomonadota*. **(D)** Genera whose relative abundance was significantly increased in the EA group, **(E)** of which relative abundance of *Lactobacillus*, *Dubosiella*, and *Bifidobacterium* in the EA group was >0.05. ($n = 6$, Metastats, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

To examine whether the gut microbiota correlates with PD clinical features, and also to determine whether the extensive changes in the gut microbiota and clinical features of PD following EA treatment are associated, we further analyzed the relationship between the gut microbiota and motor functions using Spearman's correlation, based on above 68 genera (Supplementary Table S2) and the representative parameters for behavioral deficits in the Model group rats, the pole and rotarod test. The correlation analyses indicated that 11 and 6 genera were significantly correlated with the pole and rotarod test, respectively (Supplementary Table S3). Interestingly, *Lactobacillus*, *Escherichia-Shigella*, *Morganella*, *Adlercreutzia*, *Candidatus Soleaferrea*, *sulfate-reducing bacterium KNH* were correlated with the rotarod and pole test (Figure 4).

EA changes the functionality of the gut microbiota in rotenone-induced PD rats

To gain insight into the functional categories and pathways associated with EA treatment in PD rats, we used PICRUSt2 to compare the function of gut microbiota between the three groups.

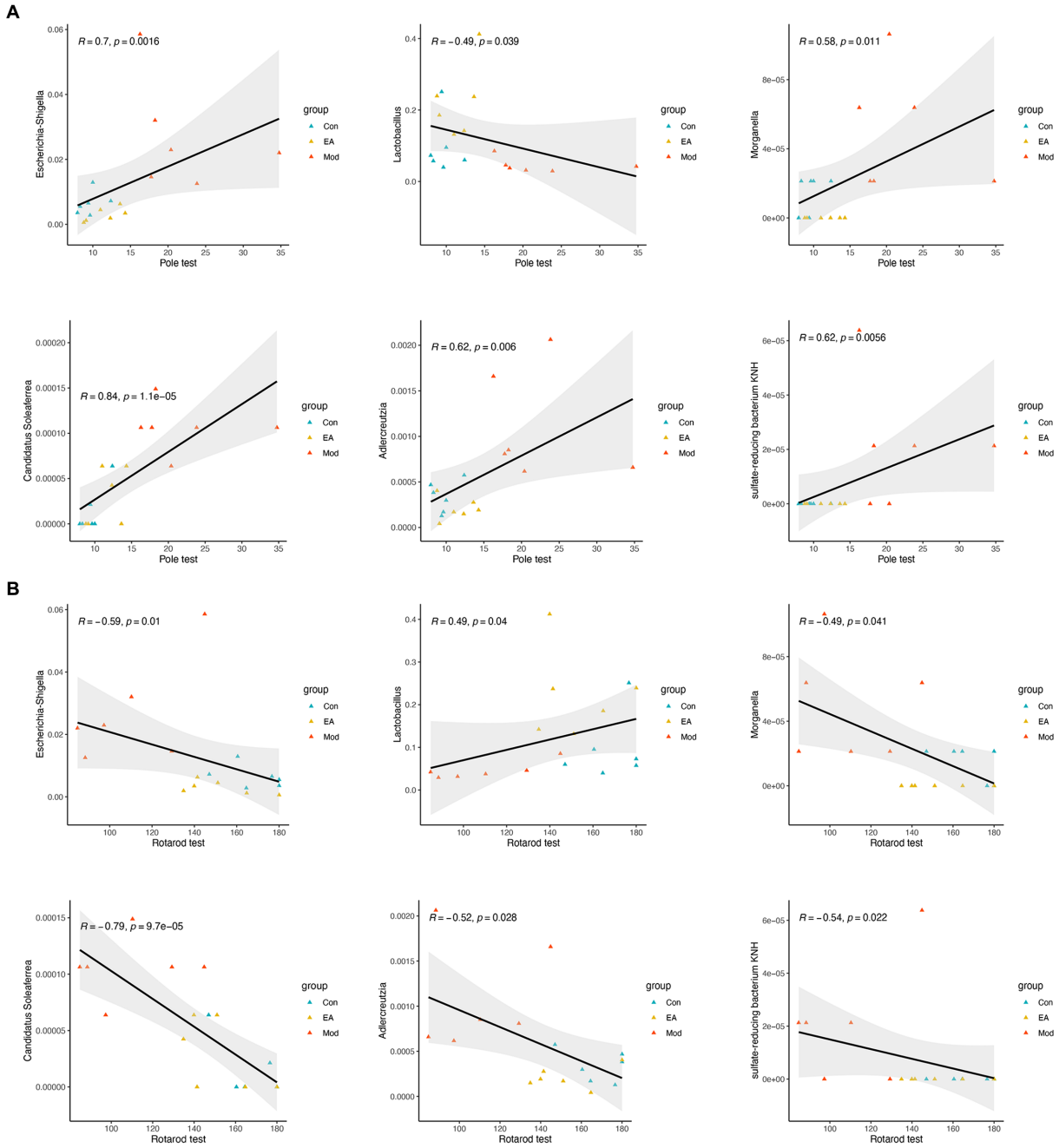

FIGURE 4
Correlation between the bacteria of genus and the behavior functions. Spearman's correlation analyses between the relative abundance of gut microbiota at the genus level and the value in **(A)** pole or **(B)** rotarod test, respectively. Significant correlations were determined based on |Spearman $r| > 0.5$ and $p < 0.05$.

A total of 89 KEGG pathways at level 3 were significantly altered in the Model or EA group ($p < 0.05$), and many of the predicted functional differences were in metabolic pathways (Supplementary Table S4). Based on the grade 2 KEGG pathway findings, the immune system, lipid metabolism, and signal transduction were disturbed in the model group of rats. EA affected 16 signaling pathways, 5 of which belonged to metabolic pathways, occupying 1/3 of the total proportion. The immune system and lipid metabolism were both associated with the pathogenesis of PD and the intervention role of EA (Figure 5A). Therefore, we performed further analysis of immune system and lipid metabolism based on grade 3 KEGG pathways. The results show that 4 pathways, including Biosynthesis of unsaturated fatty acids, primary bile acid biosynthesis, secondary bile acid biosynthesis, and NOD-like receptor signaling pathway were disturbed in the model group, and EA affects 8 signaling pathways, among which Biosynthesis of unsaturated fatty acids, primary bile acid biosynthesis, secondary bile acid biosynthesis, and NOD-like receptor signaling pathway is related both to the pathogenesis of PD and the intervention effect of EA (Supplementary Table S4; Figures 5B–E).

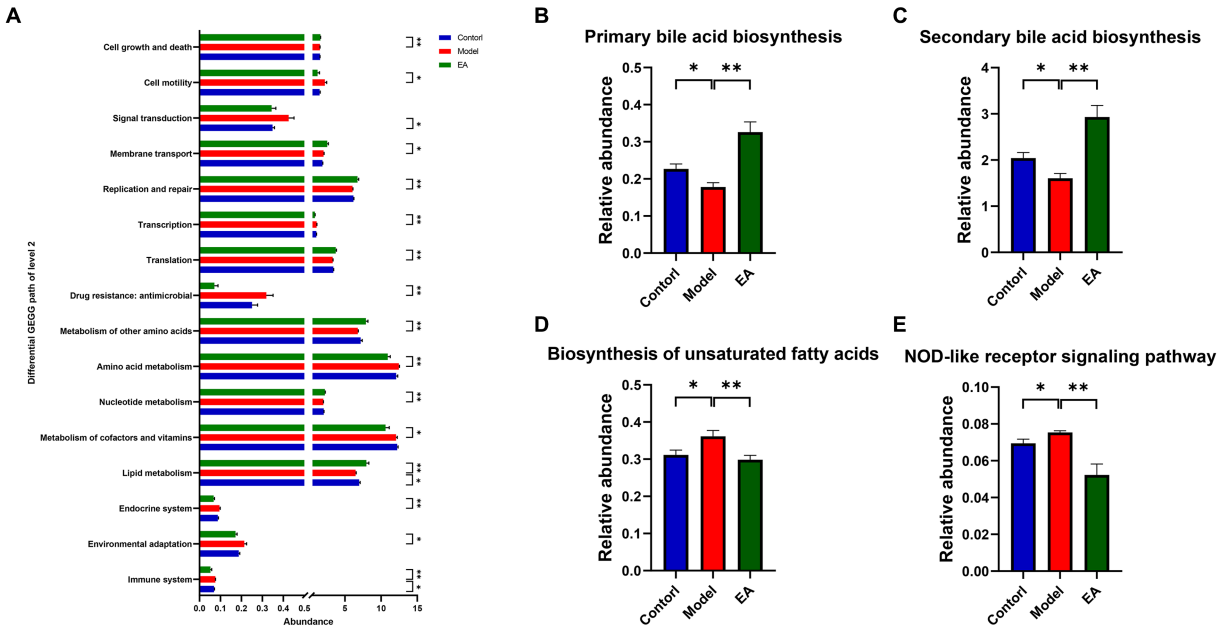

FIGURE 5
Microbial gene functions annotation on KEGG. **(A)** Differential KEGG path of level 2. **(B–E)** The relative abundance of the predicted KEGG pathways among the Control, Model, and EA groups. ($n = 6$, Wilcoxon rank sum test, $^{*}p < 0.05$, $^{**}p < 0.01$).

Fecal metabolomics analysis revealed that EA regulates abnormal metabolic patterns in PD rats

Microbial-derived metabolites affect the host through multiple pathways. Increasing evidence suggests that some metabolites of the gut microbiota can enter the bloodstream, with important effects on the physiology and behavior of the host (Sarkar et al., 2016; Martin et al., 2018). Next, we investigated the host metabolic profile in the same samples as the 16S rRNA analysis by liquid chromatography-mass spectrometry (LC/MS) and explored the relationship between microbiota and metabolites. Principal component analysis showed that the fecal samples of rats from the model group and the control group were significantly separated along the horizontal axis. The model group is more separated within the group than the control and EA groups and does not form well clusters (Figure 6A). According to the PLS-DA and the orthogonal partial least squares discriminant analysis (OPLS-DA) (Figures 6B,C), there was a large degree of separation between the EA and model groups, indicating different metabolic patterns. There are 57 metabolic sets that were significantly different between the two groups (Figure 6D), including 17 lipid metabolic pathways such as butyrate metabolism, fatty acid biosynthesis and degradation, and glycerophospholipid metabolism.

Screening the differential metabolites between EA and model groups by VIP > 1 and $p < 0.05$, a total of 474 differential metabolites were obtained, of which 363 increased and 111 decreased compared with the model group (Figure 6E). Fold Change (FC) values were performed and 92 metabolites changed 2-fold and 10 metabolites changed 3-fold (Figure 6E; Supplementary Table S2). Secondary screening of lipid components among the differential metabolites was performed, and a total of 58 differential lipids were obtained, of which 44 increased and 14 decreased compared to the model group (Figure 6F), with most lipid metabolites showing an elevated state. To explore the potential relationship between gut microbiota changes and metabolites, a correlation matrix was generated using Spearman correlation (Figure 6G). Most of the lipids were negatively correlated with *Escherichia–Shigella* and positively correlated with *Lactobacillus*, *Dubosiella,* and *Bifidobacterium*, with the exception of a few such as PA (18:0/16:0) and PE [18:2(9Z,12Z)/20:2(11Z,14Z)].

EA reversed the lipid peroxidation of SNpc by regulating plasmalogen

Based on the above results, we focused on abnormal lipid metabolism. Serum lipidomics analysis indicated that the serum lipids of the Control group and the Model group showed completely different characteristics. Specifically, the two groups showed obvious separation trend along the horizontal axis, and the Model group did not form a good clustering., while EA treatment could partially reverse this trend (Figure 7A). The volcano map showed that the lipid type with the highest proportion and the most significant difference in metabolites between the Model group and the EA group was FA (Figure 7B). Further analysis revealed that rotenone intervention reduced the concentration of almost all detected PUFA types (Figure 7C) which was in line with the prediction of microbial function above (Figure 5D), and EA treatment reversed this change. In addition, changes in lysophospholipids also attracted our attention (Figures 7B,D), which may be attributed to the significantly reduced serum PUFA content. Compared with the Control group, plasmenylethanolamine (pPE) content in the Model group was significantly increased, while ethanolamine lysoglycerophospholipid (LPE) content showed an increasing trend without statistical difference (this portion of the data is not displayed here). This indicates that

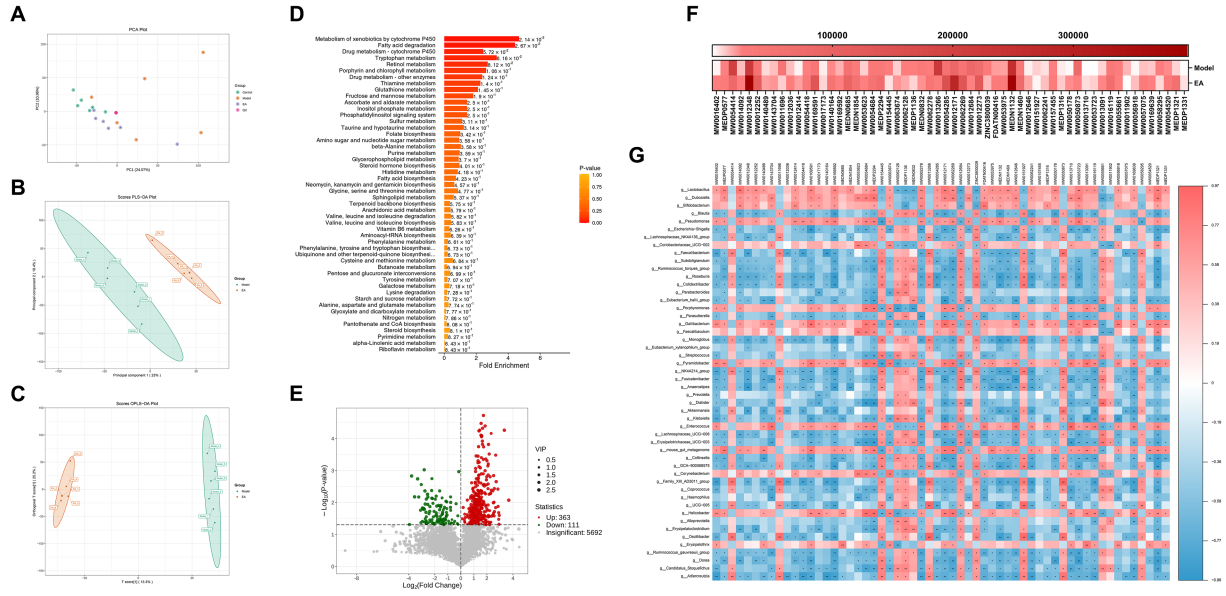

FIGURE 6
Fecal metabolomics analysis. **(A)** PCA of the control, model and EA group. **(B,C)** PLS-DA and OPLS-DA showed an obvious separation trend of fecal metabolites in the model and EA group. **(D)** The first 50 differential metabolisms were selected by MSEA enrichment analysis. **(E)** The volcanic map shows different metabolites between the EA and Model groups. **(F)** 58 lipid components among the differential metabolites. **(G)** Correlations between fecal microbiota and metabolites.

under oxidative stress, the body generated more plasmalogen species to prevent increased oxidative stress, and thereby lipid peroxidation.

This did not seem to be consistent with the basic pathological features of PD, so we further examined pPE and LPE levels in SNpc. Compared with Control group, the content of pPE in SNpc in Model group was significantly reduced, while the content of LPE was significantly increased. The EA intervention increased both pPE and LPE levels (Figure 7D). We hypothesized that this trend was due to the presence of lipid peroxidation, for oxidative stress was already present in SNpc, and that the EA intervention acted as an antioxidant by increasing phospholipid concentrations. To verify this hypothesis, we detected lipid peroxidation products in SNpc. The results showed that the contents of 4-hydroxy-2(E)-hexenal (HHN), 4-hydroxy-2(E)-nondinenal (HNDE), and 4-hydroxy-2(E)-nonenal (HNE) in SNpc in Model group were significantly increased, and EA treatment reduced their contents (Figure 7E), which verifies our conjecture above. The different trends of pPE and LPE in SNpc and serum suggest that the lack of antioxidant capacity in SNpc may be the reason for the preferential damage of dopaminergic neurons in the oxidative stress state compared with the systemic state.

Endotoxin-mediated systemic inflammation associates gut microbiota and dopamine neuron damage

To further determine the role that gut microbiota plays in EA regulation of lipid peroxidation in SNpc, we analyzed the relationship between the 68 screened genera (Supplementary Table S2) and TH^+ neurons or the lipid peroxidation product, 4-hydroxyalkenals (4-HNE), in SNpc, respectively. The correlation analyses indicated that 4 and 34 genera were significantly correlated with TH^+ neurons and 4-HNE, respectively (Supplementary Table S6). Three genera, *Morganella*, *Escherichia-Shigella*, and *Adlercreutzia*, two of which belong to the *Pseudomonadota* (previously known as *Proteobacteria*), were correlated with both TH^+ neurons (Figure 8A) and 4-HNE (Figure 8B), which positively correlated with 4-HNE and negatively with TH^+ neurons. The inflammatory response caused by the release of LPS from Gram-negative bacteria has long been constituted as an important etiologic factor in PD, and different routes and modes of LPS administration can cause various parkinsonian symptoms (Jia et al., 2023). The up-regulation of NOD-like receptor signaling pathways (Figure 5E) in the Model group also suggests the presence of LPS-mediated inflammatory cascades. Then we tested serum LPS and inflammatory factors. Rats in the Model group showed generalized elevated LPS (Figure 8C) and inflammatory responses (Figure 8D) and EA reversed this change.

Discussion

Gut microbial dysbiosis and alteration of microbial metabolites are involved in the development of PD (Sun et al., 2018b). Dysbiosis in the composition and abundance of gut microbiota can affect both the enteric nervous system (ENS) and the CNS in PD pathogenesis. Substantial evidence supported the functional effects of microbiota on the bi-directional communications between gut and brain, indicating the existence of a microbiome-gut-brain axis that induces neuroinflammation and deficiency of dopaminergic neurons and thereby leading to PD (Wang et al., 2021). Alterations in the diversity and composition of gut microbiota and microbial products are found both in PD patients and PD-related animal models. Previous study has shown that acupuncture can induce normalization of gut microbial dysbiosis as well as inhibition of neuroinflammation in the SN and the

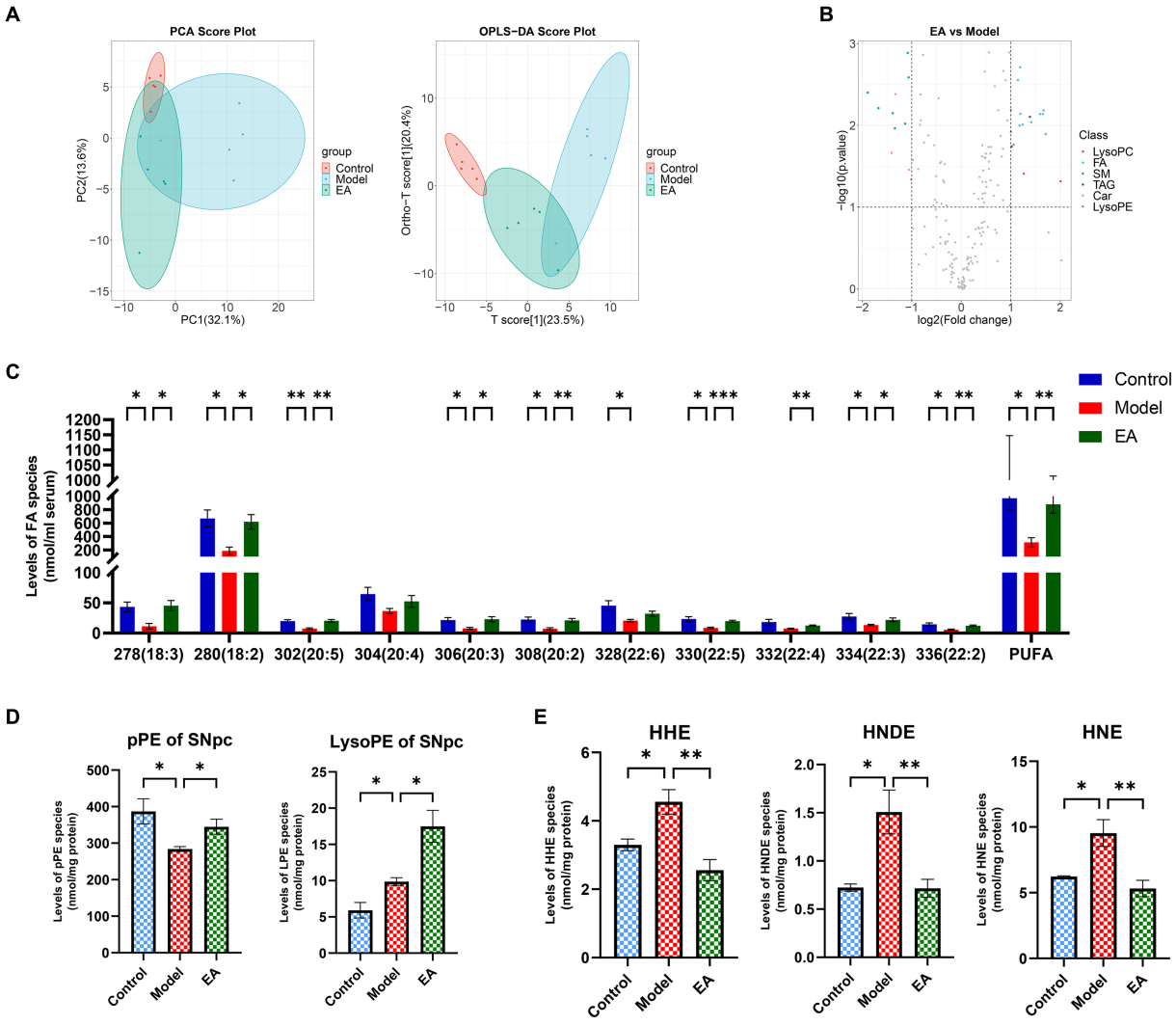

FIGURE 7
Representative comparison of lipidomics data of serum and SNpc. **(A)** PCA and OPLS-DA analysis of serum in the control, model and EA group. **(B)** The volcanic map shows different serum lipid metabolites between the EA and Model groups. Lipidomics analysis of **(C)** polyunsaturated fatty acids (PUFAs) of serum, **(D)** plasmalogen PE (i.e., plasmenylethanolamine, pPE) and ethanolamine lysoglycerophospholipid (LPE) of SNpc, and (E)4-hydroxyalkenal species of SNpc presented in lipid extracts was conducted by using multi-dimensional mass spectrometry-based shotgun lipidomics ($n = 5$, Student t-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). The prefix "p" in **(D)** stands for plasmalogen PE species which consist of a vinyl ether linkage of the aliphatic chain at the sn-1 position. **(E)** HHN, HNDE, and HNE denote 4-hydroxy-2(E)-hexenal, 4-hydroxy-2(E)-nondinenal, and 4-hydroxy-2(E)-nonenal, respectively.

ST, which are associated with PD phenotypes (Jang et al., 2020). However, the intermediate link between regulation of intestinal microbiome disorder and improvement of pathological changes of CNS by acupuncture is still unclear.

Gut microbiology studies uncover that improvement of PD motor symptoms is tied to microbiota recovery

Acupuncture has been considered a potential nondrug therapy for PD patients. Growing evidences showed that acupuncture has been used to improve brain functions and inflammation in neurological disorders such as PD (Tamtaji et al., 2019a), and also to recover the gastrointestinal dysfunctions in various metabolic disorders (Si et al., 2018). Similarly, these studies suggest that gut microbiota may be a target for acupuncture treatment, thereby playing a crucial role in mutual regulation of the brain-gut axis (Wei et al., 2019). In the recent study, acupuncture improves PD symptoms and regulates gut microbial composition in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice model (Jang et al., 2020). Another study reported that EA may alleviate behavioral defects via modulation of gut microbiota and suppression of inflammation in the SNpc of mice with PD (Han et al., 2021).

In this study, the β-diversity analysis emerges a significant difference in the gut microbiota (Figures 2D,E). There was a little overlap in the distribution of the Model and Control groups. EA-treated rats formed a unique cluster that was separate from the control and PD rats, indicating that the EA treatment completely altered the composition of the gut microbiota, which is also well

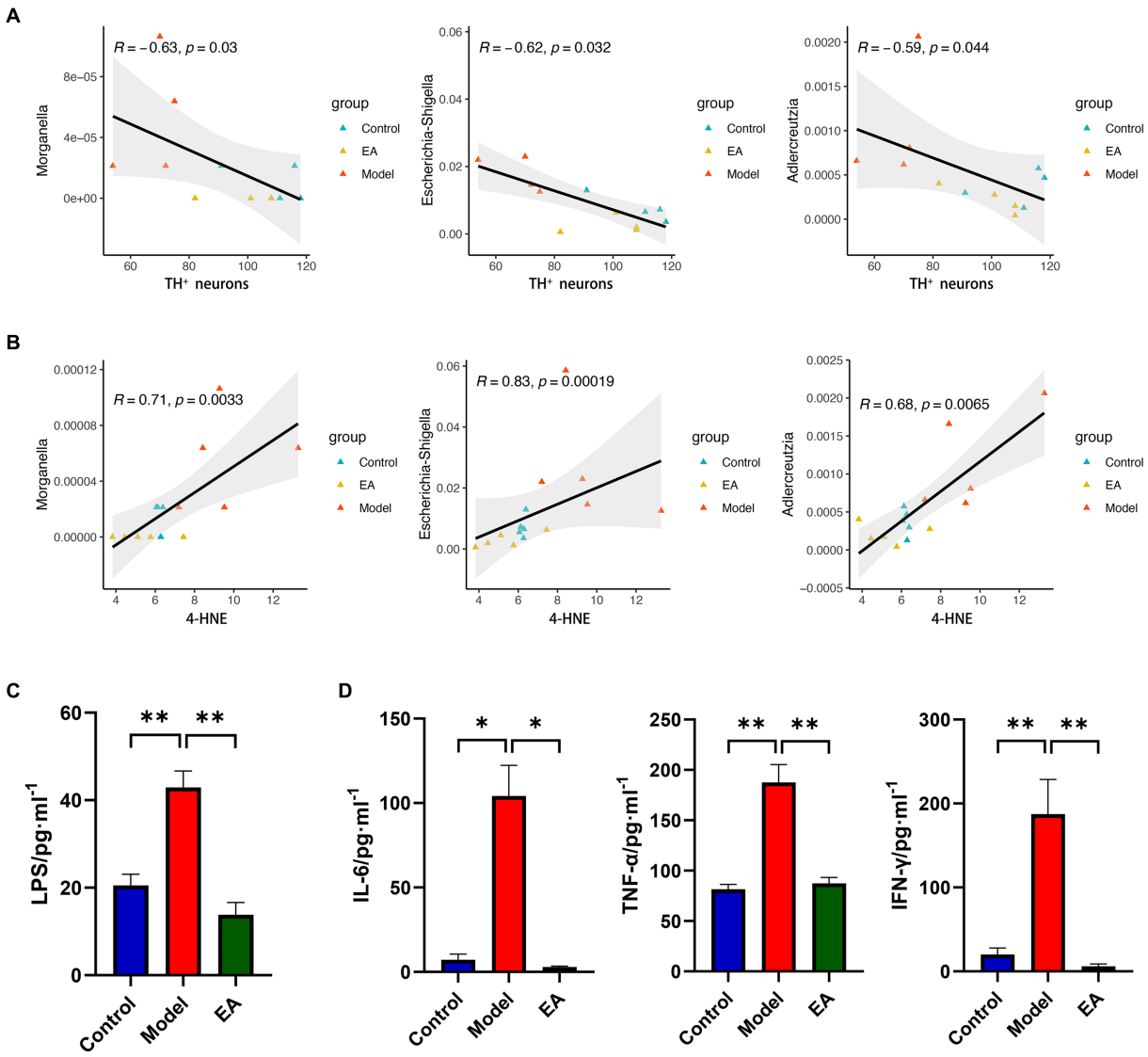

FIGURE 8
Bacterial endotoxin links lipid peroxidation with TH+ neuronal damage. Spearman's correlation analyses between the relative abundance of gut microbiota at the genus level and the value of **(A)** TH⁺ neurons or **(B)** 4-HNE, respectively. Significant correlations were determined based on |Spearman *r*| > 0.5 and $p < 0.05$. Serum LPS **(C)** and inflammatory responses **(D)** in each group ($n = 3–4$, Student *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

corroborated by the change in genus level (Supplementary Table S2; Figure 3). The EA intervention significantly altered 68 genera, much more than the 15 genera treated with rotenone. In addition to reversing changes in genus levels in the vast majority of the model groups, most of the alterations appeared to be specific to the EA treatment. In terms of α-diversity analysis, the richness (Figure 2A) and diversity (Figures 2B,C) of gut microbiota in rotenone-induced PD rats were significantly altered, that ACE, Shannon and Simpson indices were significantly increased compared with those in the Control group (For the Simpson Index, we used Simpson's Index of Diversity 1-D to deal with it intuitively). EA reversed the change of microbiota richness and diversity index in Model group. This finding does not fit with the conventional wisdom that the richness and diversity of gut microbiota tend to decline during disease states. However, among all the PD patient gut microbiota studies that reported significant differences in alpha diversity, the vast majority (five out of six) had significantly increased gut microbiota richness (Chen et al., 2021). Similar animal results have been reported for changes in the gut microbiota of Parkinson's disease (Cao et al., 2023). What led to the counterintuitive changes in model group diversity and richness, and what do the unique changes resulting from the EA treatments mean (are those 53 genera changes serving to adjust for gut microbiome disruption)? These need to be concluded by species-specific analyses.

At the phylum level, there is evidence that the *Pseudomonadota* (previously *Proteobacteria*) is a marker of dysbiosis in the gut microbiome, with pro-inflammatory characteristics, which generally overexpressed in gastrointestinal and immune diseases (Shin et al., 2015). *Pseudomonadota* contributes to the elevated luminal lipopolysaccharide (LPS) levels in PD, which may impair intestinal integrity (Nighot et al., 2017). LPS-triggered immune process can affect the CNS through the gut-brain axis, activating microglia and

leading to dopaminergic neuron death (Deng et al., 2020). However, the abundance of *Pseudomonadota* did not change significantly at the phylum level, so we further looked at changes at the genus level. Preliminary analysis shows that *Bacillota* (previously known as *Firmicutes*) has the maximum number of species with significant differences between the Control and Model groups, not *Pseudomonadota* (Supplementary Table S2; Figure 3A). One step further, different genera with opposite trends in Model group and EA group were screened out, and the abundance of *Escherichia–Shigella*, which belonging to *Pseudomonadota*, was absolutely dominant (Figure 3B). *Morganella*, belongs to the same phylum, *Pseudomonadota*, was associated with major depression and genetic damage (Qin et al., 2022). Moreover, we showed that the abundances of the genera *Escherichia–Shigella*, *Lactobacillus,* and *Dubosiella* were highly correlated with behavioral metrics of motor deficits (both pole and rotarod tests) (Figure 4). Concerning motor symptoms, it has been observed that the pro-inflammatory taxa *Escherichia* and *Serratia* are also prevalent in non-tremor dominant patients with PD (Vascellari et al., 2021). Additionally, *Escherichia coli* and *Salmonella* produce bacterial amyloids (Yan et al., 2020), which positively promoted α-syn pathology in the gut and brain (Sampson et al., 2020). This is in line with our observation of higher *Escherichia–Shigella* abundance in PD rats.

On the other hand, the genera with significant increase in EA group were screened out, in which the relative abundance of *Lactobacillus*, *Dubosiella,* and *Bifidobacterium* exceeded 0.05. Reportedly, *Lactobacillus* is generally considered beneficial and often included in probiotic mixtures (Skrzydlo-Radomanska et al., 2020; Romano et al., 2021), which could represent a compensatory mechanism to restore intestinal homeostasis. Previous studies have shown that acupuncture can protect the gut microbiota by promoting the growth of *Lactobacillus* and inhibiting the proliferation of *Pseudomonadota* and *Escherichia-Shigella*, thereby exerting a therapeutic effect on many diseases such as PD (Han et al., 2021), Alzheimer's disease (Zhang Y. et al., 2022), depression (Wang et al., 2020), and ulcerative colitis (Song et al., 2020). The studies of gut microbiota in PD appear complex and inconsistent, although the consistent or contradictory results compared with other studies (Vascellari et al., 2020). Short-chain fatty acids (SCFA) and their production bacteria were found to be generally reduced in the intestines of PD patients, which may interact with increased intestinal mucosal permeability and endotoxin exposure (Yang et al., 2019). *Dubosiella* and its metabolite butyrate protect neurons from LPS-induced damage by inhibiting microglia-mediated inflammation and oxidative stress through the GPR109A/Nrf2/HO-1 pathway (Zhang H. et al., 2022). Although there was no difference in the relative abundance of *Bifidobacterium* among the three groups (Kruskal–Wallis one-way ANOVA), considering that this difference may only appear in EA (according to Metastats, *Bifidobacterium* has a significant difference between EA group and Model group) and its own special role, *Bifidobacterium* has also been included in the differential genus. Both clinical and cellular experiments have found that *Lactobacillus* and *Bifidobacterium* can reduce inflammation and oxidative stress and improve clinical symptoms in patients with Parkinson's disease (Magistrelli et al., 2019; Tamtaji et al., 2019b). One investigation showed that *Bifidobacteria* showed a significant advantage after mosapride intervention, which may be related to increased intestinal motility and reduced plasma endotoxin levels with mosapride (Chen et al., 2022). EA at ST25 has also been shown to improve intestinal peristaltic capacity and ENS function (Song et al., 2022). Combined with our results on microbiome analyses, it can be hypothesized that EA may inhibit neuroinflammation, protect DA neurons and improve motor impairment in PD rats by improving the relative abundance of gut microbiota, such as inhibition of *Escherichia–Shigella* by increasing the abundance of *Lactobacillus*, *Dubosiella,* and *Bifidobacteria*.

EA regulates gut microbiota functions associated with lipid metabolism and endotoxin-mediated systemic inflammation

PICRUSt2-predicted functional KEGG pathway analysis revealed abnormal functional pathways in rotenone-treated rats, especially those related to lipid metabolism. Previous studies on microbiota function in human Parkinson's disease patients have focused on loss of fatty acid metabolism and dysregulation of lipid metabolism, especially bile acid metabolism (Li et al., 2021), which is surprisingly consistent with our results (Figures 5B,C). Bile acids interact with their receptors to inhibit apoptosis, inflammation, and oxidative stress (Wahlstrom et al., 2016). However, regardless of its intermediate pathways, as a direct inducement of neuronal damage, the inflammation and oxidative stress have always been of concern in the central pathology of Parkinson's disease. NOD-like receptors are associated with a variety of inflammatory diseases (Chen et al., 2009), and several of their family members are involved in the immune response process in *Shigella* and LPS (Girardin et al., 2001; Kim et al., 2008). The regulation of NOD-like receptor signaling pathways (Figure 5E), serum LPS (Figure 8C) and inflammatory factors (Figure 8D) suggests that EA may modulate systemic inflammation through gut microbiome and innate immune system.

Alterations in lipid species due to dysregulation of lipid-metabolizing enzymes may directly promote PD pathology (Xicoy et al., 2019). In particular, the hallmark pathological protein in PD, α-syn, has lipid membrane functions, and the endosome-lysosomal system and synaptic signaling pathways in PD are critically dependent on lipid dynamics (Galper et al., 2022). Moreover, PD genetics suggests that dysregulation of lipid homeostasis may contribute to the development of disease (Fanning et al., 2020; Fais et al., 2021). For example, β-glucocerebrosidase (GBA), which encodes glucocerebrosidase, a lysosomal hydrolase of lipid metabolism, is the most common genetic factor that increases the risk of PD (Aharon-Peretz et al., 2004). Thus, lipids are a promising research avenue to understand the etiology of PD as well as potential pharmacodynamic biomarkers of PD. Lipid metabolism is mainly regulated by nutrients like sugars and fatty acids. Studies have shown that therapies targeting the gut microbiota can improve metabolic function (Koutnikova et al., 2019). However, several reports have shown that lipid levels are relevant with the gut microbiota composition and microbial metabolites (Fu et al., 2015; Just et al., 2018). The influence of the gut microbiota on host lipid metabolism may be mediated through microbial metabolites generated by the gut microbiota, including SCFA, secondary bile acids, trimethylamine and LPS (Schoeler and Caesar, 2019). In addition, microbiota-induced changes in the lipid composition of the host cell membrane can affect signaling pathways,

and the resulting downstream products can affect host local tissue and systemic immunity and metabolism (Brown et al., 2023). Gut dysbiosis is associated with metabolic disorders, and there exists a causal relationship between microbial function and metabolic disorders. However, the mechanistic links between gut microbiota and lipid metabolism and how the specific lipids affect microbial profile remains elusive.

Recent investigations have suggested that acupuncture also modulated various intestinal microbial metabolites and metabolic pathways (Yang et al., 2022). According to our study, the KEGG pathway enrichment analysis performed on metabolic pathway showed that differential lipid metabolism pathways were significantly enriched between PD and EA groups. To further explore the relationship between gut microbiota and metabolites differences, we identified a total of 474 metabolites of gut microbiota with significant differences were detected in EA-treated rats compared with PD rats (Figure 6E). Among these metabolites, lipid metabolism, such as biosynthesis of unsaturated fatty acids, fatty acid and bile acid was differentially expressed (Supplementary Table S5). Furthermore, we selected the first 50 differential metabolisms by MSEA enrichment analysis (Figure 6D). EA particularly affected dopamine 3-O-sulfate, L-Gulonolactone, and S-adenosyl-L-homocysteine (Supplementary Table S5), which were reported from previous studies involved in the response to treatment in PD patients, respectively (Bronaugh et al., 1975; Muller and Kuhn, 2006; Harrison et al., 2008). Additionally, we identified those lipids derived from microbiota, including β-Glycerophosphoric acid, 4-Hydroxybenzoic acid, and 4-Methoxycinnamic acid (Supplementary Table S5). Recent studies of brain-penetrating polyphenolic acids, namely, 3-hydroxybenzoic acid (3-HBA), 4-hydroxybenzoic acid (4-HBA), 3,4-dihydroxybenzoic acid (3,4-diHBA), and 3-(3-hydroxyphenyl) propionic acid (3-HPPA), show ability to effectively modulate the development to PD-type neuropathy and the progression of α-synucleinopathy (Ho et al., 2019; Ono et al., 2020).

To further explore the correlation between the significantly different intestinal microbiota and lipid metabolism, we conducted Spearman correlation analysis. Our results showed that Most of the lipids were negatively correlated with *Escherichia-Shigella* and positively correlated with *Lactobacillus*, *Dubosiella* and *Bifidobacterium* (Figure 6G). Based on these results, we speculated that a possible correlation between the altered lipids metabolism and gut microbiota compositions would bring new avenues for targeting PD pathological process, which may be an important pathway for acupuncture to regulate the correlation between the microorganisms and metabolomics.

EA reversal of SNpc lipid peroxidation is associated with correction of endotoxin-mediated systemic inflammation

In addition to serving as essential structural components of the cellular membranes, plasmalogens play many crucial roles in cellular functions, including reservoirs for second messengers and working as endogenous antioxidants (Nagan and Zoeller, 2001; Braverman and Moser, 2012). It has been demonstrated that aberrant metabolism of plasmalogens is closely associated with insulin resistance (Tonks et al., 2016), atherosclerosis (Rasmiena et al., 2015), neurodegeneration (i.e., Alzheimer's disease, Parkinson's disease) (Han et al., 2005; Han, 2010), and aging (Lessig and Fuchs, 2009), etc. Decreased plasmalogen contents with increased choline and ethanolamine lysoglycerophospholipids (particularly those containing PUFAs) indicate the increased oxidative stress (Gross, 1984). Our results show that pPE content in the Model group was significantly increased, while LPE content showed an increasing trend without statistical difference. This indicates that under oxidative stress, the body generated more plasmalogen to resist oxidation, so there is no systemic lipid peroxidation. However, the antioxidant capacity of SNpc does not seem to be effective. Significantly lower pPE and higher LPE levels (Figure 7D) suggest obvious oxidative stress in SNpc, and elevated 4-hydroxyalkenal species (Figure 7E) confirm the presence of lipid peroxidation. After EA intervention, both pPE and LPE were increased, indicating that EA intervention increased plasmalogens synthesis to combat oxidative stress, and lipid peroxidation was also controlled.

Moreover, correlation analyses also verified strong correlations of *Escherichia-Shigella* and *Morganella* with motor symptoms and SNpc lipid peroxidation. Dysbiosis of the microbiota leads to increased intestinal permeability, allowing the LPS product to be transferred from the intestinal lumen into the host circulation (Rietschel et al., 1994; Tilg et al., 2016), which corroborates with our results (Figure 8C). Different routes and modes of LPS administration can cause a variety of Parkinson's disease symptoms. An animal experiment showed that injection of LPS into the rat substantia nigra activated microglia, triggered an inflammatory response, increased the expression of pro-inflammatory cytokines, and altered the activity of oxidative stress markers, inducing the characterization of a PD model (Sharma and Nehru, 2015). Intraperitoneal injection of LPS increases α-syn expression and intestinal permeability in the large intestine (Kelly et al., 2014). Thus, LPS is widely used in models of neuroinflammation and oxidative stress in Parkinson's disease (Deng and Bobrovskaya, 2022). It has been shown that combinations of bacteria and their products rather than specific bacteria appear to be responsible for the specific folding of α-syn (Stolzenberg et al., 2017; Manfredsson et al., 2018; Terada et al., 2018). Therefore, the study of bacterial clusters with similar functional characteristics, such as LPS leakage, is more relevant than simply focusing on specific bacterial species. Future studies should not be limited to characterizing pathogenic bacteria, such as *Escherichia–Shigella*, or beneficial bacteria, such as *Lactobacillus*.

Due to conditions, no colony transplantation or *in vitro* culture experiments were performed to clarify that modulation of the gut microbiota is a necessary way for EA to intervene in the motor symptoms of PD. For example, EA at ST25 may also inhibit LPS-induced inflammation exertion by activating NPY^+ peripheral sympathetic neurons projecting to immune organs such as the spleen However, Prof. Qiu-Fu Ma 's study showed that the activation of the sympathetic anti-inflammatory pathway by EA at ST25 is time-sensitive. In concrete terms, if high-intensity EA stimulation at ST25 is given before the onset of LPS-induced systemic inflammation, it can exert a β2-noradrenergic receptor-mediated anti-inflammatory effect through the activation of peripheral NPY^+ sympathetic neurons; whereas, applying the same abdominal acupoints and stimulation intensities after the induction of LPS-induced inflammation, it exhibits a markedly pro-inflammatory effect, which is mainly due to the fact that LPS can induce an increase in expression of inflammation-promoting α2-adrenergic receptor (Liu et al., 2020). This somehow

suggests that there must be other powerful and effective anti-inflammatory pathways. Considering the site specificity of ST25 and its ability to regulate intestinal motility functions (Song et al., 2022), there exists a particular value in the study of gut microbiota. Relative to acute inflammatory diseases such as sepsis, gut microbiota modulation, a mild and long-lasting anti-inflammatory tool, is better suited for chronic inflammatory diseases as opposed to autonomic anti-inflammatory pathways.

Conclusion

This study suggests that the improvement of motor function in PD model by EA may be mediated in part by restoring the gut microbiota. Its intermediate processes involve circulating endotoxins and inflammatory mediators, SNpc oxidative stress and lipid peroxidation. In addition, the specific effect of EA on gut microbiota at the genus level may explain its powerful anti-Parkinson effect on PD model. It is suggested that the gut-microbiome – brain axis may be a potential mechanism of EA treatment for the PD rats.

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 at: https://www.ncbi.nlm.nih.gov/, PRJNA958618.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of the Nanjing University of Traditional Chinese Medicine, Nanjing, China (permission no. 202112A047). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

X-mH: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft. L-z-xS: Conceptualization, Investigation, Methodology, Project administration, Writing – original draft. Z-zZ: Data curation, Software, Visualization, Writing – original draft. XR: Data curation, Writing – review & editing. H-cL: Methodology, Writing – review & editing. ZY: Conceptualization, Investigation, Methodology, Writing – review & editing. LH: Conceptualization, Investigation, Methodology, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1358525/full#supplementary-material

SUPPLEMENTARY TABLE S1
Goods coverage of all samples.

SUPPLEMENTARY TABLE S2
List of bacterial genera with significant differences in relative abundance between gut microbiota of Control, Model and EA groups.

SUPPLEMENTARY TABLE S3
Spearman correlation analysis of 68 genera and motor functions.

SUPPLEMENTARY TABLE S4
List of PICRUSt2-predicted functional KEGG pathways.

SUPPLEMENTARY TABLE S5
List of differential metabolites Information.

SUPPLEMENTARY TABLE S6
Spearman correlation analysis of 68 genera and TH+ neurons or 4-HNE.

SUPPLEMENTARY DATA SHEET 1
Figure S1: Evaluation of modelling.

SUPPLEMENTARY DATA SHEET 2
Figure S2: 16S rRNAseq Rarefaction curve, Shannon-Wiener curve, species accumulation curves.

SUPPLEMENTARY DATA SHEET 3
Figure S3: Diversity analysis of intestinal flora.

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Microbiota profiling reveals alteration of gut microbial neurotransmitters in a mouse model of autism-associated 16p11.2 microduplication

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The gut-brain axis is evident in modulating neuropsychiatric diseases including autism spectrum disorder (ASD). Chromosomal 16p11.2 microduplication 16p11.2$^{dp/+}$ is among the most prevalent genetic copy number variations (CNV) linked with ASD. However, the implications of gut microbiota status underlying the development of ASD-like impairments induced by 16p11.2$^{dp/+}$ remains unclear. To address this, we initially investigated a mouse model of 16p11.2$^{dp/+}$, which exhibits social novelty deficit and repetitive behavior characteristic of ASD. Subsequently, we conducted a comparative analysis of the gut microbial community and metabolomic profiles between 16p11.2$^{dp/+}$ and their wild-type counterparts using 16S rRNA sequencing and liquid chromatography-mass spectrometry (LC/MS). Our microbiota analysis revealed structural dysbiosis in 16p11.2$^{dp/+}$ mice, characterized by reduced biodiversity and alterations in species abundance, as indicated by α/β-diversity analysis. Specifically, we observed reduced relative abundances of *Faecalibaculum* and *Romboutsia*, accompanied by an increase in *Turicibacter* and *Prevotellaceae UCG_001* in 16p11.2$^{dp/+}$ group. Metabolomic analysis identified 19 significantly altered metabolites and unveiled enriched amino acid metabolism pathways. Notably, a disruption in the predominantly histamine-centered neurotransmitter network was observed in 16p11.2$^{dp/+}$ mice. Collectively, our findings delineate potential alterations and correlations among the gut microbiota and microbial neurotransmitters in 16p11.2$^{dp/+}$ mice, providing new insights into the pathogenesis of and treatment for 16p11.2 CNV-associated ASD.

KEYWORDS

ASD, 16p11.2, gut microbiota, 16S rRNA, metabolomic, histamine, neurotransmitter

Introduction

The gut microbiota comprises an intricate community of microorganisms residing in the gastrointestinal tract (McCallum and Tropini, 2023). Over the past decade, scientific research has shed light on a robust bidirectional communication network linking the gastrointestinal system and the central nervous system, commonly acknowledged as the microbiota-gut-brain axis (Agirman and Hsiao, 2021). Disruptions in the gut microbiome and their consequent influence on the gut-brain axis surfaced as significant factors in the onset of neurodevelopmental disorders, such as Autism spectrum disorder (ASD) (Wang Q. et al., 2023), Parkinson's disease (Nie and Ge, 2023), depression (Donoso et al., 2023), and Alzheimer's disease (Zhu et al., 2023).

ASD is an immensely varied neurodevelopmental condition typified by recurrent stereotypical behaviors and impairments in social interaction (Weiss et al., 2008). Recent epidemiological findings indicate a prevalence rate of 2.3% among children and adolescents in the United States in 2018 (Li et al., 2022). Notably, individuals diagnosed with ASD frequently manifest gastrointestinal symptoms, including diarrhea, constipation, and gaseousness, alongside the core features of the disorder (Fulceri et al., 2016; Restrepo et al., 2020). Recent investigations have underscored the substantial influence of the gut microbiota on both gastrointestinal manifestations and neurodevelopmental aspects in individuals with ASD. Intestinal microorganisms have the capacity to synthesize neurotransmitters, thereby impacting the microbiota-gut-brain axis (Srikantha and Mohajeri, 2019). Specific microbial species, such as commensal bacteria *Bacteroides vulgatus*, *Eggerthella lenta*, and *Clostridium botulinum*, could influence the onset and development of ASD by altering glutamate-glutamine metabolism, reducing cortisol, and disrupting the metabolism of aromatic amino acids (Wang et al., 2019). Notably, previous studies revealed the relation of the pathogenic bacterium *Clostridium botulinum* to gastrointestinal symptoms in ASD (Ding et al., 2017) and discussed its potential role in promoting ASD through secreted toxins, which affect the expression of the GTPase RHO family (Argou-Cardozo and Zeidan-Chulia, 2018), although the difference in toxicology leading to fatal disease versus ASD remains unclear. Furthermore, fetal histamine concentrations have been linked to the presence of diarrhea symptoms in individuals displaying elevated IFN-γ levels within the ASD cohort (Xu et al., 2023). Moreover, disruptions in the gut microbiota may disturb neurotransmitter regulation, potentially contributing to the development of neurodevelopmental disorders. For example, anthocyanins have been demonstrated to increase the abundance of *Lactobaccillales*, stimulating enterochromaffin (EC) cells, enhancing the availability of tryptophan for serotonin synthesis. This effect leads to improved social interactions and reduced repetitive behaviors in mice with ASD induced by valproic acid exposure (Serra et al., 2022).

Within individuals diagnosed with ASD, one of the most prevalent genetic variations is the 16p11.2 copy number variants (CNVs), affecting approximately 1% of ASD cases (Kumar et al., 2008; Marshall et al., 2008). This genetic locus spans approximately 600 kb and encompasses 29 protein-coding genes. Microduplications or microdeletions within this region have been linked to a spectrum of disorders, including neurological conditions like ASD (Fernandez et al., 2010) and schizophrenia (Chang et al., 2021), as well as metabolic irregularities, hematological conditions, skeletal anomalies, genitourinary abnormalities, and sleep disturbances (Wu et al., 2015; Verbitsky et al., 2018; Crawford et al., 2019; Giannuzzi et al., 2019; Kamara et al., 2021; Wang et al., 2022; Hanssen et al., 2023). Nevertheless, the precise mechanisms by which 16p11.2 CNVs influences the onset of ASD remain inadequately understood (Rein and Yan, 2020). Autism is a multifaceted disorder, with genetic, environmental, and gut microbiota factors all at play (Sauer et al., 2021). It's crucial to understand how the 16p11.2 genetic variant influences autism's onset and/or progression, not just through genetic mechanisms, but also through non-genetic factors. While research has been conducted revolving around 16p11.2 microdeletion syndrom and gut microbiota, the specific effects of 16p11.2 microduplications on gut microbiota, and how these changes might relate to autistic symptom, are still unclear.

The 16p11.2$^{dp/+}$ mouse model characterized by a microduplication within the 7Slx1b-Sept1 region, provides an effective tool for investigating the functional implications of 16p11.2$^{dp/+}$. To explore the intricate and multifaceted connections between 16p11.2$^{dp/+}$ and gut microbiota within the context of ASD-like symptoms, we initially confirmed the presence of core ASD-related behaviors in 16p11.2$^{dp/+}$ mice. Following this, we conducted an analysis of the gut microbiota and metabolites in fecal samples collected from 16p11.2$^{dp/+}$ mice and wild-type (WT) mice, using 16S rRNA sequencing and liquid chromatography-mass spectrometry (LC/MS). Our 16S rRNA data generated a total of 915 amplicon sequence variants (ASVs) from 16p11.2$^{dp/+}$ mice and 10 WT mice. Additionally, we performed a metabolomics assessment, identifying a total of 458 metabolites in these samples. Furthermore, we conducted a correlation analysis between fecal metabolites and individual bacteria to explore the relationships between these distinct microbial species and fecal metabolites.

Materials and methods

Animal

16p11.2$^{dp/+}$ mice, characterized by a heterozygous duplication of mouse chromosome 7F3 that is syntenic to the human 16p11.2 locus, were procured from Jackson Laboratory (Stock No: 013129). These mice were then crossbred with WT C57BL/6 background female mice (Horev et al., 2011). To maintain the colony, breeding involved crosses between WT females and 16p11.2$^{dp/+}$ males. For the control group, offspring of WT males were employed. A total of 10–11 mice per group were involved in the study. All mice were provided with sterile food and autoclaved water *ad libitum* and subjected to a 12-h light cycle. The study was conducted in strict adherence to the ethical guidelines and protocols outlined by the Institutional Animal Care and Use Committee at Southern University of Science and Technology.

Behavioral testing

Three-chamber test

The social behavior test was adapted from experiments previously described (Sgritta et al., 2019). The three-chamber apparatus consisted of a rectangular box (60×40×20 cm, L×W×H) divided into three interconnected chambers. Mice were habituated for 10 min in the empty apparatus. Sociability was then assessed over a 10-min period during

which the mice had the option to interact with either an empty cage or a stranger mouse (M). Subsequently, preference for social novelty was tested for 10 min by introducing another stranger mouse (Nov). The time spent by the test mouse interacting with the empty cage, Fam mouse or Nov mouse was recorded and measured using the EthoVision XT 10 software package. The human observers were blind to the treatment group.

Open field test

The open field test was adapted from experiments we previously described (Jiang et al., 2022). Mice were placed in a box ($40 \times 40 \times 40\,cm^3$) and allowed to explore freely for 10 min. The interior $20 \times 20\,cm^2$ area was defined as center area. Distance traveled, velocity of movement, and duration in the center area were recorded and automatically analyzed by Noldus EthoVision XT10 (Noldus Information Technology; Leesburg, VA, United States).

Self-grooming test

The mice were placed in an empty cage containing corncob bedding for 5 minutes to acclimate. Each mouse then recorded how long it spent grooming itself over the next 10 minutes. Self-grooming includes wiping the face, scratching/rubbing the head and ears, and grooming the whole body.

Elevated plus maze

The maze was composed of two open and two closed arms intersecting each other, elevated 1 m above the floor. The Elevated plus maze was equipped with a video tracking system, and the videos were analyzed by Noldus EthoVision XT10 software. The test mice were placed in the center facing the open arm, and their activity was measured for 5 min. The total time spent in open arms, center area and closed arms was recorded.

Fecal sample collection

After a feeding period of 90 days, we collected fresh fecal samples from both 16p11.2$^{dp/+}$ male mice and their WT counterparts. For each mouse, we collected at least two tubes of samples, with each tube containing approximately 200 mg of fecal matter.

DNA extraction and 16S rRNA sequencing

Genomic DNA was isolated from fecal samples of mice utilizing the hexadecyltrimethylammonium bromide (CTAB) technique, as outlined in the study conducted by the pervious study (Kang et al., 2019). In brief, we extracted total microbial DNA of superior quality using the E.Z.N.A Stool DNA Kit (manufactured by Omega Bio-Tek, Inc., Norcross, GA, United States), adhering to the guidelines provided by the manufacturer. The amplification of the V3-V4 regions of the 16S rRNA gene was carried out using PCR, involving an initial step at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 60 s, and a final extension step at 72°C for 5 min. Universal bacterial primers, 341F (5'-CCTAYGGGRBGCASCAG-3′) and 806R (5'-GGACTACNN GGGTATCTAAT-3′) were used for this purpose. For the preparation of sequencing libraries, the NEB Next®Ultra™DNA Library Prep Kit for Illumina (NEB, USA) was employed following the manufacturer's guidelines, and index codes were incorporated. Library quality was assessed using the Qubit@ 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2,100 system. Ultimately, the library underwent sequencing on a HiSeq2500 PE250 platform, generating paired-end reads of 250 base pairs.

Fecal metabolomic analysis

Metabolites in stool samples were determined by LC-MS. Briefly, 800 µL of a methanol-acetonitrile solution (1:1, v/v) was added to 80 mg of fecal sample, vortexed, and then centrifuged. The samples were incubated on ice for 10 min and centrifuged at 14,000 rpm at 4°C for 20 min, after which the supernatant was retained and stored at −80°C. For Ultra High Performance Liquid Chromatography (UHPLC)-MS/MS analysis, we used an Agilent 1,290 Infinity LC system (Agilent, 1.7 µm, 2.1 mm × 100 mm) to perform chromatographic separation of the samples at a constant temperature of 25°C and an AB Triple TOF 6600 series mass spectrometer (AB SCIEX) to detect eluted metabolites. The raw data files were converted into .mzXML format by ProteoWizard, and then the XCMS program[1] was used to perform peak alignment and quantification for each metabolite. Subsequently, peak intensities were normalized to the total spectral intensity.

Data analysis and statistic tests

The data from behavioral tests for the two groups underwent t-test for analysis. In the case of the three-chamber test results, a two-way ANOVA was employed, followed by Bonferroni's multiple-comparisons test for further analysis.

The standard amplicon sequencing data analyses were carried out using QIIME 2 and its DADA2 plugin. These analyses included sequence quality control, the generation of feature tables and representative sequences, taxonomy assignment of representative sequences against the SILVA SSURef NR99 version 138.1 database, and the construction of phylogenetic trees. α-diversity and β-diversity analyses were performed using the R package vegan. To assess the significant differences in α-diversity between 16p11.2$^{dp/+}$ and WT mice, the Tukey HSD test integrated into the alpha_boxplot function of the R package amplicon was utilized. Principal Coordinates Analysis (PCoA) was applied based on the Bray-Curtis distance using the R package vegan to identify the community compositions in all samples. The analysis of differential taxa was conducted using LEfSe. KEGG pathway predictions were performed using PICRUSt2. The differential abundance of phylum, genus, and functional modules between any two groups was assessed using the Wilcoxon rank-sum test. The p-values were adjusted for multiple testing with the Benjamini-Hochberg method to control the false discovery rate (FDR).

Partial least squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were employed to visualize and compare the metabolites in each group, with metabolic variations between the groups detected using a permutation testing algorithm. Significant differential metabolites were identified using a threshold of p value (t-test) < 0.05 and VIP > 1, and clustering blot maps were created. The correlation between microbes and metabolites was analyzed using Spearman correlation analysis, with significance set at $p < 0.05$.

1 https://xcmsonline.scripps.edu

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Results

16p11.2$^{dp/+}$ mice exhibit social novelty deficits and repetitive behaviors

To investigate if 16p11.2$^{dp/+}$ mice recapitulate clinical characteristics seen in humans, we conducted behavioral tests on 8-10-week-old male 16p11.2$^{dp/+}$ mice and age-matched WT mice. We tested ASD-related behaviors such as social skills, repetitive behaviors, and anxious behaviors. In the three-chamber test, assessing sociability and social recognition, both 16p11.2$^{dp/+}$ and WT mice showed a preference for a chamber with a stranger mouse (M) over an empty cage (E) (Figures 1A–C). Interestingly, during the social novelty phase, the 16p11.2$^{dp/+}$ mice exhibited a significantly lower preference for novel mice (Nov) compared to that of WT mice. This suggests a potential defect in social novelty preference in 16p11.2$^{dp/+}$ mice (Figures 1D–F). In addition to social deficits, repetitive behaviors are another core symptom of ASD. To assess the repetitive behaviors of 16p11.2$^{dp/+}$ mice, we observed the frequency of grooming within a 10-minute period in their home cage. Notably, 16p11.2$^{dp/+}$ mice engaged significantly more in self-grooming, an indicative repetitive behavior (Figures 1G,H). Given that individuals with ASD often exhibit certain anxiety-like behaviors, we sought to test whether 16p11.2$^{dp/+}$ mice display similar behaviors. We measured motor and anxiety-like behavior in mice through open field tests (Figures 1I–K) and an elevated plus maze (Supplementary Figure S1). However, these

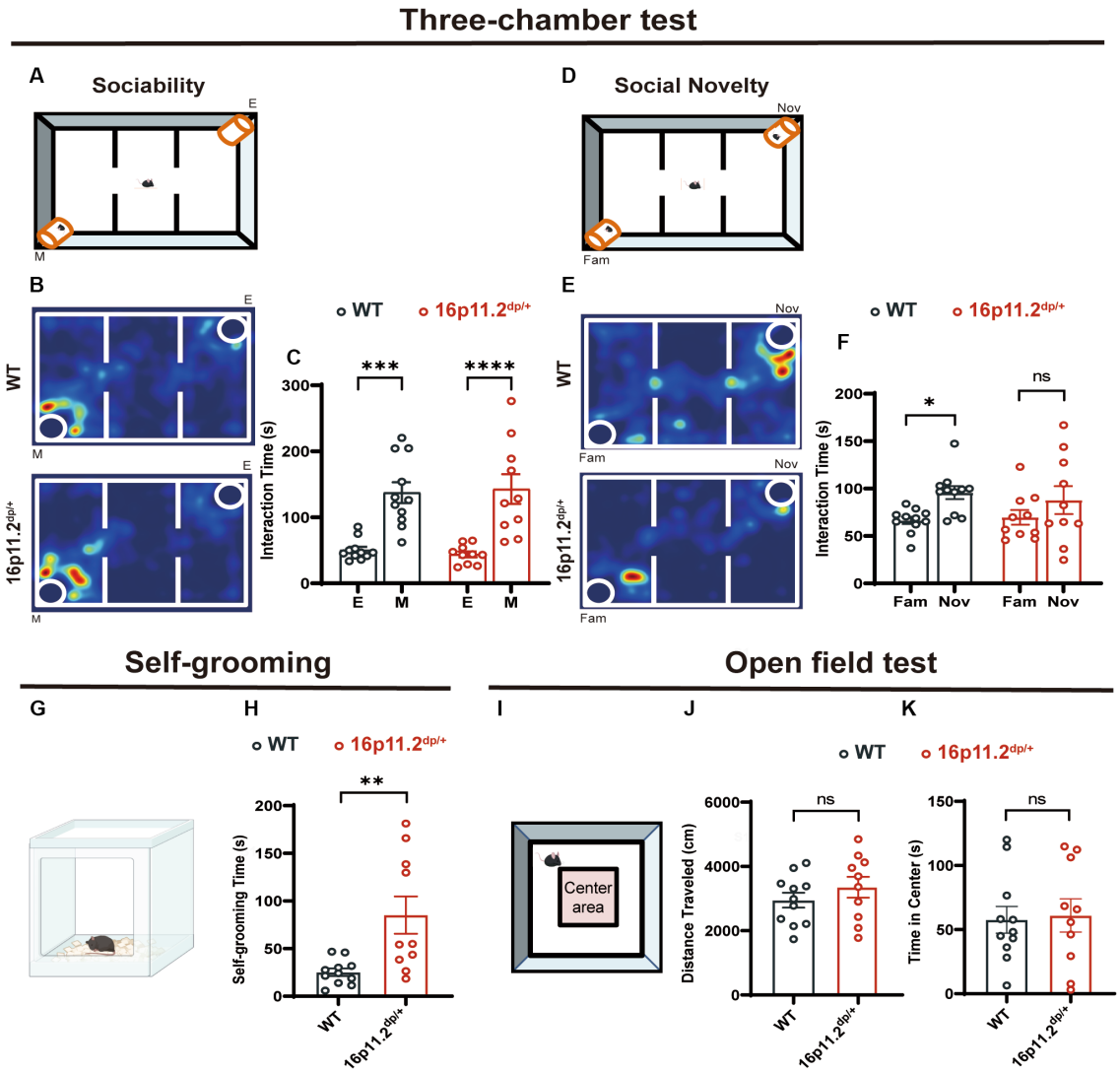

FIGURE 1
16p11.2$^{dp/+}$ mice showed social novelty deficits and repetitive behaviors. **(A,D)** Illustrations of the three-chamber test (E: empty cage; M: stranger mouse; Fam: Familiar mouse; Nov: novel stranger mouse). **(B)** Representational heat maps during the sociability phase. **(C)** The duration spent interacting with a mouse (social) and an empty cage (non-social) during 10 min. **(E)** Representational heat maps during the social novelty phase. **(F)** 16p11.2$^{dp/+}$ mice displayed reduced preference for the novel social mouse compared to WT mice. **(G)** Illustration of the mouse self-grooming experiment. **(H)** Self-grooming time in 16p11.2$^{dp/+}$ mice was significantly increased. **(I)** Diagram representing the open field test. **(J,K)** No significant differences were observed in the distance traveled and time spent in the central area between 16p11.2$^{dp/+}$ mice and WT mice. Statistical significance is indicated as follows: Data is presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.001$, ns: not significant, WT: n = 11; 16p11.2$^{dp/+}$: n = 10.

experiments did not reveal any significant anxiety-like behavior in 16p11.2$^{dp/+}$ mice. In summary, our findings indicate that 16p11.2$^{dp/+}$ mice display deficits in social novelty recognition and engage in stereotyped repetitive behaviors reminiscent of ASD.

Altered gut microbiota in 16p11.2$^{dp/+}$ mice compared to WT mice

To unearth possible gut microbiota distinctions in 16p11.2$^{dp/+}$ mice versus their WT counterparts, potentially underpinning the observed behavioral defects, we conducted 16S rRNA sequencing. A total of 919 amplicon sequence variants (ASVs) were identified, with 915 remaining after the removal of 4 that were unassigned to bacteria. As depicted in Figure 2A, the Chao1 index and Shannon index were significantly lower in the 16p11.2$^{dp/+}$ group compared to the WT group. The same trend was also observed in other α-diversity estimation methods, including ACE index (Supplementary Figure S2A), and richness (Supplementary Figure S2B). The results suggest that the 16p11.2$^{dp/+}$ mice exhibited lower biodiversity and species abundance in comparison to the WT mice. A PCoA was conducted to assess the degree of similarity between microbial communities in the two groups using Bray–Curtis distance metrics (Figure 2B). The analysis revealed that the microbiota composition of the 16p11.2$^{dp/+}$ group exhibited significant distinctions from that of the WT group. We performed a comprehensive analysis of bacterial composition at multiple taxonomic levels. The 16p11.2$^{dp/+}$ mice and WT mice displayed different microbial profiles at both the phylum and genus levels (Supplementary Figures S2D,E). Notably, compared to the WT group, the 16p11.2$^{dp/+}$ group was characterized by higher *Bacteroidetes* levels and a decreasing trend in the *Firmicutes*/*Bacteroidetes* ratio, although this trend did not reach statistical significance (Supplementary Figure S2C).

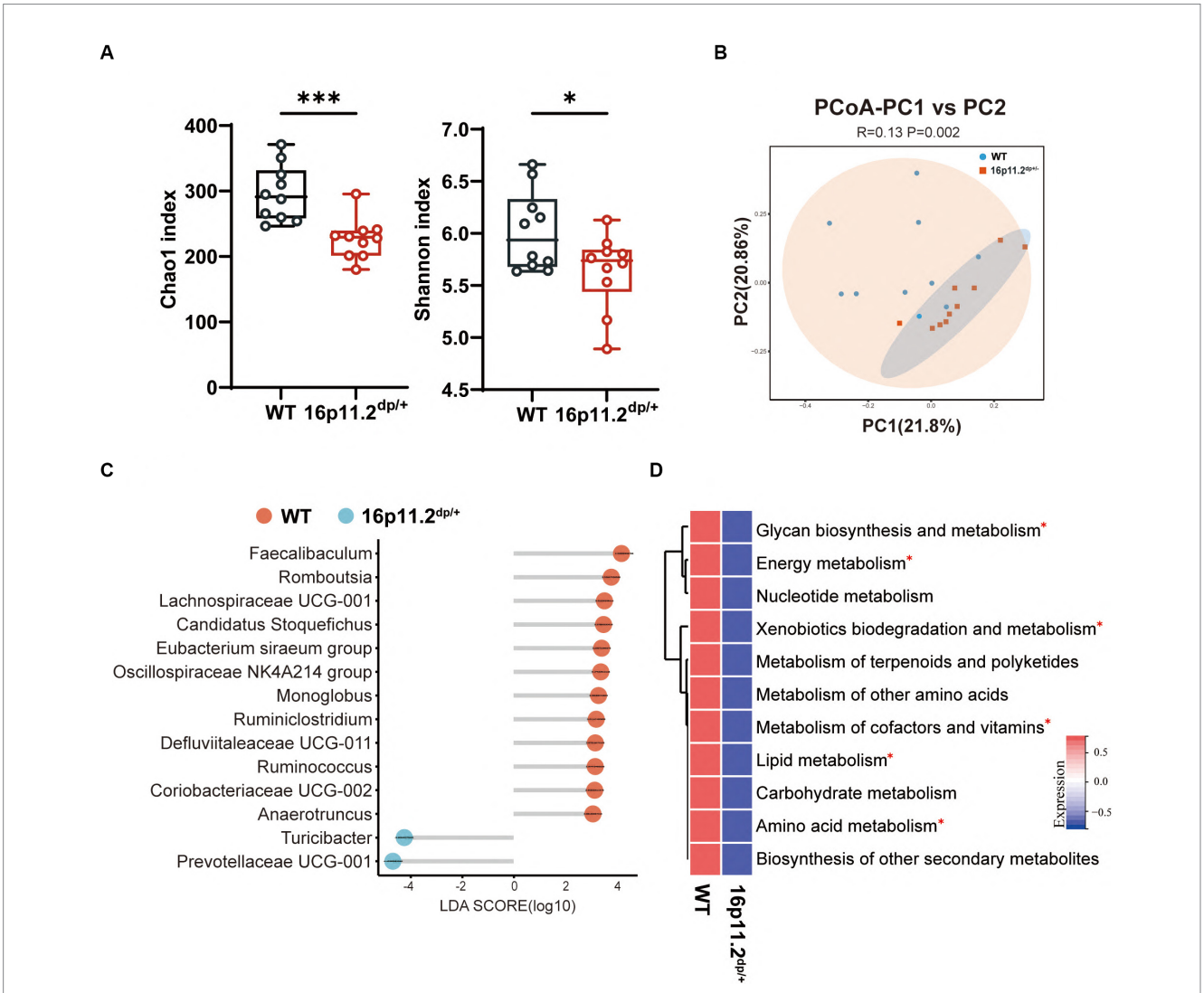

FIGURE 2
Analysis of microbial composition and functional implication in 16p11.2$^{dp/+}$ mice versus WT mice. **(A)** Alpha diversity metrics, including Chao1 and Shannon indices, were calculated using fecal samples collected from 16p11.2$^{dp/+}$ mice and WT mice. t-test, *p<0.05, ***p<0.001. **(B)** Beta diversity was assessed through a principal coordinates analysis (PCoA) plot comparing the 16p11.2$^{dp/+}$ and WT groups. PERMANOVA was used, p<0.05. **(C)** Linear Discriminant Analysis (LDA) scores for differentially abundant bacterial taxa at the genus level between 16p11.2$^{dp/+}$ and WT groups (LDA > 2, p<0.05, FDR<0.05). Red bars represent taxa enriched in WT group, while green bars represent taxa enriched in 16p11.2$^{dp/+}$ group. **(D)** Average abundance of KEGG pathways that were differentially enriched in 16p11.2$^{dp/+}$ and WT mice at the level 2. Significance indicated by t-test, *FDR < 0.05. All experimental sections are based on analyses of 10 mice per group.

Next, we employed the LEfSe method to examine genus-level differences in gut microbiota between two groups of mice (LDA score > 2.0, $p < 0.05$). The lollipop plot unveiled a noteworthy alteration in the microbiota of 16p11.2$^{dp/+}$ mice, marked by heightened levels of *Turicibacter* and *Prevotellaceae UCG_001*, and diminished levels of *Faecalibaculum* and *Romboutsia* (Figure 2C).

To gain preliminary insight into the functional changes in the gut microbial community of 16p11.2$^{dp/+}$ group, we conducted a KEGG analysis using our 16S rRNA sequencing data. We began by identifying distinct KEGG orthologous markers from the differential taxa between the 16p11.2$^{dp/+}$ and WT groups. These markers, representing functional bacterial genes, indicated variations in the functional capabilities of the gut microbiota between the two groups. We then mapped these markers to their associated metabolic pathways in the KEGG database. This revealed disruptions in the 16p11.2$^{dp/+}$ group in several pathways, including amino acid metabolism, lipid metabolism, energy metabolism, glycan biosynthesis and metabolism, and xenobiotics biodegradation and metabolism. Additionally, we employed the *omixerRPM r-package (v0.3.2)* to realign KEGG orthologs with the annotation of Gut-Brain Modules (GBMs) (Lou et al., 2022). In keeping with the findings from KEGG, we identified a plethora of differential pathways from GBMs, belonging to amino acid metabolism, lipid metabolism, energy metabolism (Supplementary Figure S2F).

Taken together, the above results reveal substantial changes in the gut microbiota of 16p11.2$^{dp/+}$ mice and underscore the predictive potential for disruptions in metabolic pathways using 16S rRNA data.

Altered metabolic profile in 16p11.2$^{dp/+}$ mice compared to WT mice

Microbial metabolites can impact the host's physiology and behavior via various pathways, with some metabolites even entering the bloodstream. Hence, we explored the metabolic profile of the same samples as those used for the 16S rRNA analysis through LC/MS. The fecal samples from the 16p11.2$^{dp/+}$ and WT groups were effectively distinguished based on the results of PLS-DA and OPLS-DA (Figures 3A,B). Subsequently, our metabolite profiling identified 19 significantly altered metabolites, with histamine showing the most substantial variations (Figure 3C and Supplementary Table S1).

To infer the potential functional changes in the gut microbiota of 16p11.2$^{dp/+}$ mice, we conducted a KEGG analysis. We mapped the identified metabolites to their corresponding KEGG orthologous markers that represent the enzymes involved in the metabolic reactions. This analysis revealed significant differences in thirteen KEGG pathways between the two groups, spanning metabolism, genetic information processing, and environmental information processing. Notably, the category of metabolism exhibited the highest enrichment of significantly altered metabolites (Figure 3D).

Further, we conducted an in-depth exploration of the interrelationships of these metabolites through Spearman correlation analysis, which is visually depicted in a heatmap (Figure 3E). In summary, our metabolomics analysis unveiled 19 differentially regulated metabolites of significant relevance, and demonstrated enrichment in multiple KEGG pathways associated with metabolism.

Alteration of neurotransmitter network activity primarily liked to histamine metabolism in 16p11.2$^{dp/+}$ mice

Given the intricate interplay between gut microbiota and intestinal metabolites, both of which frequently contribute to disease pathogenesis, we performed a comprehensive combined analysis of these factors. Initially, we conducted an integrated analysis of 16S rRNA and metabolomics data at the KEGG pathway Level 2, revealing intersections in five pathways (Figure 4A). Subsequently, while analyzing the distinctive metabolites linked to these five pathways, we revealed a neurotransmitter network involving histamine, 5-HT, and dopamine. Histamine primarily serves as the central hub within this network, with metabolic connections to other neurotransmitters (Figure 4B). Notably, vitamin B6 (Ferrante and Mercogliano, 2023) and bile acids (Ku, 2014) have the capacity to boost the enzymatic activity of histidine decarboxylase (HDC), a pivotal enzyme in histamine synthesis, and their metabolic profiles exhibit notable dynamism. Further, to identify metabolites and bacteria closely linked to histamine, we conducted a correlation analysis between histamine and other significantly variant metabolites, uncovering a profound association with metabolites like Coproporphyrin III and 1-Stearoyl-rac-glycerol (Figure 4C). Moreover, we delved into the relationships between metabolites and various bacterial genera at the genus level, notably *Coriobacteriaceae UCG_002*, *Defluviitaleaceae UCG_011*, and *Oscillospiraceae NK4A214 group*, thereby mapping their roles within the microbial community (Figure 4D). In summary, the disruptions observed in histamine metabolism in 16p11.2$^{dp/+}$ mice are intricately linked with the metabolic activities of gut bacteria.

Discussion

Our research reveals alterations in the gut microbiome and microbial neurotransmitter profiles linked to ASD traits. Comparing with wild-type mice, we observed reduced gut microbial diversity and specific shifts in microbial population abundances correlated with ASD-like behaviors. Moreover, our metabolomic analysis highlighted significant changes in metabolites related to amino acid pathways, further suggesting a potential link between the gut microbiota and ASD-like behaviors. These comprehensive results underscore the gut-brain axis's role in ASD pathogenesis, offering new perspectives for future research.

Building on our findings, the 16p11.2$^{dp/+}$ mouse model further illustrates ASD-like symptoms. This specific CNV, with a notable percentage of cases occurring *de novo* (Rein and Yan, 2020), is significantly linked to ASD, schizophrenia, and intellectual disability. Behavioral assessments revealed core ASD symptoms such as social deficits and repetitive behaviors, consistent with previous studies on this model (Rein et al., 2021). Moreover, our evaluation of open arm exploration time and performance in the elevated plus maze revealed no discernible changes in 16p11.2$^{dp/+}$ mice. Previous studies have suggested that 16p11.2$^{dp/+}$ mice may demonstrate a reduction in open arm time when tested in the elevated plus maze, but this effect may vary depending on gender (Bristow et al., 2020). Our study highlights the typical ASD-related features exhibited by the 16p11.2$^{dp/+}$ microduplication and suggests that 16p11.2$^{dp/+}$ mice could serve as a valuable model for ASD-related research.

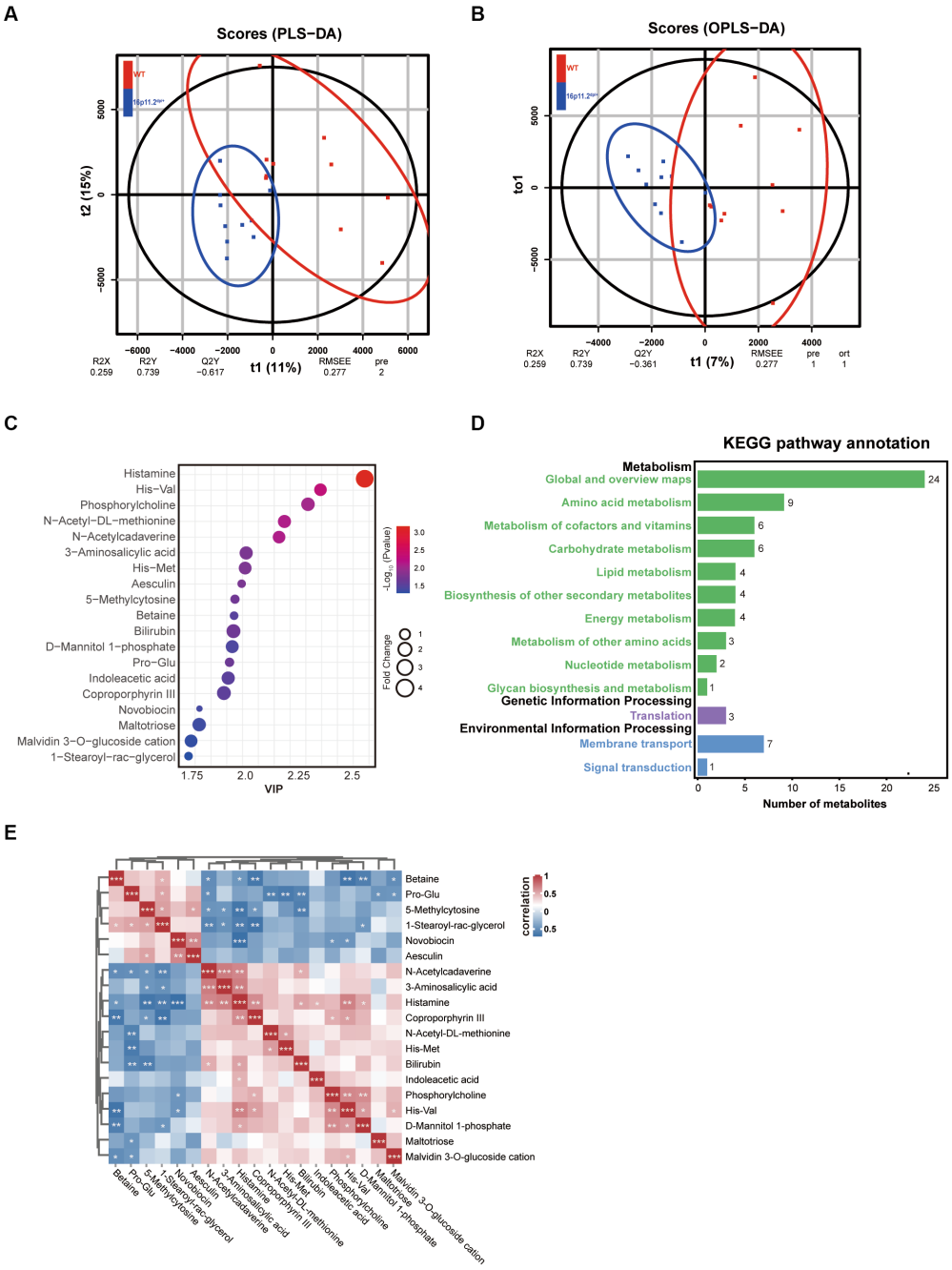

FIGURE 3
Altered gut microbial metabolic profile in 16p11.2$^{dp/+}$ mice compared to WT mice. **(A)** Clustering analysis using partial least-squares discriminant analysis (PLS-DA). **(B)** Clustering analysis using orthogonal partial least-squares discriminant analysis (OPLS-DA). **(C)** Bubble chart representing 19 significantly different metabolites between 16p11.2$^{dp/+}$ and WT groups. Metabolites with >1.5-fold changes, VIP ≥ 1, and $p < 0.05$ (t-test) are displayed. **(D)** Thirteen KEGG pathways exhibited significant differences between 16p11.2$^{dp/+}$ and WT groups. **(E)** Spearman's correlation analysis of significantly different metabolites, with red indicating a positive correlation and blue indicating a negative correlation. The intensity of color reflects the strength of the correlation. Statistical significance is represented as follows: Data is presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant, n = 10 per group.

To delve into the gut microbial implications under the overarching brain-related dysfunction, we employed 16S rRNA sequencing to investigate alterations in the gut microbiota composition of 16p11.2$^{dp/+}$ mice. Utilizing α-diversity metrics like observed species richness, Chao1, ACE, and Shannon indices, alongside β-diversity analysis through PCoA, we observed a biodiversity decrease in the 16p11.2$^{dp/+}$ mice, aligning with prior ASD studies (Gilbert et al., 2013; Golubeva et al., 2017; Kang et al., 2017; Lim et al., 2017; Kang et al., 2018; Sharon et al., 2019; Wang et al., 2019). Our analysis of phylum-level composition, particularly the *Firmicutes* and *Bacteroidetes*, showed variations that reflect the complex nature of gut microbiota dynamics observed in other studies, without specifying distinct trends (Tomova

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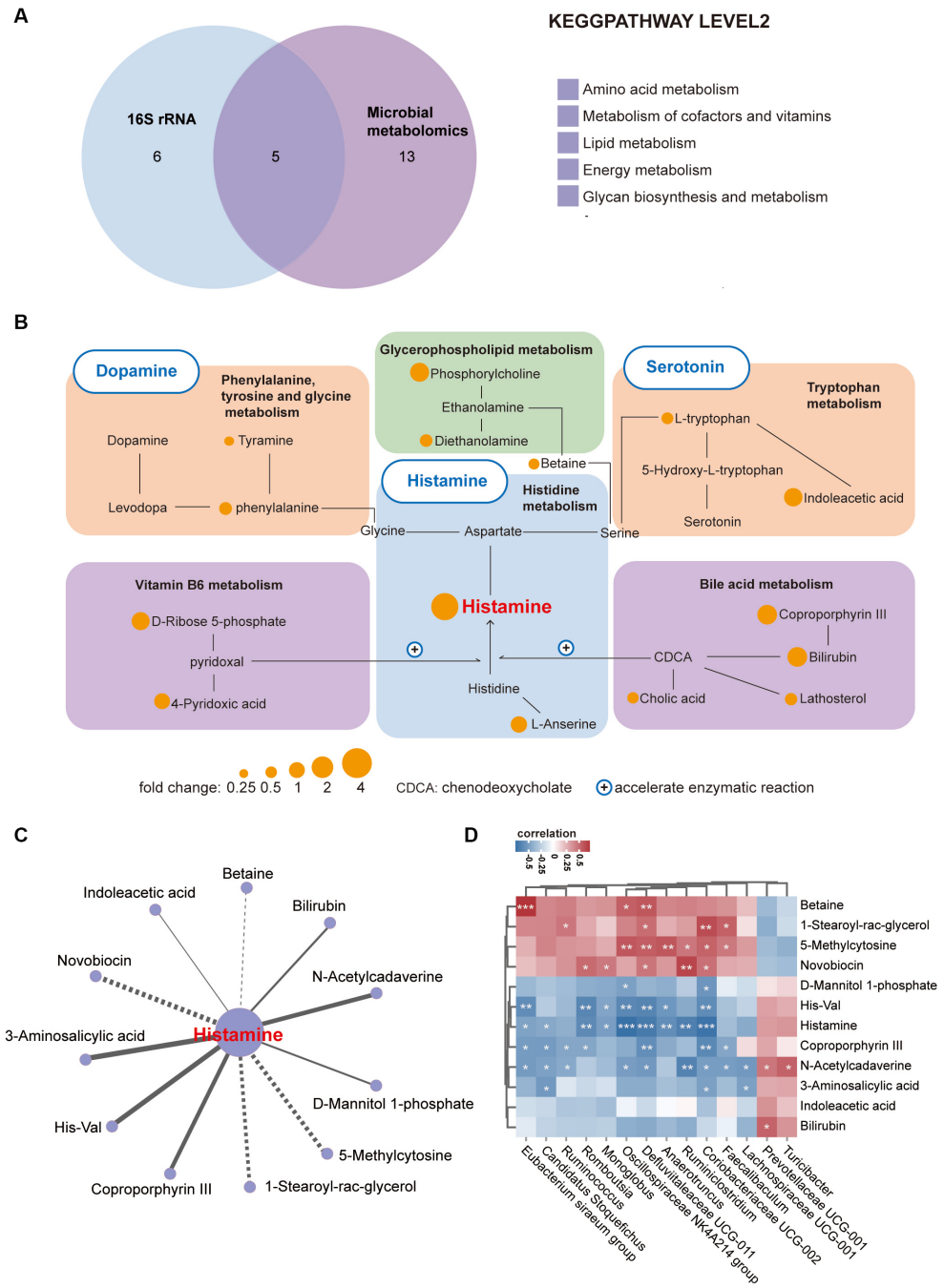

FIGURE 4
Enrichment of a neurotransmitter network centered on histamine in 16p11.2$^{dp/+}$ mice. **(A)** Venn diagram illustrates the number of altered KEGG pathways shared between 16p11.2$^{dp/+}$ and WT mice at the level 2. **(B)** Differentially expressed metabolites associated with the neurotransmitter network between the 16p11.2$^{dp/+}$ and WT groups (Fold change >1.24, VIP ≥ 1, $p < 0.1$, t-test). **(C)** Network analysis linking histamine with significant differential metabolites. Lines connecting metabolites indicate the direction of their association with each genus of microbe, represented by solid lines (positive) or dotted lines (negative) ($|r| > 0.4$, $p < 0.05$). **(D)** Spearman's correlation between differentially abundant species at the genus level, and metabolites associated with histamine metabolism. The correlation effect is indicated by a color gradient from blue (negative correlation) to red (positive correlation). Statistical significance is indicated as follows: Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, assessed using the t-test. n = 10 per group.

et al., 2015; Strati et al., 2017; Zhang et al., 2018). At the genus level, we observed an increase in *Turicibacter* and *Prevotellaceae UCG_001*, and a decrease in *Faecalibaculum* and *Romboutsia*. These observations align with prior studies of fecal microbiome analyses in autistic children, albeit with some nuances. For example, *Turicibacter* has been implicated in ASD-related behaviors, as demonstrated by symptom improvement in VPA mouse models following fecal microbiota transplantation (Wang J. et al., 2023). Additionally, research has shown an inverse correlation between *Turicibacter* abundance and sex hormones, potentially offering insights into the prevalence of ASD in

females (Wu et al., 2022). In a high-fat diet-induced ASD mouse model, metformin was found to enhance *Faecalibacterium*, leading to a positive correlation with sociability and components of the 5-HT pathway (Deng et al., 2022a). *Prevotellaceae UCG_001* and *Faecalibaculum* are known to be associated with the presence of short-chain fatty acids (Kang et al., 2023), which have been implicated in the development of ASD (Mac Fabe, 2015). Given the limitations of 16S rRNA sequencing in fully characterizing the microbial community, we plan to conduct metagenomic analysis in the future to analyze at various taxonomic levels (such as species, genus, and phylum). This effort aims to identify specific species involved in the regulation of ASD-like behavior, thereby enhancing our understanding of the microbiome's impact on ASD, and expanding upon the insights gained from our current research findings.

The potential influence of gut microbiota on ASD through the modulation of intestinal metabolites presents an intriguing area of investigation. In this study, we conducted a comprehensive analysis of fecal metabolites using LC/MS, identifying 19 metabolites with significant differences, notably histamine. These metabolites have diverse functions related to metabolism, genetic information processing, and environmental information processing. Remarkably, the most enriched pathway among these metabolites is amino acid metabolism, as indicated by KEGG analysis. We established the functional links between gut microbiota and metabolites in 16p11.2$^{dp/+}$ mice. Through integrating KEGG analysis of 16S rRNA and metabolomics data, we unveiled an altered neurotransmitter network in 16p11.2$^{dp/+}$ mice. This finding is in keeping with emerging evidence suggesting that children with ASD often exhibit irregular neurotransmitter levels, including histamine, serotonin, dopamine, and gamma-aminobutyric acid (GABA) (Montgomery et al., 2018; Strandwitz, 2018; De Palma et al., 2022; Montanari et al., 2022), known to be influenced by gut bacteria (Dinan and Cryan, 2017; Chen et al., 2021; Dicks, 2022). Our study revealed disruptions in the neurotransmitter network, with histamine being the most significantly altered core neurotransmitter among them.

Histamine, a pivotal neurotransmitter, has been implicated in neurological disorders (Nuutinen and Panula, 2010; Scammell et al., 2019), including ASD (Fernandez et al., 2012; Wright et al., 2017). Previous research has highlighted elevated blood histamine levels in children diagnosed with ASD (Rashaid et al., 2022). The effects of histamine primarily occur through its interaction with various histamine receptors, notably H1R, H2R, H3R, and H4R. Extensive literature suggests that targeting H2R in the brains of individuals with ASD and H3R in mouse brains shows promise in alleviating ASD symptoms (Linday, 1997; Linday et al., 2001; Eissa et al., 2020; Venkatachalam et al., 2021). Furthermore, histamine plays a role in enhancing the transmission of other neurotransmitters, including serotonin, norepinephrine, and dopamine in the brain (Flik et al., 2015). Histamine within the intestinal lumen has the potential to be absorbed into the bloodstream (Tidmarsh, 1932), where it can serve multiple functions, including the regulation of neurological disorders. Additionally, histamine is associated with various gastrointestinal disorders, such as irritable bowel syndrome (Barbara et al., 2004) and inflammatory bowel diseases (Dvornikova et al., 2023). Gut histamine can stimulates gastric acid secretion via H2R, induce abdominal discomfort through H4R activation (De Palma et al., 2022), and enhance intestinal motility (Mochizuki, 1953), leading to diarrhea (Smolinska et al., 2022), a common clinical symptom in ASD.

The level of histamine in the intestinal tract is maintained through a dynamic equilibrium that involves its synthesis, degradation, absorption, and secretion processes. Gut bacteria can influence histamine levels in feces by regulating histamine secretion (Sanchez-Perez et al., 2022), expediting synthesis process via HDC (Lucas et al., 2008) and promoting degradation through histamine N-methyltransferase (HNMT) and diamine oxidase (DAO) (Shi et al., 2022). Our analysis of the correlation between histamine and bacterial species revealed an array of bacteria distinct from those previously reported to influence histamine, either through production [e.g., *Lactobacillus*, *Enterococcus*, *Klebsiella* (Krell et al., 2021; De Palma et al., 2022)] or by affecting histamine levels [e.g., *Pseudomonas aeruginosa*, *Escherichia coli* (Fiorani et al., 2023)]. Therefore, we postulate that the augmented levels of histamine in the intestinal cavity of 16p11.2$^{dp/+}$ mice are less likely to be significantly influenced by those known histamine-influencing microbiomes, but more likely attributed to host synthesis and/or secretion. Additionally, our GBMs analysis enabled the identification of histamine degradation pathways within samples from both groups under study. However, due to the insufficient sequencing depth of 16S rRNA, as well as the still-evolving GBMs, precision of the analysis was compromised. Even though the statistical values of histamine degradation pathways in the 16p11.2$^{dp/+}$ group showed a noticeable decline compared to the WT group, it did not culminate in a statistically significant difference (Supplementary Table S2). In this send, it is possible that altered histamine metabolism might influenced the abundance of some, if not all, bacteria identified in our study. Intriguingly, the levels of these bacteria were inextricably linked to a plethora of bioclinical implications, including autistic symptom (Ye et al., 2021; Deng et al., 2022b), intestinal inflammation (Gu et al., 2022), gut neurotransmitter production (Deng et al., 2022b; Wang Z. J. et al., 2023), and bile acid metabolism (Jian et al., 2022).

Based on these findings, we propose that the 16p11.2 CNV is closely associated with which are manifested as increased intestinal histamine levels, imbalances in gut microbiota and metabolites, potentially contributing to the development of ASD. Emerging evidence indicates that the genes MAPK3 and MAZ, located in the 16p11.2 region, are implicated in the control of degranulation in intestinal mast cells, leading to histamine release (Bilotta et al., 2021; Gou et al., 2023). Elevated histamine levels in the lumen have the potential to induce intestinal inflammation by triggering mast cell activation (De Palma et al., 2022), potentially resulting in its degranulation. Future research warrants the investigation on (1) the potential morphological and molecular changes of mast cells resulting from defects in 16p11.2-associated genes; (2) the impact of these changes on specific gut microbiomes and metabolism; (3) the causal mechanisms linking histamine to 16p11.2-driven autistic behaviors; and (4) adopting metagenomic sequencing for future material reanalysis and annotation.

Conclusion

In conclusion, our investigation has unveiled the manifestation of gut dysbiosis in 16p11.2$^{dp/+}$ mice across diverse taxonomic levels.

Notably, these mice exhibit diminished microbial diversity and a perturbed structure within their fecal microbiota. Furthermore, our integrated analysis of gut microbiota and metabolites has elucidated the aberrant feature of a predominant neurotransmitter network primarily centered around histamine. These findings provide novel insights into the pathogenesis and potential therapeutic avenues for 16p11.2 CNV-associated ASD, with a particular focus on targeting gut neurotransmitter metabolism.

Data availability statement

The raw data of the 16S rRNAseq is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1056406. The microbial metabolomics data presented in the study are included in the Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The experimental procedures involving animals were all approved by the Institutional Animal Care and Use Committee at the Southern University of Science and Technology (SUSTC-2019-174). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZF: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. XY: Conceptualization, Data curation, Writing – review & editing. YJ: Conceptualization, Formal analysis, Writing – review & editing. XinlM: Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing. HuaL: Methodology, Software, Visualization, Writing – review & editing. YY: Methodology, Software, Visualization, Writing – review & editing. JC: Methodology, Software, Visualization, Writing – review & editing. ZC: Methodology, Software, Visualization, Writing – review & editing. HuiL: Data curation, Project administration, Writing – review & editing. XZ: Data curation, Project administration, Writing – review & editing. XinjM: Supervision, Validation, Writing – review & editing. NL: Supervision, Validation, Writing – review & editing, Funding acquisition. DW: Supervision, Validation, Writing – review & editing. JJ: Supervision, Validation, Writing – review & editing.

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Conflict of interest

XinlM and HuaL were employed by Guangdong Perfect Life Health Science and Technology Research Institute Co., Ltd.

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1331130/full#supplementary-material

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Electroacupuncture regulates gut microbiota to reduce depressive-like behavior in rats

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Background and objectives: Growing studies show that gut microbiota is closely associated with depression. Acupuncture treatment could regulate the gut microbiota of many diseases. Here, we aim to observe the effect of electroacupuncture (EA) on gut microbiota in rats that showed depressive-like behavior.

Materials and methods: The rats were randomly divided into normal group, chronic unpredictable mild stress model (CUMS) group, CUMS+electroacupuncture (EA) group, and CUMS+sham-electroacupuncture (Sham) group. The CUMS+EA rats were treated with EA stimulation at bilateral Zusanli (ST36) and Tianshu (ST25) acupoints for 2weeks (0.7mA, 2/100Hz, 30min/day). The rats in the sham EA group were treated with the same conditions without inserting needles and electrical stimulation. Behavioral tests were conducted by forced swimming test (FST), open field test (OFT), and sucrose preference test (SPT) to assess depression-like behavior in rats. The relative abundance of intestinal bacteria in rat feces was detected by 16S rRNA analysis. The expression of calcitonin-gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), somatostatin (SST), and adrenocorticotropic hormone (ACTH) in serum was detected by ELISA kit, and VIP, CGRP, and SST in the colon were detected by qRT-PCR and Western blot.

Results: Chronic unpredictable mild stress model rats exhibited depressive-like behaviors and had differential abundance vs. control rats. CUMS significantly decreased the relative abundance of Bifidobacterium and Streptococcus at the genus level, CGRP in plasma ($p < 0.05$), and significantly increased the intestine propulsion rate, the mRNA and protein expression of VIP, SST, and mRNA in the colon, and ATCH in plasma ($p < 0.05$). EA rats with microbial profiles were distinct from CUMS rats. EA markedly reduced the depressive-like behaviors, significantly increased the intestine propulsion rate, the relative abundance of Bacteroidetes, Proteobacteria, and Actinobacteria at the phylum level, Bifidobacterium and Streptococcus at the genus level, and VIP and CGRP in plasma ($p < 0.05$), and significantly decreased Firmicutes, the ratio of Firmicutes to Bacteroidetes at the phylum level, ACTH and SST in plasma, and SST mRNA in the colon ($p < 0.05$).

Conclusion: The antidepressant effect of EA at ST36 and ST25 is related to regulating intestinal flora and the neurotransmitter system. Our study suggests that EA contributes to the improvement of depression, and gut microbiota may be one of the mechanisms of EA effect.

KEYWORDS

electroacupuncture, depression, gut microbiota, neurotransmitter, rats

1 Introduction

Depression is a common mental illness worldwide that affects more than 300 million people of all ages, according to the World Health Organization (WHO) (GBD 2017, 2018). Antidepressant medication as tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) has also produced significant side effects that patients do not tolerate (Malhi and Mann, 2018). Therefore, antidepressant non-pharmacological therapy has gotten more and more attention.

Acupuncture, as a traditional Chinese Medicine, has a good therapeutic effect on treating depression for thousands of years. Accumulating evidence also showed that acupuncture or electroacupuncture (EA) could be a non-pharmacological therapy for treating different kinds of depression, such as depression-related insomnia (Dong et al., 2017), postpartum depression (Li et al., 2018), depression in methamphetamine (MA) addicts during abstinence, and promote rehabilitation of patients (Zeng et al., 2018), post-stroke depression (PSD) (Zhang et al., 2019), and perimenopausal depression (Xiao et al., 2019). Acupuncture combined with antidepressant medications shows a better therapeutic effect than SSRI therapy alone (Chan et al., 2015). A meta-analysis showed that acupuncture may decrease Hamilton Rating Scale (HAMD) for Depression. At the same time, no significant effects on clinical response, Edinburgh Postnatal Depression Scale (EPDS), and serum estradiol levels were observed (Li et al., 2019). However, the underlying mechanism of effect of acupuncture for depression is not precise.

A growing body of research evidence suggests that depression has strongly associated with gut microbiome disorder (Winter et al., 2018). The microbiota-depleted rats that received the fecal microbiota from the depressed patients showed depressive-like behavior and physiological feature characteristic (Kelly et al., 2016). The bidirectional regulation between the brain and the gut microbiome mediates by the enteric nervous system–vagus nerve–central nervous system (Carabotti et al., 2015), the hypothalamic–pituitary–adrenal (HPA) axis (Misiak et al., 2020), Neuroendocrine-Immune (Liu et al., 2020), and neuroendocrine (Zalar et al., 2018). There are many ways of brain–gut interaction: probiotics may provide a useful novel treatment method for neuropathological disorders and/or as an adjunct treatment for psychiatric disorders (Vlainić et al., 2016; Paiva et al., 2020).

The emerging field of research focused on the development of preventive and therapeutic interventions for depression targeting the gut microbiota (Dash et al., 2015). Psychobiotics containing a variety of Lactobacillus and Bifidobacterium have been shown to reduce anxiety, improve mood, and enhance cognitive function in both healthy people and patient populations (Butler et al., 2019). *Bifidobacterium adolescentis* NK98 and *Lactobacillus reuteri* NK33 may relieve colitis and depression by improving intestinal microecological dysbiosis (Han et al., 2020). *Bifidobacterium breve* CCFM1025 also showed significant antidepressant like effect in mice (Tian et al., 2020). Another study demonstrated that probiotics improve mental flexibility and change gut microbiota at the same time in healthy elderly (Kim et al., 2021). The above data suggest that some prebiotics have a positive effect on the central nervous system (CNS) and may therefore play an important role in regulating depression.

The ancient classic "Lingshu meridians" clearly points out that the stomach meridian of Foot-Yangming could treat mental diseases. The study on the regularity of acupoint selection and the compatibility in the treatment of postpartum depression showed that the main acupoints were selected including Zusanli (ST36) (Zheng et al., 2023), but the mechanism is unclear. The aim of present study was to observe whether the antidepressant effect of EA was regulated by intestinal flora, so we selected the acupoints on the stomach meridian of Foot-Yangming. ST36 and Tianshu (ST 25) have been proven to regulate gut microbiota. Mild moxibustion at bilateral ST 25 and ST36 significantly increased the relative DNA abundances of Bifidobacterium and Lactobacillus but decreased that of *Escherichia coli* in rats with irritable bowel syndrome (IBS) (Bao et al., 2019). Mild moxibustion at the bilateral Shangjuxu (ST37) and ST25 significantly increased alpha diversity, downregulated the abundance of Bacteroides and Prevotella, and upregulated the abundance of Lactobacillus for the irritable bowel syndrome (IBS) rats (Wang et al., 2018). ST 25 and ST36 are on the stomach meridian of Foot-Yangming, which mainly treats gastrointestinal and mental disorders. Therefore, in the present study, ST36 that can be used as an anti-depression acupoint on the stomach channel of Zyangming and ST 25, a common point that can regulate intestinal flora, were selected to observe the regulatory effect of EA on the intestinal tract in depressed rats induced by chronic unpredictable mild stress (CUMS). It was speculated that the gut microbiota may play an important role in the process of the antidepressant effect of EA on depressed rats.

2 Materials and methods

2.1 Animal preparation

All protocols were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences (D2019-02-11-2).

Male Sprague–Dawley rats (180–200 g, $n = 24$) were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences [license number: SCXK (Jing) 2014–0013]. All rats were subjected to a 12–12-h light–dark cycle with controlled temperature (22 ± 2°C) and humidity environment (50%), allowing them to freely get food and water. The health condition of animals was inspected daily. After 1 week of adaptation, the rats were randomly divided into four groups ($n = 6$/group): normal, chronic unpredictable mild stress model (CUMS, model), CUMS + electroacupuncture (EA), and CUMS + sham-electroacupuncture (Sham EA). Behavioral tests, including forced swimming test (FST), open field test (OFT), and sucrose preference test (SPT), were administered before and after 28 days of the CUMS procedure and after 2 weeks of EA treatment (Figure 1A).

2.2 The chronic unpredictable mild stress procedure

The CUMS model in the present experiment was a validated model of depression and adapted from other researchers (Zhang et al.,

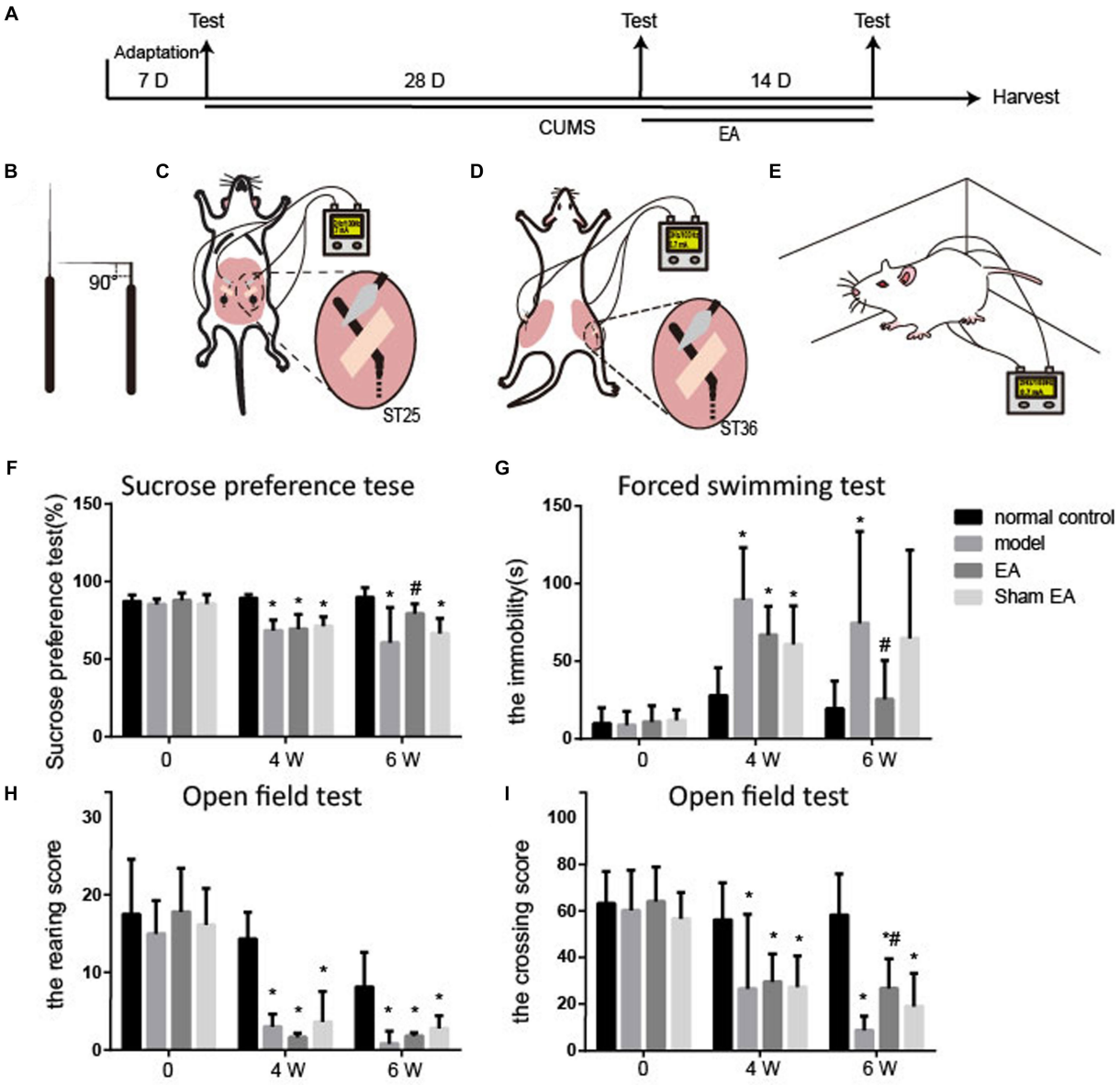

FIGURE 1
EA reduced depressive-like behaviors in CUMS rats (n = 6). **(A)** The experimental procedures of CUMS, EA treatment, and behavioral measurements. **(B–E)** Schematic drawing of the process of EA treatment. **(B)** The acupuncture was fold. **(C)** The bent acupuncture needle was inserted and fixed at the location of ST25. **(D)** The bent acupuncture needle was inserted and fixed at the location of st36. **(E)** The free moving rats during the EA treatment. **(F)** Sucrose preference test. **(G)** Forced swimming test. **(H,I)** Open field test. The effect of EA at ST 25 and ST36 on sucrose preference test, open field test, and forced swimming test in CUMS rats (mean ± SD).*$p < 0.01$, vs. the normal control group; $^{\#}p < 0.05$, vs. the model group.

2016) with a minor modification. Each animal in CUMS group and CUMS+EA and CUMS+ sham group was socially isolated in a separate cage and administered the following different stressors for 42 days: swimming in ice water for 10 min (at 0°C); food deprivation for 24 h; electric foot shock for 30 times (2 mA for 10 s every 50 s); clipping tails for 1 min (1 cm from the base of the tail); water deprivation for 24 h; housing in a wet padding for 24 h (200 mL of water per cage); 45°cage tilted for 14 h; light–dark cycle reversal for 24 h; and physical restraint for 3 h. Rats received one of these stimulus each day, and the same stimulus was not applied consecutively over 2 days. The normal group is not disturbed, except for necessary procedures such as regular cage cleaning.

2.3 EA treatment

The rats from the EA group underwent EA stimulation at bilateral ST36 and ST25 acupoints. The hair of the location of acupoints of ST36 and ST25 was removed off for the fixation of acupuncture needles. The sterile acupuncture needles (0.3 mm × 13.0 mm, Suzhou Medical Appliance Factory, Jiangsu, China) at 5 mm were fold into 90° (Figure 1B) and inserted into the ST36 and ST25 acupoints at the depth of 5 mm, according to the location of each acupoint. The upper bent part of the sterile needle handles were fixed onto the surface of the rats' skin with an adhesive tape (Figures 1C,D). The needle handles were connected with an electrical stimulator (Hanshi-100A; Nanjing

Jisheng Medical Technology Company, Nanjing, China) and stimulated at an alternating frequency of 2/100 Hz for 30 min and an intensity of 0.7 mA each day for 2 weeks, while the rats were awake and free moving in a cage (Figure 1E). The rats were observed during the process of entire EA treatment. The acupuncture needle rarely falls off under this situation. However, if the acupuncture needle does fall off during the process of electroacupuncture treatment, the acupuncture needle would be re-inserted into the acupoints and re-fix with adhesive tape again to ensure EA treatment for 30 min of each experimental animal. The rats from the sham EA group were given the same conditions without a needle inserted and electrical stimulation.

ST36 is located approximately 5 mm below the fibular and on the posterolateral side of the hind limb knee joint (Li, 2007); ST25 is located on 5 mm lateral to the intersection of upper 2/3 and the lower 1/3, connecting the xiphoid process with the upper edge of the pubic symphysis (Yu et al., 2016).

2.4 Assessment of depressive-like behavior

For all behavioral tests, rats were transported in their home cages to the testing room at least 1 day before testing to get acclimated to the testing environment. A video tracking system was used to record the behavior of the rats. The experimenters were blinded to group identity during the experiment and quantitative analyses.

2.5 Sucrose preference test

Before the SPT, the rats were trained to drink sucrose solution (1%) for 24 h and then were given one bottle of 1% sucrose solution and one bottle of pure water for another 24 h. Then, the rats were deprived of water for 24 h, following 1-h test that the each rat was given a bottle of 1% sucrose solution and a bottle of pure water. The two bottles were weighed and recorded before and after the testing. Sucrose preference was calculated by the following formula: sucrose preference (%) = sucrose intake/(sucrose intake + water intake) × 100%.

2.6 Open field test

The open field test (OFT) measured the rats' exploratory behavior and general activity. The rats were placed in the center of the bottom of the apparatus (100 cm × 100 cm × 40 cm black chamber, with 25 equal-size squares marked by white lines) and freely explore the surrounding for 3 min. The numbers of crossing (squares crossed by limb) and rearing (hind limb standing times) were counted during the 3-min period. Before each test, the inner wall and bottom surface of the apparatus were wiped with 75% alcohol to avoid any residual information from the previous rats.

2.7 Forced swimming test

Forced swimming test is one of the most commonly used assays for evaluating the depressive-like behavior in small animals such as rodents to reflect behavioral despair (Yankelevitch-Yahav et al., 2015). Rats were placed in a pressure environment filled with water (a cylinder container with 45 cm height, 25 cm diameter) at 23 ± 1°C and forced to swim for 15 min. After 24 h, the rats were placed in the same water for 5 min. The swimming, struggling, and immobility time were recorded.

2.8 Gastric emptying test

After fasting for 24 h, the rats were gavaged and fed with a liquid test meal (2 mL) that contained a non-absorbable marker phenolic red (50 mg/dL). The rats were deep anesthesia with pentobarbital sodium (35 mg/kg, i.p.) and then sacrificed. After ligating the cardia and pylorus, the stomach was removed, and gastric contents were rinsed with distilled water. The distilled water was added to 20 mL into the gastric solution, and 20 mL of 0.5 mol/L NAOH was added and mixed. After 1 h, 5 mL supernatant was collected and added 0.5 mL trichloroacetic acid (20%). The mixed liquid was centrifuged for 10 min with a radius of 3,500 r/min; then, the absorbance value (OD) was detected at 560 nm. The standard OD of phenol red was generated with 20 mL phenolic red (50 mg/dL) at 560 nm. Gastric emptying rate (%) was calculated as (1 − measured phenol red OD/standard phenol red OD) × 100%.

2.9 Detection of small intestine propulsion rate

After the rats were sacrificed, the whole small intestine was removed and straightly put on ice to observe the length of the small intestine, dyed red with phenol red. If the end of the dyed red of the small intestine becomes purple after dropping a small amount of 0.5 mol/L NAOH solution, it is where phenol red arrives. Then, a small amount of NAOH solution was added before and after the purplish red area to determine the farthest location of phenol red reached. The distances from the pylorus to ileocecal length (A) and from the pylorus to phenol red stained red end (a) were measured. The small intestine propulsion rate was (%) = a/A × 100%.

2.10 Sample collection

Blood and colon were collected after the rats were sacrificed. The serum was centrifuged and separated at 3,000 rpm for 10 min at 4°C and then stored at −20°C. Colon and hypothalamus samples were collected immediately and stored under −80°C.

2.11 16S rRNA analysis of fecal samples

The fecal collected samples were stored into 1.5 mL tubes under −80°C. The total DNA of fecal samples was extracted using the E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Inc., United States). The V3-V4 region of the 16S rRNA gene of bacteria was amplified from the fecal DNA extracts by modified universal bacterial primer pairs 515F (5'-TCGTCG GCAGCGTCAGATG TGTATAAGAGACAGGT GCCAGCMGCCGCGGTAA-3′) and 806R (5'-GTCTCGTGGGCT CGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAA T-3′) with Illumina adaptor overhang sequences. The amplification

TABLE 1 Primer sequences.

Primer	Sequence(5′–3′)	Length (bp)
Sst forward	CGAGCCCAACCAGACAGAG	225
Sst reverse	TTTGGAGGAGAGGGATCAGAG	
VIP forward	TGATGTGTCCAGAAATGCCA	123
VIP reverse	CTACTGCTGATTCGTTTGCC	
GAPDH forward	CCTTCCGTGTTCCTACCCC	131
GAPDH reverse	GCCCAGGATGCCCTTTAGTG	

program consisted of one pre-denaturation cycle for 5 min at 95°C, 28 denaturation cycles for 45 s at 95°C, annealing for the 50s at 55°C, extension for 45 s at 72°C, and finally 1 extension cycle for 10 min at 72°C. After that, the PCR products were purified by Agencourt AMPure XP (Beckman Coulter, Inc., United States) and sequenced using an Illumina MiSeq sequencing platform (Illumina, San Diego, CA, United States).

Use QIIME (v1.8.0) software to divide the data into different samples. The sequences were merged and overlapped relationship using Pear (v0.9.6) software to by the overlapping relationship. Then, the sequences with lengths less than 230 bp were removed by Vsearch (v2.7.1) software, and the de-chimera sequences were compared by the uchime method according to the Gold Database. Afterward, operational taxonomic units (OTUs) clustering of high-quality sequences using the UPARSE algorithm of Vsearch (v2.7.1) sofware. The BLAST algorithm was used to compare the OTU representative sequence with the Silva138 data. According to OTU and its abundance results, the QIIME (v1.8.0) software and R (v3.6.0) software were used to calculate and plot the α-diversity index, respectively. The histogram of species composition was analyzed by R (v3.6.0) software. The β-diversity distance matrix was calculated by the QIIME (v1.8.0) software, and the PCoA and NMDS were analyzed by the R (v3.6.0) software on the basis of the distance matrix. Sequence data supporting the results of this study are available at the National Center for Biotechnology Information with the main entry code PRJNA924098.

2.12 Western blot analysis

The total protein was extracted from the tissue by protein lysate containing protease and phosphatase inhibitors (Roche, Shanghai, China) using a tissue homogenizer. An equal amount of protein in each sample was subjected to 5 or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 90/160 V for 60 min and then electro-transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, United States) at 90 mA for 150 min. The 5% bovine serum albumin (BSA, Amresco, Solon, OH, United States) solution was used to block at room temperature for 60 min. Then, the membranes were incubated with first antibody vasoactive intestinal peptide (VIP) (1: 500; ab8556, Abcam, Cambridge, United Kingdom), calcitonin-gene-related peptide (CGRP) (1:500; ab47027, Abcam, Cambridge, United Kingdom), and GAPDH (1: 20,000, TDY042, Tiandeyue Biological Technology Company, Beijing, China) overnight at 4°C. After incubated with secondary antibody [donkey anti-goat IgG 1:10,000 diluted or donkey anti-rabbit Immunoglobulin (Ig)G 1:20,000 diluted; Jackson Immuno Research Laboratories, West Grove, PA, United States] at room temperature for 1 h, the membranes were developed by an enhanced chemiluminescence (ECL) detection system. The TotalLab Quant analysis software (TotalLab Limited, Newcastle upon Tyne, United Kingdom) was used to scan and quantify the blots for densitometric analyses. The primer sequences are shown in Table 1.

2.13 Enzyme-linked immunosorbent assay

The concentrations of vasoactive intestinal peptide (VIP), CGRP, somatostatin (SS), and ACTH in serum were detected by ELISA kit (R&D Systems, United States) as the protocol provided by the manufacturer's instructions.

2.14 Quantitative real-time PCR

The total RNA was extracted from tissue with reagent (Life Technologies, Carlsbad, California) and reverse-transcribed to cDNA by Prime Script TM Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). Quantitative real-time (QRT) PCR was performed using cDNA as template by the QRT-PCR detection systems (ABI7500, Applied Biosystems, United States). The qPCR reaction system conditions were 95°C 30 s; 95°C 5 s, 60°C 10 s, and 72°C 15 s, with a total of 40 cycles. Using GAPDH as internal reference, the relative expression of mRNA of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.15 Statistical analysis

Measurement data are expressed as the mean ± SD, and SPSS17.0 software (SPSS, Chicago, IL, United States) was used for data analysis. Data between groups were compared using one-way ANOVA and the least significant difference test. LSD test was used for post-test when variance was equal, and Games-Howell test was used for post-test when variance was not equal. $p < 0.05$ indicated that the difference was statistically significant.

3 Results

3.1 EA significantly alleviated the depressive-like behaviors

As shown in Figure 1, no differences in sucrose preference, the immobility time, and the rearing or the crossing score were observed between the four groups before the CUMS procedure.

Compared with the normal control group, the rearing and crossing score, and sucrose preference were observably decreased ($p < 0.05$), and the immobility time markedly increased ($p < 0.05$, Figure 1B) in the model, EA, and sham groups after CUMS modeling for 4 weeks. It is suggested that the rats showed depressive-like behaviors after CUMS modeling.

The sucrose preference and the crossing score were upregulated in the EA group ($p < 0.05$, Figures 1D,E), and the sham group also

exhibited an increase in sucrose preference and the crossing score, but there were no significances ($p > 0.05$) after 2 weeks of treatment. The immobility time in EA or sham group was observably increased, compared to the model group ($p < 0.05$, Figure 1C). The rearing score was not changed after EA treatment. EA observably alleviated the CUMS-induced depressive-like behaviors.

3.2 EA reduced the intestine propulsion rate but not the gastric emptying rate

The gastric emptying rate has no difference between the different groups ($p > 0.05$, Figure 2A). Compared with normal control group, the intestine propulsion rate was markedly increased in the model group ($p < 0.05$). EA significantly decreased the intestinal propulsion rate ($p < 0.05$, Figure 2B), and the sham EA had little effect on the intestinal propulsion rate ($p > 0.05$).

3.3 EA had little effect on the intestinal microbial diversity

Single sample diversity analysis reflected the abundance and diversity of the microbial community. Alpha-diversity metrics refer to the species richness, diversity, and evenness under the local uniform biological environment, which was also known as intrabiological diversity. To evaluate the α-diversity of intestinal microflora, the microbial community richness was characterized by the Chao1 index and observed species index, the diversity of the microbial community was characterized by the Shannon index, and the evolution-based diversity of microbial communities was characterized by the PD-whole-tree index. There were no obvious differences among the four groups according to alpha diversity ($p > 0.05$, Figures 3A–D). It is indicated that CUMS model and EA have little effect on intestinal microbial diversity.

3.4 EA changed the microbial flora

We used non-metric multidimensional scaling (NMDS) analyses and principal coordinate analysis (PCoA), which examine relationships between ecologic communities, such as microbial communities, to determine whether those OTUs identified to differentiating rats in a different groups (Figure 4). The higher the coincidence rate between the two groups, the more similar the composition of the gut microbiota between the two groups, and the lower the coincidence rate between the two groups, the farther the distance between them. The results showed that the composition of the intestinal microflora was different between the two groups of rat. PCoA was used to assess whether there are clusters or groupings in the data. PCoA allows to observe the similarities and differences between samples. PCoA indicated the presence of four groups (Figure 4A). Based on the PCoA, the samples were labeled as control (control rats with microbial profiles distinct from model rats and EA rats), model (model rats with microbial profiles distinct from EA rats), Sham EA (sham EA rats with similar microbial composition as EA rats and model rats), and EA (EA rats with microbial profiles distinct from control rats and model rats; Figure 4A). NMDS analysis identified that the model rats have differential abundance vs. control rats and the samples were completely divided into two distinct groups, with EA rats vs. model rats (Figure 4B).

3.5 EA regulated the relative abundance of gut microbiota at the phylum level

The relative abundance of phylum shows differences in samples from different groups (Figure 5A). Compared with the normal control group, the relative abundance of Bacteroidetes, Firmicutes, and others has no significant difference at the phylum level in the model group ($p > 0.05$, Figures 5B–F). Compared with the model group, the relative abundance of Bacteroidetes, Proteobacteria, and Actinobacteria significantly increased ($p < 0.05$, Figures 5B,E,F), Firmicutes significantly decreased ($p < 0.05$, Figure 5C), and the ratio of Firmicutes to Bacteroidetes significantly reduced ($p < 0.05$, Figure 5D).

3.6 EA regulated the relative abundance of gut microbiota at the genus level

The relative abundance of the genus shows differences in samples from different groups (Figure 6A). Compared with the normal control group, the relative abundance of Bifidobacterium and Streptococcus was

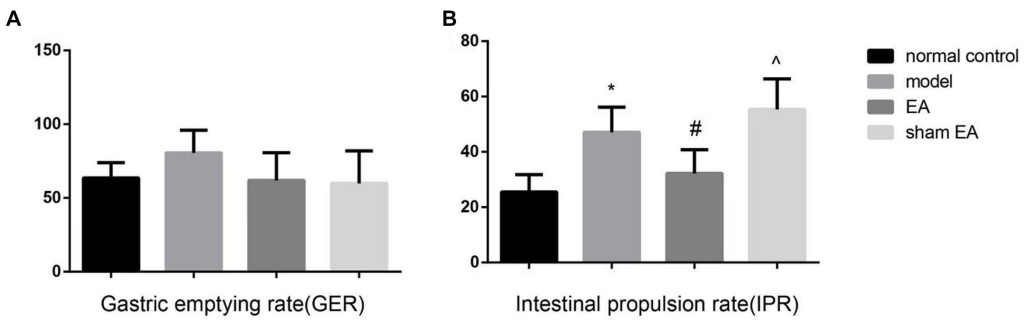

FIGURE 2
The gastric emptying rate (GER) and intestine propulsion rate (IPR) in the different groups ($n = 6$). **(A)** The GER in the different groups. **(B)** The IPR in the different groups. $^{*}p < 0.01$, vs the normal control group; $^{\#}p < 0.05$, vs the model group, $^{\wedge}p < 0.01$, vs the EA group.

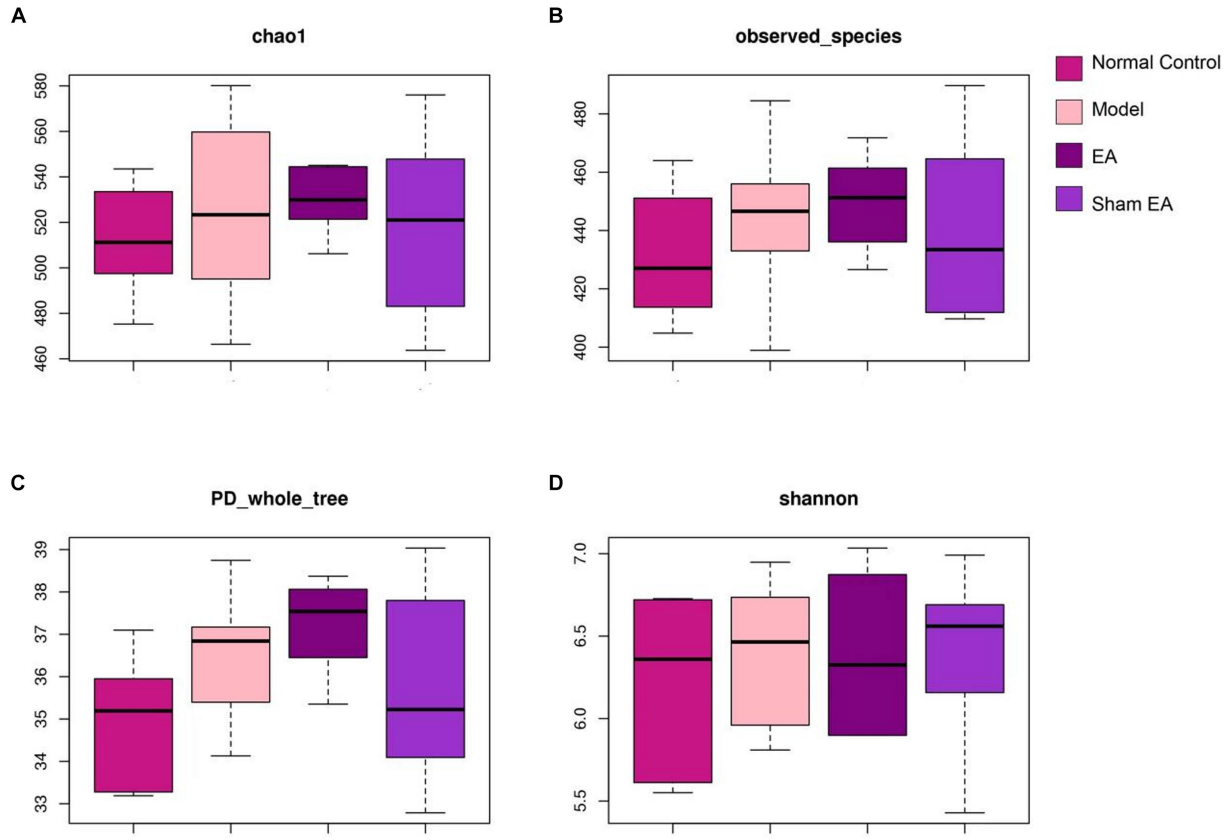

FIGURE 3
16S rRNA gene sequencing reveals that EA had no effect on the α-diversity of intestinal microflora including **(A)** chao1 index, **(B)** observed species index, **(C)** PD whole tree and **(D)** shannon index ($n = 6$).

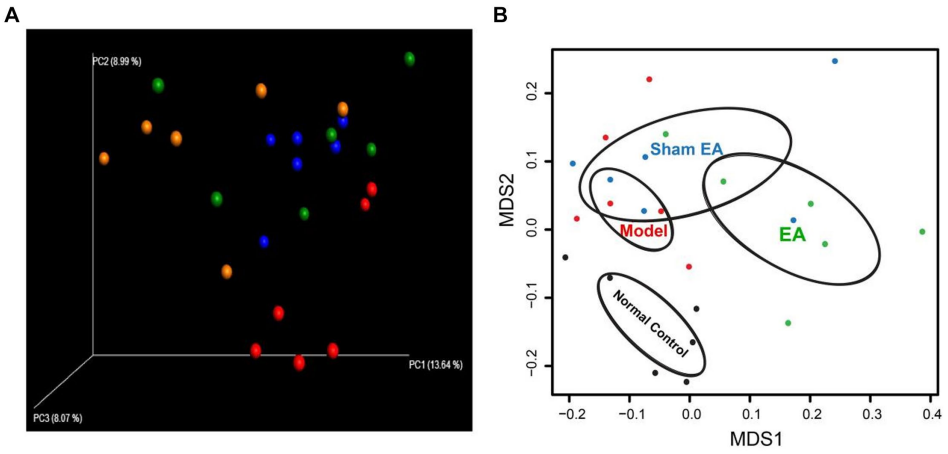

FIGURE 4
16S rRNA gene sequencing reveals changes to microbial flora in EA rats ($n = 6$). **(A)** Three-dimensional principal coordinate analysis (PCoA) of unweighted UniFrac distances showed an obvious difference in gut microbiota composition between EA rats and model rats. Red: Normal Control, Blue: Model, Orange: EA, and Green: Sham EA. **(B)** NMDS based on weighted UniFrac distance between samples of rats in different groups. Black: Normal Control, Red: Model, Green: EA, and Blue: Sham EA.

significantly decreased at the genus level in the model group ($P<0.05$, Figure 6B). The relative abundance of Bifidobacterium and Streptococcus significantly increased after EA treatment ($p<0.05$, Figure 6B). There was no significant difference in the relative abundance of Lactobacillus at the genus level among different groups ($p>0.05$, Figure 6B).

3.7 EA regulated the expression of neuroendocrine in the colon and plasma

Compared with the normal control group, the expressions of VIP protein and VIP mRNA increased significantly in the model group

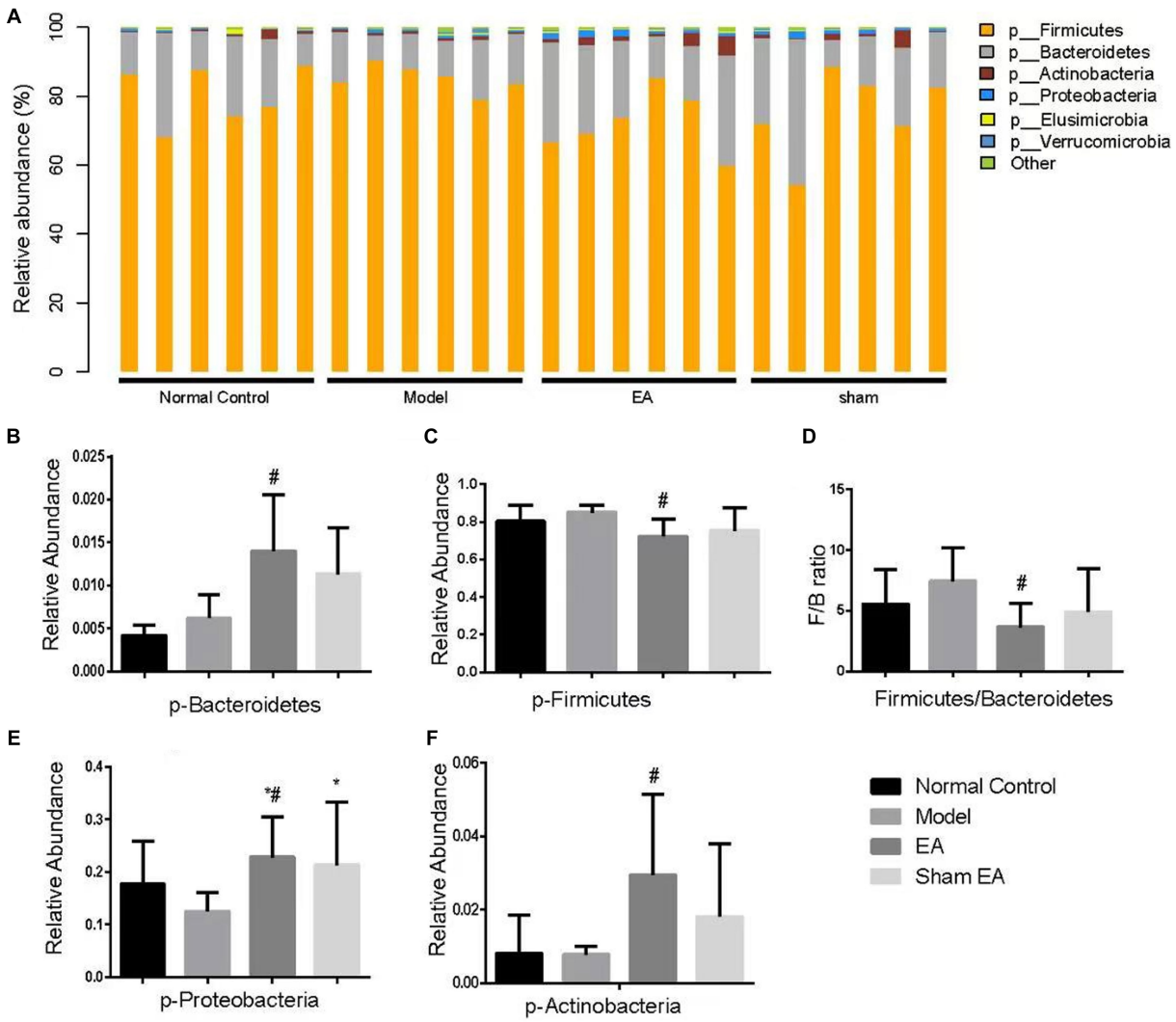

FIGURE 5
Comparison of gut microbiota composition at the phylum level in rats from each group ($n = 6$). **(A)** Relative abundances of the phylum present in samples from different groups of rats. **(B–F)** The relative abundances of Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria were significantly changed in EA rats as compared with model rats. $^{*}p < 0.01$, vs. the normal control group; $^{\#}p < 0.05$, vs. the model group, and $^{\wedge}p < 0.01$, vs. the EA group.

($p < 0.05$, Figures 7A,B) in the colon and the expression of VIP decreased markedly in plasma ($p < 0.05$, Figure 7C). Compared with the model group, the expressions of VIP protein and VIP mRNA decreased in the EA group ($p > 0.05$, Figures 7A,B) in the colon, and the expression of VIP significantly increased in the EA and sham EA group in plasma ($p < 0.05$, Figure 7C).

The expressions of CGRP in the colon and plasma decreased slightly ($p > 0.05$, Figure 7D) and markedly ($p < 0.05$, Figure 7E) in the model group compared to the normal control group, respectively. The expression of the CGRP significantly increased ($p < 0.05$) in the colon and plasma. Compared with the EA group, the expression of CGRP in plasma decreased significantly ($p < 0.05$) in the sham EA group, compared to the model group. The expressions of CGRP in the colon and plasma after sham EA intervention were still lower than that of the normal control group ($p < 0.05$).

Compared to the normal control group, after CUMS modeling, the expression of ACTH increased significantly ($p < 0.05$, Figure 7F) in plasma. The expression of ACTH in EA and sham EA groups was lower than in the model group ($p < 0.05$, Figure 7F).

Compared with the normal control group, the relative expression of SST mRNA in the colon and the expression of SST in plasma increased decreased markedly ($p < 0.05$, Figure 7G) and slightly ($p < 0.05$, Figure 7H) in the model group. The relative expression of SS mRNA in the colon and the expression of SS significantly decreased ($p < 0.05$, Figures 7G,H) in the EA group, compared with the model group.

4 Discussion

Acupuncture, a highly favored complementary therapy, is widely recognized for its potential benefits in treating depression. A meta-analysis of randomized trials revealed that acupuncture had a positive impact on the HAMD score, particularly when combined with antidepressant medications. This combination therapy not only

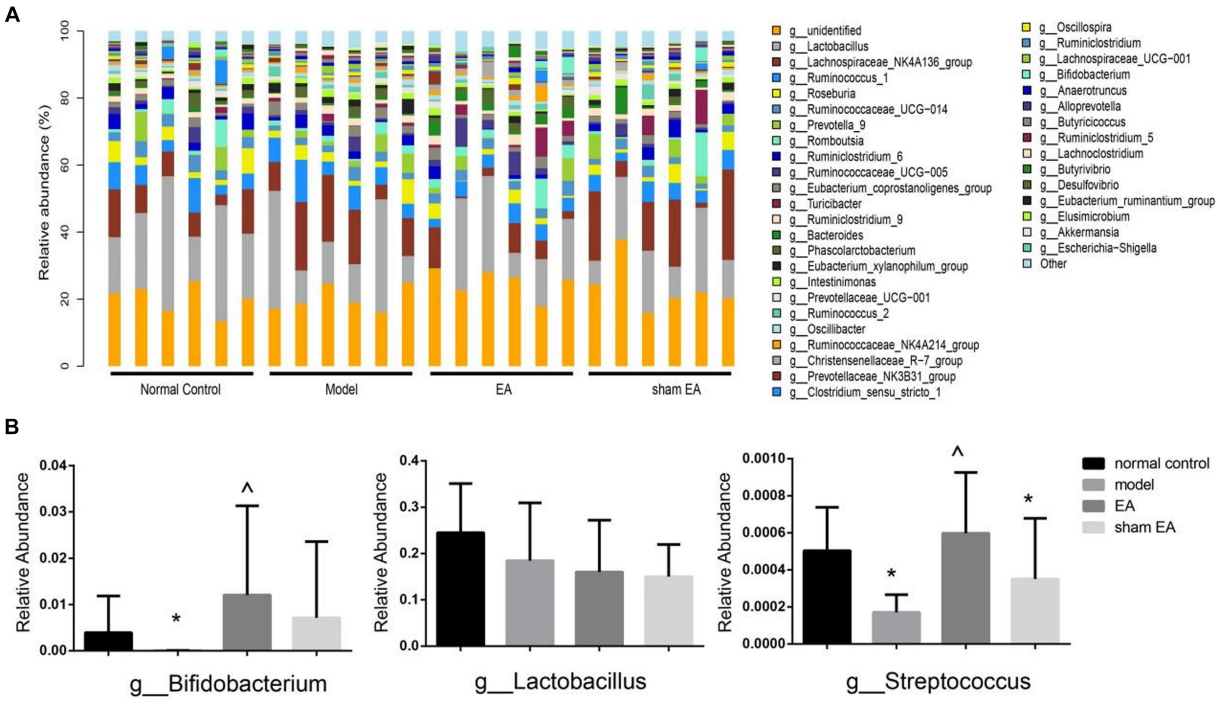

FIGURE 6
Comparison of gut microbiota composition at the genus level in rats from each group ($n = 6$). **(A)** Relative abundances of the genus present in samples from different group rats. **(B)** The relative abundances of Bifidobacterium, Lactobacillus, and Streptococcus in a different group. $^{*}p < 0.01$, vs. the normal control group; $^{\#}p < 0.05$, vs. the model group, and $^{\wedge}p < 0.01$, vs. the EA group.

improved the severity of depression and sleep quality but also demonstrated an early onset of action. Furthermore, the treatment was found to be safe and well-tolerated throughout the initial 6-week period (Chan et al., 2015; Dong et al., 2017). For postpartum depression and depression, acupuncture treatment could significantly reduce HAMD scores (Li et al., 2019). Accumulating evidence suggests that acupuncture affects depression, but its under mechanism is still unclear, which limits it to guide future clinical directions (Smith et al., 2018). In the present study, we conducted a series of research on the effect of acupuncture on depressive rats, using measures related to depressive disorder, including observation of behavior, neuropeptides, and alterations. After 4 weeks of CUMS modeling, the rats showed significant depressive behaviors, and EA treatment of ST36-ST25 for 2 weeks improved the depressive behaviors as the sucrose preference, immobility time, and crossing score increased significantly. However, there was no such effect in the sham EA group.

In recent years, an increasing amount of research has provided support for the notion that imbalances in gut microbiota and microbiota–gut–brain dysfunction may contribute to the development of major depression (Liang et al., 2018). The gut microbiota may significantly impact the development of depression through the brain–gut–microbiota axis, affecting both neural and immune systems (Chang et al., 2022). Using clinical samples, a notable disparity was observed in the gut microbiota compositions between individuals suffering from major depressive disorder (MDD) and healthy controls. MDD patients displayed substantial alterations in the relative abundance of phyla—Firmicutes, Actinobacteria, and Bacteroidetes (Zheng et al., 2016). In the rat model of depression induced by chronic variable stress (CVS), significant alterations in the gut microbiota were observed, particularly at the phylum and genus levels (Yu et al., 2017). In our findings, the genus-level analysis revealed a slight increase in the relative abundance of phyla—Bacteroidetes and the Firmicutes/Bacteroidetes ratio following CUMS modeling.

Electroacupuncture had a substantial impact on the gut microbiota composition in obese abdominal rats. This impact was primarily characterized by a decrease in the Firmicutes/Bacteroidetes ratio and an increase in Prevotella abundance (Wang et al., 2019). Acupuncture at acupoints GB34 and ST36 changed the relative abundance of 18 genera in Parkinson's disease (PD) rats: Butyricimonas, Holdemania, Frisingicoccus, Gracilibacter, Phocea, and Aestuariispira exhibited substantial correlations with both anxiety and motor functions (Jang et al., 2020), but there is little research to observe the effect of acupuncture or electroacupuncture on the gut microbiota in depressive rats. In this study, EA treatment had little effect on the diversity of intestinal microflora but had some effect on the relative abundance in CUMS rats. The application of EA treatment led to a noteworthy rise in the proportion of Bacteroidetes, Proteobacteria, and Actinobacteria, while decreasing the ratio of Firmicutes/Bacteroidetes.

The gut microbiota and the central nervous system communicate bidirectionally via the microbiota–gut–brain axis (Du et al., 2020). Therefore, the gut microbiota can be targeted for both prevention and treatment of depression; this includes the possibility of probiotic supplementation (Ansari et al., 2020; Paiva et al., 2020). Microbiome-based treatments such as probiotics are suggested for depression to cultivate helpful bacteria in the gut (Mörkl et al., 2020). Thus, we also observed the changes in the probiotics after EA treatment for depressive rats. The relative abundance of g-Bifidobacterium and g-Streptococcus significantly decreased after CUMS modeling, and EA treatment

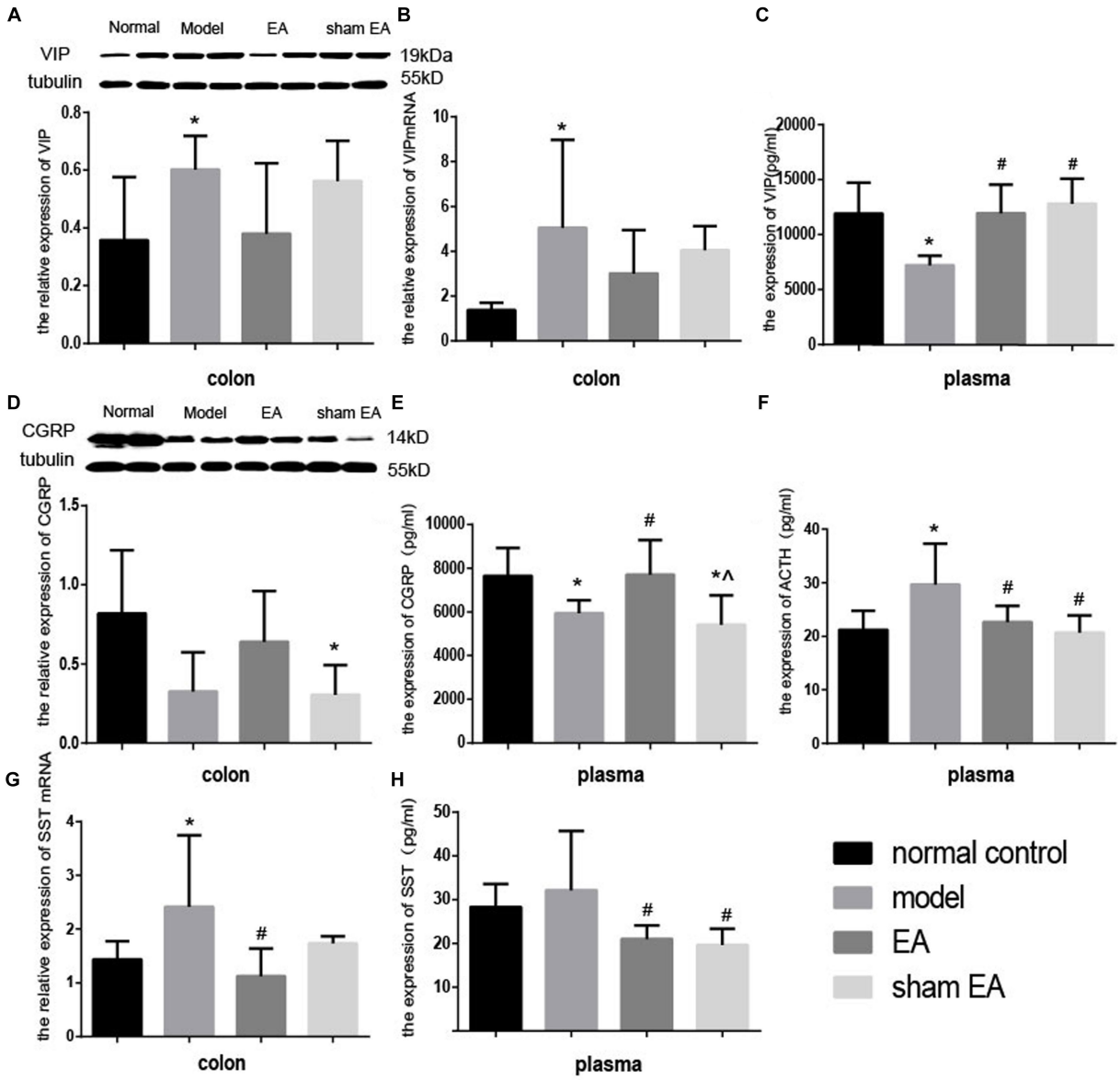

FIGURE 7
Effect of EA on the expression of VIP CGRP, ACTH, and SST in colon tissues and plasma ($n = 6$). *$p < 0.01$, vs. the normal control group; #$p < 0.05$, vs. the model group, ^$p < 0.01$, vs. the EA group.

markedly increased the relative abundance of g-Bifidobacterium and g-Streptococcus. A 16-week, double-blind, randomized, placebo-controlled trial showed that probiotic supplements containing *Lactobacillus helveticus* and Bifidobacterium long might be effective in alleviating symptoms of depression (Wallace et al., 2020). Hence, it is proposed that EA's antidepressant impact correlates with the adjustment of the proportionate occurrence of probiotics, including g-Streptococcus and g-Bifidobacterium that mitigate depressive behaviors.

Depression causes intestinal flora disorders and is associated with neuroendocrine disorders. Neuropeptides such as substance P, CGRP, SST, and adrenocorticotropin-releasing factor released by specialized cells within the gastrointestinal tract are likely to play an important role in the bidirectional signaling between the gut and brain (Holzer and Farzi, 2014). Chronic stress can cause injury to the duodenum and reduce VIP levels in the plasma and duodenum (Huang et al., 2007). Chronic mild stress can increase SST expression levels in plasma (Faron-Górecka et al., 2016). Anxiety-depressive states could result in altered SS and VIP secretion, which in turn affects gastrointestinal motility and function (Han, 2013). These changes in neuropeptides may be caused by altered regulation of gut microbes. Fecal microbiota from patients with depression increased hippocampal ACTH (Liu et al., 2020). Probiotics can increase the relative abundance of Lactobacillus and Bifidobacterium and decrease the level of serum ACTH (Li et al., 2021). Triple viable Bifidobacterium plus smecta reduced the serum levels of VIP and improved the clinical symptoms with diarrhea (Zhao, 2015). Those peptides are strong modulation of neuroinflammation, have also been found to exert direct antimicrobial effects, and they thought to be in involved in the bidirectional relationship between the gut microbiota and the host (including gut-brain axis) (Aresti Sanz and El, 2019). Also, these peptides are thought to have direct antimicrobial effects, modulate

neuroinflammation, and are therefore able to orchestrate changes in the gut microbial community and participate in the bidirectional relationship between the gut and the brain (Holzer and Farzi, 2014). In the present research, EA treatment downregulated the increased SST mRNA in colon and SST in plasma, and ACTH in the plasma which may be induced by the increased expression of probiotics (Bifidobacterium). The mice showed depression-like behavior and decreased CGRP levels, and intracerebroventricular CGRP administration has anti-depressive effect (Hashikawa-Hobara et al., 2015). This is consistent with our results that EA treatment upregulated the protein expression of CGRP in the colon and CGRP in plasma.

Gastric emptying rate and intestinal propulsion rate are important manifestations of gastrointestinal motility. Germ-free mouse studies have demonstrated that the presence of a gastrointestinal microbiota is crucial for maintaining normal gastrointestinal motility (Dimidi et al., 2017). Specifically, in the absence of gut microbiota, there is an increase in gastric emptying and gut transit time compared to wild-type mice (Abrams and Bishop, 1967; Iwai et al., 1973). Multi-probiotics could significantly improve the major depressive disorder (MDD) patients' gastrointestinal functions (Tian et al., 2023). Our research findings showed that EA significantly reduces the intestine propulsion rate and slightly reduces the gastric emptying rate which may be directly induced in part by the gut microbiota including Bifidobacterium. Neuropeptides including VIP, SST, and ACTH also regulate gastrointestinal motility. VIP is a gastrointestinal hormone that can inhibit gastrointestinal motility and gastric emptying and reduce gastrointestinal motility and excitability. CGRP can regulate intestinal sensory function, inhibit gastric acid secretion, and reduce gastric emptying. EA treatment increased the expression of VIP in plasma, the protein expression of CGRP in the colon, and CGRP in plasma that may induce the intestine propulsion rate and the gastric emptying.

Our study proves that EA treatment is a promising non-pharmacological treatment method to alleviate depressive symptoms, provides new evidence for the selection of acupoints for clinical treatment of depression, and provides reference for improving the efficacy of clinical treatment of depression. It is hoped that it can open up ideas for clinicians.

5 Limitations

The present study has identified a correlation between the antidepressant properties of EA treatment and its modulation of the intestinal microbiota in a rodent model of depression. However, to elucidate the underlying neuroanatomical mechanisms that underpin this relationship, additional investigations are warranted. Further research endeavors should delve into the intricate interplay between the central nervous system, the gut–brain axis, and the microbiota–gut–brain axis, aiming to unravel the intricate neural circuitry and signaling pathways involved. Such studies may shed light on the specific regions, neural networks, and neurotransmitter systems that are implicated in the antidepressant effects of EA and its influence on gut microbial composition.

6 Conclusion

In our study, we observe that EA at ST36 and ST25 could significantly alleviate depression-like behavior induced by CUMS in rats. This antidepressant effect may be related to the regulation of gut microbiota and neurotransmitters.

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 approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences (D2019-02-11-2). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JW: Writing – original draft. HZ: Data curation, Writing – review & editing. XS: Data curation, Writing – review & editing. JuZ: Data curation, Writing – review & editing. JiaZ: Data curation, Writing – review & editing. JinZ: Writing – review & editing. SL: Writing – review & editing. PR: Writing – review & editing.

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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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Improving precision management of anxiety disorders: a Mendelian randomization study targeting specific gut microbiota and associated metabolites

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Background: There is growing evidence of associations between the gut microbiota and anxiety disorders, where changes in gut microbiotas may affect brain function and behavior via the microbiota-gut-brain axis. However, population-level studies offering a higher level of evidence for causality are lacking. Our aim was to investigate the specific gut microbiota and associated metabolites that are closely related to anxiety disorders to provide mechanistic insights and novel management perspectives for anxiety disorders.

Method: This study used summary-level data from publicly available Genome-Wide Association Studies (GWAS) for 119 bacterial genera and the phenotype "All anxiety disorders" to reveal the causal effects of gut microbiota on anxiety disorders and identify specific bacterial genera associated with anxiety disorders. A two-sample, bidirectional Mendelian randomization (MR) design was deployed, followed by comprehensive sensitivity analyses to validate the robustness of results. We further conducted multivariable MR (MVMR) analysis to investigate the potential impact of neurotransmitter-associated metabolites, bacteria-associated dietary patterns, drug use or alcohol consumption, and lifestyle factors such as smoking and physical activity on the observed associations.

Results: Bidirectional MR analysis identified three bacterial genera causally related to anxiety disorders: the genus *Eubacterium nodatum group* and genus *Ruminococcaceae UCG011* were protective, while the genus *Ruminococcaceae UCG011* was associated with an increased risk of anxiety disorders. Further MVMR suggested that a metabolite-dependent mechanism, primarily driven by tryptophan, tyrosine, phenylalanine, glycine and cortisol, which is consistent with previous research findings, probably played a significant role in mediating the effects of these bacterial genera to anxiety disorders. Furthermore, modifying dietary pattern such as salt, sugar and processed meat intake, and adjusting smoking state and physical activity levels, appears to be the effective approaches for targeting specific gut microbiota to manage anxiety disorders.

Conclusion: Our findings offer potential avenues for developing precise and effective management approaches for anxiety disorders by targeting specific gut microbiota and associated metabolites.

KEYWORDS

gut microbiota and associated metabolites, anxiety disorders, Mendelian randomization, causal effects, management approaches

1 Introduction

Anxiety disorders are the most prevalent mental disorders, and they usually develop during early adulthood (Penninx et al., 2021). A recent population-based study of 275,057 adolescents aged 12–17 years from 82 countries representing the full spectrum of economic wealth across the six World Health Organization regions reported a 9% 12-month pooled prevalence of anxiety disorders (Biswas et al., 2020). Across all age groups in the US, anxiety disorders have a lifetime prevalence of approximately 34% (Szuhany and Simon, 2022). In creating an environment where many individuals experienced heightened levels of anxiety and stress, the recent COVID-19 pandemic also further exacerbated mental health issues globally, producing an additional 76.2 million cases of anxiety disorders worldwide, representing a 25.6% increase in pre-pandemic levels (COVID-19 Mental Disorders Collaborators, 2021). Individuals with anxiety disorders face an increased likelihood of developing cardiovascular diseases, respiratory disorders, gastrointestinal problems, and other chronic conditions (Penninx et al., 2021; Szuhany and Simon, 2022). They are also at increased risk of depression, substance use disorders, suicidal ideation, and impaired cognitive functioning, including memory, attention, and decision-making difficulties (Penninx et al., 2021; Szuhany and Simon, 2022). Thus, anxiety disorders have emerged as a significant health concern, contributing significantly to the global disease burden (COVID-19 Mental Disorders Collaborators, 2021).

First-line therapies for anxiety disorders, including medication and cognitive behavioral therapy (CBT), have limitations. Medications may cause side effects, and long-term use can result in tolerance and dependence (Penninx et al., 2021; Szuhany and Simon, 2022). CBT requires a significant time and energy commitment, can be costly to administer, and may not be suitable for all patients, often necessitating combination with other therapies (Penninx et al., 2021; Szuhany and Simon, 2022). Anxiety disorders are multifactorial, with both psychosomatic and genetic components contributing to the underlying pathogenesis (Penninx et al., 2021; Szuhany and Simon, 2022), and the precise mechanisms underlying these disorders are complex and still not completely understood. Advances in genetics, particularly genome-wide association studies (GWAS), have improved our understanding of the causes of mental disorders (Meier et al., 2019; Koskinen and Hovatta, 2023). By investigating the complex interplay between genetic and environmental factors, we can gain new insights into the biological mechanisms driving anxiety disorders to inform the development of more effective and personalized therapies.

The gut microbiome is a vast and intricate collection of microorganisms that include bacteria, fungi, viruses, and other microbes residing in the gastrointestinal tract. This microbial community is one of the most extensive and complex communities in the human body; in fact, the microbiome usually contains significantly more cells than the human body (Li et al., 2016). The gut microbiota plays a crucial role in many biological processes including breaking down nutrients, synthesizing vitamins and essential molecules, and maintaining the integrity and stability of the intestinal barrier (Li et al., 2016). Moreover, the gut microbiota interacts with various organs and body systems, including the immune, digestive, nervous, and endocrine systems, significantly impacting human health and influencing essential physiological processes (Li et al., 2016). There is now an extensive body of evidence showing that changes in gut microbiota composition are closely related to various diseases, including metabolic disorders (Agus et al., 2021; Fan and Pedersen, 2021), inflammatory conditions (Virtue et al., 2019; Cai et al., 2022), and mental health disorders such as anxiety disorders (Nikolova et al., 2021; Simpson et al., 2021). The microbiota-gut-brain axis, which describes a pathway through which changes to the gut microbiota affect brain function and behavior, has attracted significant attention (Cryan et al., 2019; Deng et al., 2021; Socała et al., 2021). Several studies have identified a potential connection between the gut microbiota and anxiety disorders, showing that individuals with anxiety disorders exhibit distinct changes in gut microbiota composition compared with healthy individuals including reduced microbial diversity, altered bacterial taxa abundance, and impaired microbial metabolic functions (Tran et al., 2019; Lee et al., 2021; Zhu et al., 2023). In addition, communication between the gut microbiota and brain involves the autonomic nervous system and its corresponding neurotransmitters (e.g., γ-aminobutyric acid (GABA), dopamine) (Dan et al., 2020; Prochazkova et al., 2021), bacterial metabolites such as short-chain fatty acids (SCFAs) (De Vadder et al., 2014; Wang et al., 2020), and amino acid metabolites (Sun et al., 2022; Zhang et al., 2023). Metabolic derivatives also play an important role in providing key neurotransmitters, so relevant metabolites such as tryptophan, the raw material for serotonin, also known as 5-hydroxytryptamine (5-HT), could potentially link the gut microbiota to anxiety disorders (Deng et al., 2021; Tian et al., 2023). On the other hand, there is evidence that the relationship between the gut microbiota and anxiety disorders can be influenced by several confounding factors such as dietary patterns, drug or alcohol use, and lifestyle factors such as smoking and physical activity (Zhu et al., 2020; Simpson et al., 2021; Cataldi et al., 2022). Controlling these factors when investigating complex biological relationships can be challenging, limiting the potential to infer definitive associations between a biological exposure (the gut microbiota) and a clinical phenotype or disease outcome (anxiety disorders). Hence, rigorous studies that utilize controlled methodologies and that isolate these confounding variables are needed.

Mendelian randomization (MR) methods use genetic instruments, normally single nucleotide polymorphisms (SNPs), as instrumental variables (IVs) for modeling and inferring causal effects and potential associations between exposure and disease outcome. MR exploits the fact that the allocation of genotypes from parent to offspring is random, such that the association between genetic variants and outcome is not affected by common

confounding factors. Also, because heredity is irreversible, the interference of reverse causation can be excluded (Davey Smith and Hemani, 2014; Burgess et al., 2015). Thanks to large-scale GWAS of gut microbiotas in different diseases, MR has been widely used to study disease pathogenesis. Indeed, several studies using MR have now established that the gut microbiota is strongly associated with several neurological diseases including Alzheimer's disease (Zeng et al., 2023), major depressive disorder (Chen M. et al., 2022), schizophrenia (Cheng et al., 2023), and insomnia (Li et al., 2023).

In this study, using the GWAS summary statistics, MR analysis was conducted to comprehensively explore whether the gut microbiota has a causal effect on anxiety disorders. We aimed to identify specific bacterial genera linked to anxiety disorders and then investigate the potential impact of various neurotransmitter-associated metabolites, dietary patterns, drugs and alcohol use, and lifestyle factors on these associations. Understanding these relationships has implications for understanding the specific mechanisms driving anxiety disorders and developing sustainable and effective management approaches targeting the gut microbiota and associated metabolites in this common condition.

2 Materials and methods

2.1 Study design

The overall flow chart of our study design is shown in Figure 1. To uncover potential associations between gut microbiotas and anxiety disorders, as well as identify specific bacterial taxa at the genus level linked to anxiety disorders, we conducted a two-sample, bidirectional MR analysis. This analysis incorporated several methods and sensitivity analyses of publicly available summary-level GWAS data. Subsequently, we conducted multivariable MR (MVMR) analysis to investigate the potential impact of various common neurotransmitter-associated metabolites linked to anxiety disorders on these taxa-level associations to differentiate between the direct and indirect effects of gut microbiotas associated with anxiety disorders. This approach aimed to identify bacterial genera-associated metabolites that could potentially affect the risk of anxiety disorders. Moreover, MVMR analysis was employed to further explore the potential impact of bacteria-associated dietary patterns, drug and alcohol use, and lifestyle factors (smoking and physical activity) on the causal effects of the identified bacterial genera on anxiety disorders. In performing this

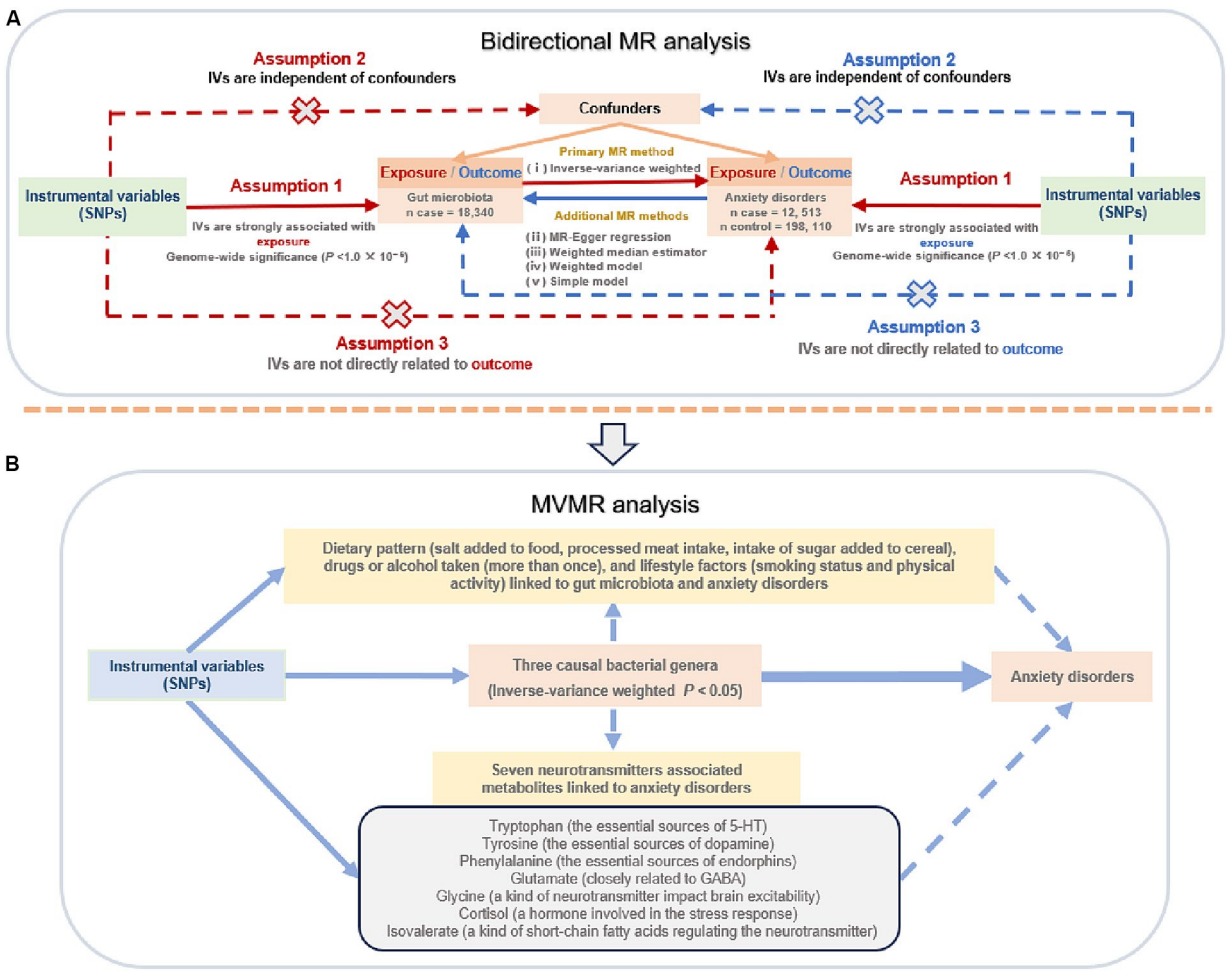

FIGURE 1
The flow chart of the study design. **(A)** Two-sample, bidirectional Mendelian randomization (MR) analysis; **(B)** multivariable MR (MVMR) analysis. SNPs: single nucleotide polymorphisms; IVs, instrumental variables; 5-HT, hydroxytryptamine; GABA: γ-aminobutyric acid.

comprehensive analysis, we aimed to provide new mechanistic insights and management perspectives for anxiety disorders.

The study was designed and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology—Mendelian Randomization (STROBE-MR) statement (Skrivankova et al., 2021a,b).

2.2 Ethics statement

This study used only publicly available GWAS data and published studies that had already undergone ethical review and received participant consent. As such, no additional ethical approval was needed.

2.3 Data sources

GWAS summary data for gut microbiotas were derived from the largest, multi-ethnic, genome-wide meta-analysis published to date on gut microbiota composition conducted by the MiBioGen consortium. This study collected whole-genome genotyping data from 18,340 individuals across 24 separate cohorts together with 16S rRNA genotyping of the participants' fecal microbiomes. Among the 24 cohorts, 20 included samples from individuals of single ancestry, with the majority of participants being of European ancestry (16 cohorts, $n = 13{,}266$) (Kurilshikov et al., 2021). Three different variable regions (V1-V2, V3-V4, and V4) of the 16S rRNA genotypes were used to profile intestinal microbial species composition, using microbiota quantitative trait loci (mbQTL) mapping to reveal genetic variants governing the abundance of bacterial taxa within the gut microbiota. Notably, the analysis revealed that these variations impacted the relative abundances of nine phyla, 16 classes, 20 orders, 35 families, and 131 genera encompassing 12 unknown genera (Kurilshikov et al., 2021). Finally, 119 genus-level taxa were included in the current study for MR analysis.

GWAS summary-level statistics for anxiety disorders were obtained from the FinnGen consortium R5 release data. The FinnGen project is an academic-industrial collaboration aiming to discover genotype–phenotype relationships in over 500,000 Finnish participants. The phenotype "All anxiety disorders" (description: "a category of psychiatric disorders characterized by anxious feelings or fear often accompanied by physical symptoms associated with anxiety") and GWAS ID "finn-b-F5_ALLANXIOUS" were adopted in the current study. This GWAS included 210,623 male and female subjects and consisted of 12,513 European cases and 198,110 healthy controls. They collected as many anxiety disorders as possible, resulting in 19 reported traits (e.g., anxiety and stress-related disorders, anxiety disorder, lifetime anxiety disorder, and ICD10 F40 F41 F42: any anxiety disorder) and 11 child traits (e.g., generalized anxiety disorder, social anxiety disorder, mixed anxiety and depressive disorder, acute stress reaction). More information on the study can be found at https://r5.risteys.finngen.fi/.

We also aimed to investigate the potential involvement of neurotransmitter-associated metabolites in the biological pathway(s) linking gut microbiotas to anxiety disorders. Through a systematic literature search and considering commonly measured serum metabolites in metabolomic studies, we identified several significant neurotransmitter-associated metabolites including tryptophan (Deng et al., 2021; Jia et al., 2024), tyrosine (Needham et al., 2022; Zhang D. et al., 2022), phenylalanine (Deng et al., 2022; Zhang D.-D. et al., 2022), glutamate (Zhao et al., 2020; Laroute et al., 2022), and glycine (Lin et al., 2021; Zhang D. et al., 2022). These metabolites have been found to closely associate with the gut-brain axis, and their dysregulation may contribute to the development of anxiety disorders. Furthermore, cortisol is often investigated alongside neurotransmitter-associated metabolites as a potential biomarker or factor in anxiety disorders (Dalile et al., 2020; Tian et al., 2023). In addition, a critical group of bacterial metabolites called short-chain fatty acids, such as isovalerate, has been found to be closely associated with anxiety disorders and was also taken into consideration in our analysis (Bayrer et al., 2023; Raman et al., 2023). We extracted genetic data for these specific human blood metabolites (i.e., tryptophan, tyrosine, phenylalanine, glutamate, glycine, cortisol, isovalerate) from the metabolomics GWAS server.[1] The most comprehensive analysis to date on the genetic loci associated with blood metabolites was of a sample of 7,824 European participants, which identified almost 2.1 million SNPs for 486 metabolites associated with human genetic variants by genome-wide association scans with high-throughput metabolic profiling (Kanehisa et al., 2012; Shin et al., 2014). Specifically, tryptophan, tyrosine, phenylalanine, and glutamate were directly associated with neurotransmitters, namely 5-HT (Deng et al., 2021; Jia et al., 2024), dopamine (Needham et al., 2022; Zhang D. et al., 2022), endorphins (Deng et al., 2022; Zhang D.-D. et al., 2022), and GABA (Zhao et al., 2020; Laroute et al., 2022). Glycine itself is a neurotransmitter (Lin et al., 2021; Zhang D. et al., 2022). Cortisol, a hormone involved in the stress response, exerts an influence on neurotransmitter activity (Dalile et al., 2020; Tian et al., 2023). Isovalerate, on the other hand, is a short-chain fatty acid that contributes to regulating neurotransmitter synthesis and release, blood–brain barrier permeability, and neuroinflammation (Bayrer et al., 2023; Raman et al., 2023).

Additionally, based on our extensive literature search, we identified potential confounding factors such as diet and lifestyle that might significantly impact both the gut microbiota and anxiety disorders (Zhu et al., 2020; Simpson et al., 2021; Cataldi et al., 2022). These factors include dietary patterns (such as salt added to food, processed meat intake, and intake of sugar added to cereal); drug and alcohol use; and lifestyle factors such as smoking status and physical activity. We therefore conducted further analysis to explore how these factors may influence the causal association between the gut microbiota and anxiety disorders. The GWAS summary statistics for dietary pattern and drug or alcohol use (more than once) were obtained from the UK Biobank release data. Lifestyle factors such as smoking and physical activity were obtained from the European Bioinformatics Institute release data. An overview of the sources of selected neurotransmitter-associated metabolites and dietary pattern, drug or alcohol use, and lifestyle factors related to the gut microbiota and anxiety disorders are shown in Supplementary Table S1.

1 https://metabolomics.helmholtz-muenchen.de/gwas/

2.4 IVs selection

As shown in Figure 1, SNPs had to meet three core assumptions for inclusion as IVs in our study: (i) correlation—the relationship between genetic variants and exposure was robust; (ii) independence—the genetic variants were independent of any confounding factors affecting exposure and outcome; and (iii) exclusion—the genetic variants influenced the risk of the outcome through exposure rather than other potential pathways (Davey Smith and Hemani, 2014; Burgess et al., 2015).

To ensure the selection of suitable SNPs as IVs from the GWAS statistics, we applied a series of quality control steps. (1) To increase the number of available genetic instruments, consistent with previous studies (Ni et al., 2021; Li et al., 2023; Yu et al., 2023), SNPs at a threshold of $p < 1.0 \times 10^{-5}$ from GWAS summary data on the exposure, such as the gut microbiota, were selected as potential IVs. (2) To avoid the effect of linkage disequilibrium (LD) between SNPs, European sample data from the 1,000 Genomes Project was used as the reference panel, and the SNPs within a clumping window size of 10,000 kb were pruned under the threshold of $R^2 < 0.001$ to mitigate LD, thus ensuring independence of each IV. (3) The SNP information associated with exposure was extracted, and palindromic SNPs (e.g., those with A/T or G/C alleles) as well as ambiguous and duplicated SNPs were eliminated after harmonizing the exposure and outcome SNPs for accurate dataset matching. (4) The remaining SNPs with positive results were searched using PhenoScanner[2] to determine whether there were potential confounders. (5) To assess the extent of weak instrument bias, we computed the F-statistic of the IVs. This statistical measure indicates the strength of the association between the IVs and the exposure. Specifically, an F-statistic ≥ 10 indicates no compelling evidence of weak instrument bias. We excluded IVs with F-statistics <10, which suggests that they were weak IVs. The formula for calculating the F-value is: $F = [(n-k-1) / k] \times [R^2 / (1-R^2)]$, where R^2 represents the proportion of exposure variance explained by the IVs, n is the sample size, and k is the number of IVs (Burgess and Thompson, 2011).

2.5 Statistical methods

2.5.1 Two-sample and bidirectional MR analysis

The inverse variance-weighted average method (IVW), the most efficient causal estimation method allowing balanced pleiotropy in MR analysis, was used as the primary analytical method for estimating causal effects of the exposures on the outcome. The IVW method assumes that all SNPs are valid instruments, and the overall effect is estimated by calculating the inverse variance-weighted mean of the effect estimates for the instrumental variables. When there is no horizontal pleiotropy bias present, the IVW method is more statistically powerful (Burgess et al., 2013). In addition to the IVW method, four additional MR methods [MR-Egger regression, weighted median estimator (WME), weighted model, simple model] served as complementary analyses to assess causal associations. The MR-Egger regression method provides unbiased estimates even in the presence of horizontal pleiotropy (Burgess and Thompson, 2017). The WME method can adjust for the effect of invalid IVs. Assuming the presence of up to half of invalid IVs, the WME method yields robust estimates (Bowden et al., 2016). When the largest number of similar individual SNP causal effect estimates are from efficient SNPs, the weighted model was consistent even if SNPs were invalid (Burgess et al., 2013). The simple mode was an unweighted mode of the empirical density function of causal estimation (Burgess et al., 2013).

To ensure the reliability of our results and eliminate interference from reverse causality, we also performed reverse MR analysis. In this analysis, we considered anxiety disorders as the "exposure" and identified bacterial genera that were causally associated with anxiety disorders as the "outcome." The methods and settings were the same as those used in forward MR.

A series of sensitivity analyses were then performed to examine the consistency of the causal association. First, Cochran's Q statistic was used to assess heterogeneity, and a p-value of <0.05 was considered to indicate the presence of significant heterogeneity in the SNP effect estimates. Second, we employed the MR-Egger intercept test and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) test to detect horizontal pleiotropy (Verbanck et al., 2018). The MR-Egger intercept test compares the MR-Egger intercept term with a value of zero, where a considerable deviation from zero suggests the presence of substantial horizontal pleiotropy. MR-PRESSO evaluates the sum of residuals from each SNP to assess the magnitude of horizontal pleiotropy, and the results of the main analysis method, IVW, can be obtained after adjusting for horizontal pleiotropy. The MR-PRESSO global test assesses the overall horizontal pleiotropy of the IVs and the MR-PRESSO outlier test assesses abnormal SNPs that lead to the existence of overall horizontal pleiotropy. The MR-Egger intercept test and MR-PRESSO global test helped to identify any potential confounding effects due to pleiotropic pathways. If the p-value of these tests was >0.05, horizontal pleiotropy was ruled out (Verbanck et al., 2018). Furthermore, we conducted a leave-one-out sensitivity analysis, progressively eliminating each individual SNP to assess the impact of potential anomalies on the overall causal effect.

2.5.2 MVMR analysis

MVMR is an extension of MR that allows SNPs to be associated with multiple exposures, provided that they are not linked to confounding variables of any exposure-outcome associations and have no direct impact on the outcome (Davey Smith and Hemani, 2014; Burgess et al., 2015). In our analysis, we accounted for relevant neurotransmitter-associated metabolites, dietary patterns, drugs and alcohol consumption, and lifestyle factors separately. Using MVMR analyses, including MVMR-IVW and MVMR-Egger, we estimated the independent and direct causal effects of identified bacterial genera on anxiety disorders (Davey Smith and Hemani, 2014; Burgess et al., 2015). This allowed us to explore potential metabolic pathways associated with neurotransmitter-associated metabolites closely linked to the gut microbiota and anxiety disorders. We also examined the potential impact of relevant dietary patterns, drugs and alcohol consumption, and lifestyle factors on the causal effects of the identified bacterial genera on anxiety disorders. Heterogeneity was assessed using Cochran's Q statistic. The parameter settings adopted for these analyses were the same as those used in the univariate MR analysis and were selected to ensure coherence and reliability across all analyses.

2 http://www.phenoscanner.medschl.cam.ac.uk/

TABLE 1 MR estimates and heterogeneity test for gut microbiota (exposure) on anxiety disorders (outcome).

Bacterial genera (exposure)	MR method	No. of SNP	Beta	SE	OR	95% CI	*p*-value	Heterogeneity test		
								Cochran's Q	Q_df	*p*-value
Eubacterium nodatum group	IVW	11	−0.103	0.038	0.902	0.837–0.972	0.007	6.018	10	0.814
	MR-Egger	11	−0.085	0.170	0.918	0.658–1.281	0.628	6.006	9	0.739
	WME	11	−0.100	0.052	0.905	0.817–1.002	0.055	–	–	–
	Weighted model	11	−0.101	0.079	0.904	0.775–1.056	0.232	–	–	–
	Simple model	11	−0.112	0.093	0.894	0.746–1.073	0.257	–	–	–
Ruminococcaceae UCG011	IVW	8	−0.099	0.044	0.905	0.831–0.987	0.023	6.154	7	0.522
	MR-Egger	8	−0.307	0.219	0.735	0.478–1.130	0.210	5.217	6	0.516
	WME	8	−0.040	0.059	0.961	0.857–1.078	0.494	–	–	–
	Weighted model	8	−0.014	0.101	0.986	0.808–1.202	0.890	–	–	–
	Simple model	8	−0.014	0.097	0.986	0.816–1.191	0.885	–	–	–
Lachnospiraceae UCG010	IVW	10	0.224	0.100	1.251	1.028–1.522	0.025	14.094	9	0.119
	MR-Egger	10	−0.008	0.312	0.992	0.538–1.827	0.979	13.077	8	0.109
	WME	10	0.233	0.115	1.262	1.007–1.582	0.044	–	–	–
	Weighted model	10	0.251	0.167	1.285	0.926–1.783	0.167	–	–	–
	Simple model	10	0.226	0.172	1.253	0.895–1.754	0.221	–	–	–

MR, Mendelian randomization; SNP, single nucleotide polymorphism; SE, standard error; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted; WME, weighted median estimator.

TABLE 2 MR-Egger intercept test and MR-PRESSO analysis for associations between gut microbiota (exposure) and anxiety disorders (outcome).

Bacterial genera (exposure)	MR-Egger intercept test		MR-PRESSO analysis					
	Egger_ intercept	*p*-value	Causal estimate	SD	*T*	*p*-value	RSSobs	Global test *p*-value
Eubacterium nodatum group	−0.003	0.917	−0.103	0.030	−3.475	0.006	7.369	0.831
Ruminococcaceae UCG011	0.028	0.370	−0.099	0.041	−2.418	0.046	8.353	0.520
Lachnospiraceae UCG010	0.019	0.453	0.224	0.100	2.238	0.052	17.978	0.123

MR-PRESSO, Mendelian randomization pleiotropy RESidual sum and outlier; SD, standard deviation; RSSobs, observed residual sum of squares.

All statistical analyses were performed using RStudio version 4.3.1. To estimate causal effects, we utilized the "TwoSampleMR" package, while the detection of horizontal pleiotropy and outliers were conducted using the "MR-PRESSO" package (Hemani et al., 2018; Verbanck et al., 2018). MVMR analysis was performed with the "MVMR" and "Mendelian Randomization" packages. The significance cutoff was set at $p < 0.05$, and the resulting MR estimates are presented as odds ratios (ORs) along with their corresponding 95% confidence intervals (CIs).

3 Results

3.1 Two-sample MR of gut microbiotas (exposure) on anxiety disorders (outcome)

Initially, 1,579 SNPs were selected as IVs for 119 bacterial genera after excluding unknown bacterial genera from the GWAS statistics (as shown in Supplementary Table S2). These IVs were then used in the forward MR analysis to investigate the potential causal effect of bacterial genera on anxiety disorders. The results of the initial MR analysis are presented in Supplementary Table S3. As shown in Table 1, the IVW estimate suggested that the genus *Eubacterium nodatum group* [OR = 0.902, 95%CI: 0.837–0.972, $p = 0.007$], and genus *Ruminococcaceae UCG011* [OR = 0.905, 95%CI: 0.831–0.987, $p = 0.023$] protected against anxiety disorders. However, the genus *Lachnospiraceae UCG010* [OR = 1.251, 95%CI: 1.028–1.522, $p = 0.025$] was associated with an increased risk of anxiety disorders.

As presented in Tables 1, 2, Cochran's Q statistic for both the MR-IVW and MR-Egger regression analyses did not show significant IV heterogeneity ($p > 0.05$ for all), indicating that heterogeneity bias was unlikely to have influenced our results. Furthermore, the results of the horizontal pleiotropy tests, including the MR-Egger intercept test and the MR-PRESSO global test, also showed no significant evidence of horizontal pleiotropy ($p > 0.05$ for all). These findings were supported by the leave-one-out plots and scatter plots shown in Figure 2.

3.2 Reverse MR analysis of anxiety disorders (exposure) on identified bacterial genera (outcome)

Reverse MR analysis was next performed to evaluate the causal effect of anxiety disorders on the three identified bacterial genera belonging to the genus *Eubacterium nodatum group*, genus *Ruminococcaceae UCG011*, and genus *Lachnospiraceae UCG010*. After selecting suitable SNPs and removing palindromic ones in Supplementary Table S4, we used 13, 14, and 15 SNPs, respectively, as potential IVs in the reverse MR analysis to investigate the potential causal effect of anxiety disorders on the following bacterial genera: *Eubacterium nodatum group*, *Ruminococcaceae UCG011*, and *Lachnospiraceae UCG010*. As shown in Table 3, reverse MR analysis

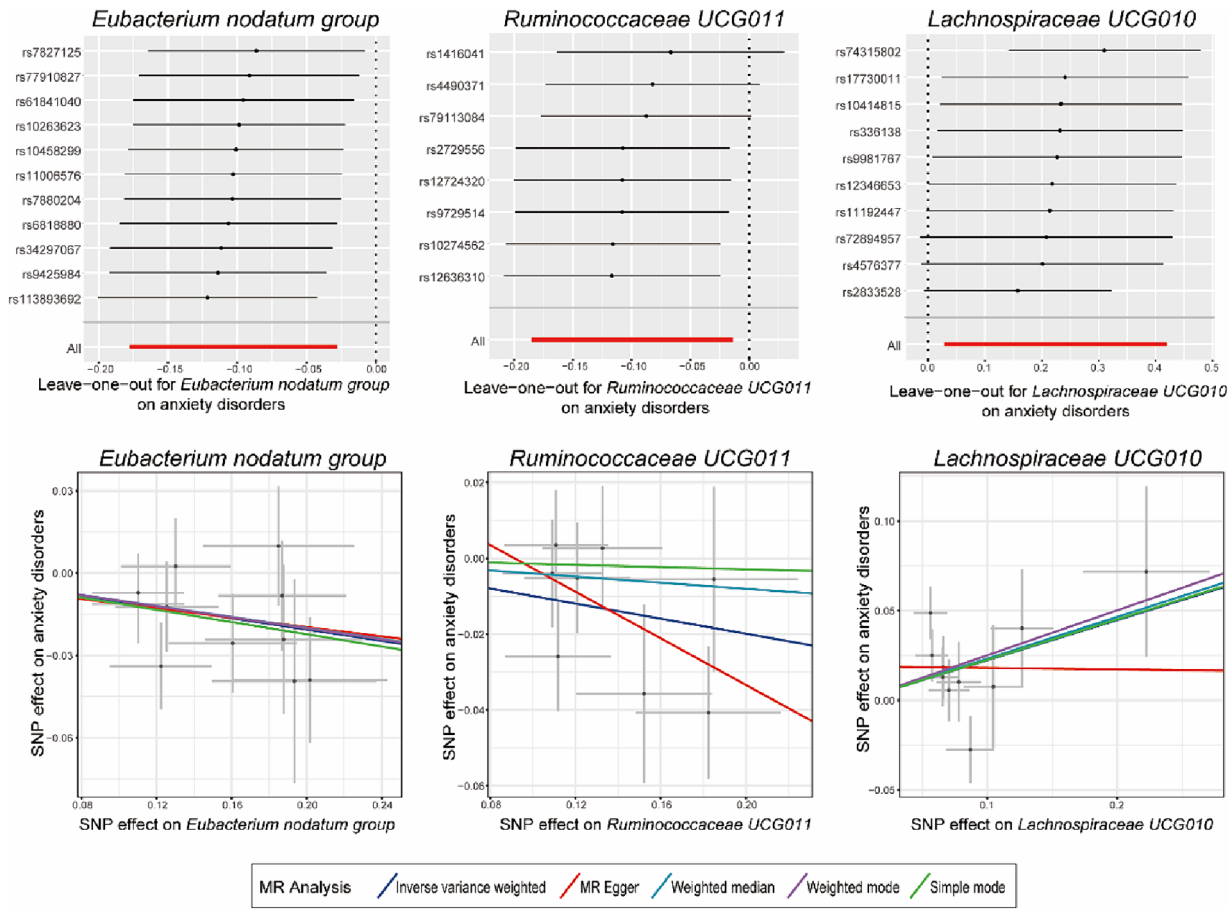

FIGURE 2
Leave-one-out plots and scatter plots for the causal effects of identified bacterial genera on the anxiety disorders. MR, Mendelian randomization; SNP, single nucleotide polymorphism; MR Egger, MR-Egger regression.

showed that there was no evidence of a causal effect of anxiety disorders on these three identified bacterial genera ($p > 0.05$ for all). As presented in Tables 3, 4, Cochran's Q statistic showed that there was no significant heterogeneity of the IVs ($p > 0.05$ for all), and the results of the MR-Egger intercept test and MR-PRESSO global test also did not detect significant horizontal pleiotropy ($p > 0.05$ for all).

3.3 MVMR to assess the effect of neurotransmitters associated metabolites on observed associations between identified bacterial genera (exposure) on anxiety disorders (outcome)

Considering the possible contribution of neurotransmitter-associated metabolites to the pathobiology of gut microbiota-associated anxiety disorders, we used MVMR for the observed significant associations (the results in Table 1) adjusted for seven common metabolites (tryptophan, tyrosine, phenylalanine, glutamate, glycine, cortisol and isovalerate). The MVMR results are reported in Table 5. When employing the MVMR-IVW method to account for the combined influence of all seven metabolites, the previously identified significant associations between identified bacterial genera and anxiety disorders were no longer significant following adjustments ($p > 0.05$ for all), and there was no significant heterogeneity in these results ($p > 0.05$ for all). Subsequently, focusing on the genus *Eubacterium nodatum group*, genus *Ruminococcaceae UCG011*, and genus *Lachnospiraceae UCG010*, we next adjusted only one metabolite at a time (Table 6). Signals of a decrease in significance of the association were detected by MVMR-IVW for genus *Eubacterium nodatum group* [$p = 0.931$ for tryptophan; $p = 0.268$ for tyrosine; $p = 0.151$ for phenylalanine; $p = 0.083$ for glycine; $p = 0.127$ for cortisol], genus *Ruminococcaceae UCG011* [$p = 0.346$ for tyrosine; $p = 0.124$ for phenylalanine; $p = 0.088$ for glycine; $p = 0.063$ for cortisol], and genus *Lachnospiraceae UCG010* [$p = 0.149$ for tryptophan]. Additionally, heterogeneity testing indicated that our MVMR estimates were unlikely biased by heterogeneity ($p > 0.05$ for all).

3.4 MVMR to assess the impact of dietary patterns, drug or alcohol consumption, and lifestyle factors on observed associations between the identified bacterial genera (exposure) and anxiety disorders (outcome)

Considering the potential impact of dietary patterns, drug or alcohol consumption, and lifestyle factors on the causal effects of the identified bacterial genera on anxiety disorders, we used MVMR for

TABLE 3 Reverse MR estimates and heterogeneity test for anxiety disorders (exposure) on identified bacterial genera (outcome).

Bacterial genera (outcome)	MR method	No. of SNP	Beta	SE	OR	95% CI	*p*-value	Heterogeneity test		
								Cochran's Q	Q_df	*p*-value
Eubacterium nodatum group	IVW	13	0.051	0.123	1.052	0.827–1.340	0.679	19.344	12	0.081
	MR-Egger	13	−0.319	0.334	0.727	0.378–1.399	0.360	17.143	11	0.104
	WME	13	0.007	0.146	1.007	0.756–1.341	0.963	–	–	–
	Weighted model	13	−0.025	0.206	0.975	0.651–1.461	0.904	–	–	–
	Simple model	13	0.112	0.237	1.119	0.702–1.781	0.645	–	–	–
Ruminococcaceae UCG011	IVW	14	0.017	0.090	1.017	0.853–1.212	0.854	6.374	13	0.931
	MR-Egger	14	−0.040	0.257	0.961	0.581–1.590	0.878	6.318	12	0.899
	WME	14	0.011	0.123	1.011	0.794–1.287	0.931	–	–	–
	Weighted model	14	−0.024	0.193	0.976	0.669–1.424	0.902	–	–	–
	Simple model	14	−0.175	0.204	0.839	0.563–1.252	0.406	–	–	–
Lachnospiraceae UCG010	IVW	15	−0.021	0.052	0.979	0.884–1.083	0.679	16.984	14	0.257
	MR-Egger	15	0.098	0.150	1.103	0.822–1.481	0.523	16.086	13	0.245
	WME	15	−0.021	0.068	0.979	0.856–1.119	0.755	–	–	–
	Weighted model	15	0.075	0.110	1.078	0.868–1.338	0.508	–	–	–
	Simple model	15	−0.062	0.123	0.940	0.739–1.196	0.622	–	–	–

MR, Mendelian randomization; SNP, single nucleotide polymorphism; SE, standard error; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted; WME, weighted median estimator.

TABLE 4 MR-Egger intercept test and MR-PRESSO analysis for associations between anxiety disorders (exposure) and identified bacterial genera (outcome).

Bacterial genera (exposure)	MR-Egger intercept test		MR-PRESSO analysis					
	Egger_ intercept	*p*-value	Causal estimate	SD	*T*	*p*-value	RSSobs	Global test *p*-value
Eubacterium nodatum group	0.036	0.260	0.051	0.123	0.413	0.687	22.516	0.100
Ruminococcaceae UCG011	0.005	0.818	0.017	0.063	0.263	0.797	7.329	0.938
Lachnospiraceae UCG010	−0.011	0.410	−0.021	0.052	−0.414	0.685	19.408	0.283

MR-PRESSO, Mendelian randomization pleiotropy RESidual sum and outlier; SD, standard deviation; RSSobs, observed residual sum of squares.

TABLE 5 MVMR results of causal links between identified bacterial genera (exposure) on anxiety disorders (outcome) after adjusting for all neurotransmitter-associated metabolites.

Bacterial genera (exposure)	Adjustment of confounder	MR method	Beta	SE	OR	95% CI	*p*-value	Heterogeneity *p*-value
Eubacterium nodatum group	Seven metabolites	MVMR-IVW	0.020	0.040	1.020	0.944–1.102	0.621	0.166
		MVMR-Egger	−0.038	0.056	0.963	0.863–1.074	0.500	0.182
Ruminococcaceae UCG011	Seven metabolites	MVMR-IVW	−0.075	0.040	0.928	0.858–1.002	0.057	0.213
		MVMR-Egger	−0.078	0.058	0.924	0.825–1.036	0.176	0.197
Lachnospiraceae UCG010	Seven metabolites	MVMR-IVW	0.083	0.082	1.087	0.925–1.277	0.312	0.185
		MVMR-Egger	0.316	0.111	1.372	1.104–1.704	0.004	0.328

MVMR, multivariable Mendelian randomization; SE, standard error; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted.

observed significant associations (the results in Table 1) adjusted for dietary patterns, drug or alcohol consumption, and lifestyle factors. We implemented adjustments for dietary pattern (salt added to food, processed meat intake, intake of sugar added to cereal), drug or alcohol consumption (more than once), and lifestyle factors (smoking status and physical activity) one at a time, and the MVMR results are reported in Table 7. Signals of a decline in the significance of association were detected in the MVMR-IVW for genus *Eubacterium nodatum group* [$p = 0.223$ for salt added to food; $p = 0.646$ for processed meat intake; $p = 0.224$ for smoking status], genus *Ruminococcaceae UCG011* [$p = 0.418$ for salt added to food; $p = 0.951$ for processed meat intake; $p = 0.748$ for smoking status; $p = 0.083$ for physical activity], and genus *Lachnospiraceae UCG010* [$p = 0.729$ for salt added to food; $p = 0.082$ for processed meat intake; $p = 0.107$ for intake of sugar added to cereal; $p = 0.844$ for smoking status; $p = 0.054$ for physical activity]. Heterogeneity testing indicated that our MVMR estimates were unlikely biased by heterogeneity (most $p > 0.05$).

4 Discussion

This study exploited the summary statistics of gut microbiota obtained from the largest GWAS meta-analysis conducted by the MiBioGen consortium, which included 18,340 individuals across 24

10.3389/fmicb.2024.1380912

TABLE 6 Multivariable MR results of causal links between identified bacterial genera (exposure) on anxiety disorders (outcome) after adjusting for specific neurotransmitter-associated metabolite.

Bacterial genera (exposure)	Adjustment of confounder	MR method	Beta	SE	OR	95% CI	*p*-value	Heterogeneity *p*-value
Eubacterium nodatum group	Tryptophan	MVMR-IVW	0.004	0.042	1.004	0.924–1.090	0.931	0.186
		MVMR-Egger	−0.031	0.059	0.969	0.864–1.088	0.595	0.182
	Tyrosine	MVMR-IVW	−0.050	0.045	0.951	0.872–1.039	0.268	0.763
		MVMR-Egger	−0.120	0.060	0.887	0.788–0.998	0.046	0.850
	Phenylalanine	MVMR-IVW	−0.075	0.052	0.928	0.838–1.028	0.151	0.325
		MVMR-Egger	−0.234	0.089	0.792	0.665–0.942	0.009	0.701
	Glutamate	MVMR-IVW	−0.098	0.049	0.907	0.823–0.998	0.046	0.955
		MVMR-Egger	−0.151	0.220	0.860	0.559–1.323	0.492	0.917
	Glycine	MVMR-IVW	−0.082	0.047	0.921	0.840–1.011	0.083	0.812
		MVMR-Egger	−0.096	0.060	0.908	0.808–1.022	0.109	0.775
	Cortisol	MVMR-IVW	−0.074	0.048	0.928	0.845–1.021	0.127	0.784
		MVMR-Egger	−0.195	0.074	0.822	0.711–0.952	0.009	0.984
	Isovalerate	MVMR-IVW	−0.109	0.049	0.897	0.815–0.986	0.025	0.851
		MVMR-Egger	−0.160	0.084	0.852	0.723–1.004	0.056	0.835
Ruminococcaceae UCG011	Tryptophan	MVMR-IVW	−0.083	0.042	0.920	0.847–0.999	0.047	0.232
		MVMR-Egger	−0.091	0.061	0.913	0.810–1.028	0.132	0.216
	Tyrosine	MVMR-IVW	−0.045	0.047	0.956	0.872–1.049	0.346	0.787
		MVMR-Egger	−0.079	0.067	0.924	0.811–1.053	0.235	0.772
	Phenylalanine	MVMR-IVW	−0.089	0.058	0.915	0.817–1.025	0.124	0.131
		MVMR-Egger	−0.113	0.116	0.893	0.711–1.120	0.328	0.089
	Glutamate	MVMR-IVW	−0.091	0.045	0.913	0.835–0.998	0.045	0.572
		MVMR-Egger	−0.066	0.105	0.936	0.762–1.150	0.529	0.463
	Glycine	MVMR-IVW	−0.074	0.043	0.929	0.853–1.011	0.088	0.655
		MVMR-Egger	−0.069	0.060	0.933	0.829–1.050	0.251	0.599
	Cortisol	MVMR-IVW	−0.084	0.045	0.919	0.841–1.005	0.063	0.536
		MVMR-Egger	−0.191	0.074	0.826	0.714–0.955	0.010	0.761
	Isovalerate	MVMR-IVW	−0.113	0.045	0.893	0.817–0.976	0.013	0.725
		MVMR-Egge	−0.055	0.091	0.946	0.792–1.131	0.543	0.690
Lachnospiraceae UCG010	Tryptophan	MVMR-IVW	0.120	0.083	1.128	0.958–1.327	0.149	0.202
		MVMR-Egger	0.317	0.108	1.373	1.110–1.697	0.003	0.329
	Tyrosine	MVMR-IVW	0.290	0.086	1.336	1.130–1.580	0.001	0.662
		MVMR-Egger	0.377	0.108	1.458	1.180–1.802	0.001	0.700
	Phenylalanine	MVMR-IVW	0.294	0.113	1.342	1.075–1.675	0.009	0.129
		MVMR-Egger	0.274	0.202	1.315	0.886–1.953	0.175	0.088
	Glutamate	MVMR-IVW	0.309	0.091	1.363	1.140–1.629	0.001	0.450
		MVMR-Egger	0.363	0.158	1.438	1.055–1.958	0.021	0.366
	Glycine	MVMR-IVW	0.278	0.087	1.321	1.115–1.565	0.001	0.601
		MVMR-Egger	0.429	0.112	1.536	1.234–1.912	0.001	0.799
	Cortisol	MVMR-IVW	0.287	0.103	1.332	1.089–1.630	0.005	0.321
		MVMR-Egge	0.377	0.141	1.458	1.105–1.923	0.008	0.316
	Isovalerate	MVMR-IVW	0.319	0.092	1.376	1.149–1.648	0.001	0.421
		MVMR-Egge	0.449	0.137	1.566	1.196–2.051	0.001	0.470

MVMR, multivariable Mendelian randomization; SE, standard error; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted.

separate cohorts. Predicted through genetic factors at a more precise genus level (utilizing a total of 1,579 SNPs for 119 bacterial genera, after excluding unknown bacterial genera), our MR study provides evidence that gut microbiota can have a causal effect on anxiety disorders. Specifically, at the genus level, *Eubacterium nodatum group* and *Ruminococcaceae UCG011* appeared to be protective, while *Ruminococcaceae UCG010* was associated with an increased risk of anxiety disorders. Reverse MR analysis and a series of sensitivity analyses also confirmed the robustness of these findings. Currently, research on the association between anxiety disorders and these identified bacterial genera are lacking. However, interventions targeting improvements in the structural composition of the gut microbiota, such as the abundance of *Eubacterium nodatum group* genus, have shown promising efficacy in the treatment of attention deficit hyperactivity disorder (Tang et al., 2022). Another study provided further evidence that remodeling the gut microbiota by

10.3389/fmicb.2024.1380912

TABLE 7 MVMR results of causal links between the identified bacterial genera (exposure) and anxiety disorders (outcome) after adjusting for dietary pattern, drug or alcohol consumption, and lifestyle factors.

Bacterial genera (exposure)	Adjustment of confounder	MR method	Beta	SE	OR	95% CI	*p*-value	Heterogeneity *p*-value
Eubacterium nodatum group	Salt added to food	MVMR-IVW	−0.042	0.034	0.959	0.897–1.026	0.223	0.049
		MVMR-Egger	−0.068	0.050	0.934	0.846–1.031	0.175	0.047
	Processed meat intake	MVMR-IVW	−0.022	0.048	0.978	0.891–1.074	0.646	5e-04
		MVMR-Egger	−0.085	0.068	0.918	0.804–1.049	0.209	6e-04
	Intake of sugar added to cereal	MVMR-IVW	−0.109	0.046	0.897	0.820–0.981	0.017	0.125
		MVMR-Egger	−0.183	0.070	0.832	0.725–0.955	0.009	0.164
	Drug or alcohol consumption	MVMR-IVW	−0.100	0.038	0.905	0.840–0.973	0.008	0.684
		MVMR-Egger	−0.040	0.059	0.961	0.857–1.078	0.500	0.739
	Smoking status	MVMR-IVW	−0.037	0.030	0.964	0.909–1.023	0.224	0.044
		MVMR-Egger	−0.033	0.047	0.967	0.883–1.060	0.480	0.041
	Physical activity	MVMR-IVW	−0.095	0.036	0.909	0.848–0.975	0.007	0.900
		MVMR-Egger	−0.139	0.047	0.871	0.793–0.955	0.004	0.921
Ruminococcaceae UCG011	Salt added to food	MVMR-IVW	−0.029	0.036	0.971	0.905–1.042	0.418	0.047
		MVMR-Egger	−0.028	0.054	0.973	0.874–1.082	0.611	0.043
	Processed meat intake	MVMR-IVW	−0.003	0.048	0.997	0.908–1.095	0.951	5e-04
		MVMR-Egger	0.001	0.064	1.001	0.884–1.133	0.992	4e-04
	Intake of sugar added to cereal	MVMR-IVW	−0.111	0.052	0.895	0.809–0.990	0.031	0.100
		MVMR-Egger	−0.113	0.085	0.893	0.756–1.055	0.183	0.074
	Drug or alcohol consumption	MVMR-IVW	−0.097	0.042	0.907	0.835–0.986	0.022	0.656
		MVMR-Egger	−0.058	0.069	0.944	0.825–1.080	0.401	0.628
	Smoking status	MVMR-IVW	−0.010	0.032	0.990	0.930–1.054	0.748	0.068
		MVMR-Egger	−0.053	0.049	0.948	0.861–1.045	0.282	0.070
	Physical activity	MVMR-IVW	−0.071	0.041	0.931	0.859–1.009	0.083	0.477
		MVMR-Egger	−0.114	0.059	0.892	0.795–1.001	0.052	0.479
Lachnospiraceae UCG010	Salt added to food	MVMR-IVW	−0.024	0.070	0.976	0.851–1.120	0.729	0.050
		MVMR-Egger	0.104	0.099	1.109	0.913–1.347	0.296	0.063
	Processed meat intake	MVMR-IVW	0.150	0.086	1.162	0.981–1.377	0.082	8e-04
		MVMR-Egger	0.053	0.11	1.054	0.837–1.327	0.654	9e-04
	Intake of sugar added to cereal	MVMR-IVW	0.177	0.110	1.194	0.962–1.482	0.107	0.088
		MVMR-Egger	0.038	0.145	1.039	0.782–1.381	0.792	0.129
	Drug or alcohol consumption	MVMR-IVW	0.230	0.085	1.259	1.065–1.489	0.007	0.220
		MVMR-Egger	0.170	0.127	1.186	0.925–1.520	0.180	0.198
	Smoking status	MVMR-IVW	0.013	0.064	1.013	0.893–1.148	0.844	0.070
		MVMR-Egger	0.042	0.095	1.043	0.866–1.257	0.656	0.066
	Physical activity	MVMR-IVW	0.161	0.083	1.174	0.997–1.383	0.054	0.231
		MVMR-Egger	0.141	0.103	1.151	0.941–1.409	0.171	0.205

MVMR, multivariable Mendelian randomization; SE, standard error; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted.

restoring dysbiosis characterized by the presence of genus *Eubacterium nodatum group* and other relevant bacterial genera effectively improved the disruption of the gut-brain barrier, thereby providing a potential therapeutic approach for the treatment of cerebral ischemic stroke (Chen et al., 2019). The genus *Ruminococcaceae UCG011* and genus *Ruminococcaceae UCG010*, belonging to family *Ruminococcaceae*, have been reported as associated with some diseases such as leukemia (Chen et al., 2023), type 2 diabetes (Li and Li, 2023), and esophageal cancer (Wang et al., 2024), but they have yet to be studies in the context of mental health disorders. However, multiple clinical studies have demonstrated that psychological distress and mental illness, such as depression and anxiety, are associated with alterations in the abundance of *Ruminococcaceae* family members (Chen et al., 2021; Chin Fatt et al., 2023), and animal studies have suggested that regulating the abundance of *Ruminococcaceae* family members with various interventions can promote the production of SCFAs and other neurochemical-related metabolites. These metabolites, in turn, modulate neurotransmitter-associated metabolism, inflammation, and the microbiota-gut-brain axis, thereby alleviating depressive and anxiety-like behaviors in different mouse models of chronic stress (Chen Y. et al., 2022; Zhou et al., 2022).

Given the limited research on the bacterial genera associated with anxiety disorders identified in our MR analysis, it is crucial to investigate the role of metabolites that are closely linked to gut microbiota and anxiety disorders. Our study utilized large-scale GWAS data and involved MVMR analysis to identify potential metabolic pathways involving neurotransmitter-associated metabolites, which are believed to facilitate communication between

the gut microbiota and the brain. Our results showed that tyrosine, phenylalanine, glycine, and cortisol played a crucial role in the protective effects of genus *Eubacterium nodatum group* and genus *Ruminococcaceae UCG011* on anxiety disorders. First, tyrosine is the precursor to dopamine, a neurotransmitter responsible for regulating brain functions related to emotions, rewards, and motivation, and the gut microbiota has been found to regulate tyrosine metabolism, which subsequently affects dopamine synthesis and release to the CNS. Imbalances in the dopamine system can interfere with brain's limbic system activities, resulting in exaggerated neuronal excitations in anxiety-related brain areas such as the amygdala and prefrontal cortex, leading to emotional instability and worsening anxiety disorders (Needham et al., 2022; Zhang D. et al., 2022). Second, phenylalanine not only serves as a precursor of tyrosine but also plays a crucial role in promoting the synthesis and secretion of endorphins in the brain. Indeed, recent studies have shown that the gut microbiota can help with the digestion and absorption of phenylalanine, and the metabolic activity of phenylalanine can be affected by an imbalanced gut microbiota, in turn affecting the synthesis and secretion of endorphins in the CNS, ultimately influencing the occurrence and severity of anxiety disorders (Deng et al., 2022; Zhang D.-D. et al., 2022). Third, glycine can effectively suppress neuronal activity and impact brain excitability. Several studies have shown that the gut microbiota can disrupt glycine metabolism and glycine receptor signaling, thereby increasing the risk of anxiety and other neuropsychiatric disorders (Lin et al., 2021; Zhang D. et al., 2022). Lastly, cortisol, a hormone secreted by the adrenal gland, plays a crucial role in stress responses. The gut microbiota affects cortisol synthesis and metabolism through the breakdown of fibers and polysaccharides to produce metabolites like SCFAs. Sustained elevation of cortisol levels not only negatively affects the hippocampus and prefrontal cortex of the brain, impairing mood regulation, decision-making, and cognitive functions through activation of microglia-induced neuroinflammation, but also alters neurotransmitter systems in the brain, such as the serotonin and GABA systems critical for emotional regulation (Dalile et al., 2020; Tian et al., 2023). In addition, only tryptophan was found to have a potential impact on the negative effect of genus *Ruminococcaceae UCG010* on anxiety disorders. Tryptophan is an essential amino acid for the synthesis of serotonin, which plays critical roles in regulating mood, behavior, and cognition. It has been shown that disruptions in the gut microbiota can have consequences on tryptophan metabolism and utilization by (1) giving rise to the production of tryptophan antagonists, and (2) regulating tryptophan transporters to reduce tryptophan reaching the brain. These processes can result in aberrant function and reduced levels of CNS serotonin, contributing to the development and continuation of anxiety disorders (Deng et al., 2021; Jia et al., 2024).

Previous studies have suggested that the gut microbiota may be affected by dietary patterns such as salt added to food, processed meat intake, and intake of sugar added to cereals; drug or alcohol consumption; and lifestyle factors such as smoking and physical activity (Zhu et al., 2020; Simpson et al., 2021; Cataldi et al., 2022). We therefore used MVMR analysis to assess the impact of these confounding variables on the observed causal associations between the identified bacterial genera and anxiety disorders. Our findings showed that excessive consumption of processed meat products and sugar and improper intake of salt (too much or too little) increased the risk of developing anxiety by regulating the gut microbiota. Regarding dietary patterns, emerging evidence suggests that unhealthy dietary can alter the gut microbiota composition, in turn disrupting metabolic functions and the immune system, thereby inducing anxiety disorders (Bear et al., 2020; Zhu et al., 2020). Additionally, we also found that controlling smoking and engaging in moderate physical activity are important lifestyle factors that reduce the risk of anxiety disorders through interactions with the identified bacterial genera. Indeed, recent studies have reported that tobacco smoke components such as nicotine are present in the gastrointestinal tract and disrupt the gut microbiota (Chen et al., 2024), interfering with vagal nerve signaling and neurotransmission, as well as inducing CNS inflammation, ultimately increasing the risk of mental health illnesses such as depression, schizophrenia, and anxiety disorders (Curtis et al., 2019; Wang et al., 2023). Furthermore, prioritizing aerobic physical activity at moderate intensity, rather than prolonged intensity, seems to be useful in regulating the diversity and richness of beneficial bacterial taxa in both humans and animals. This improves physical performance and triggers beneficial changes in the brain, including an increase in the production of neurotransmitters such as dopamine, glutamate, and GABA and reducing central inflammatory responses (Cataldi et al., 2022). Therefore, with a growing understanding of the mechanisms involved in the complex interplay between dietary and lifestyle factors, the gut microbiota, and its impact on mental illness, specific dietary and lifestyle patterns might potentially be translated into clinical practice to help prevent anxiety and mood disorders. We propose that dietary and lifestyle interventions have the dual benefit of a direct impact on gut and brain physiology and an indirect effect via the gut microbiota, and the incorporation of dietary and lifestyle interventions may prove to be an attractive and cost-effective alternative or adjuvant therapy for the clinical management of anxiety disorders.

The main strength of this study is the use of bidirectional MR analysis to investigate the causal effects and identify specific bacterial taxa at the genus level linked to anxiety disorders. This approach effectively minimizes potential confounding factors and reverse causation, providing reliable evidence for causal relationships between gut microbiotas and anxiety disorders. The use of multiple statistical models and sensitivity analyses ensured the accuracy and validity of the estimated causal effects. Moreover, applying MVMR analysis to explore the potential impact of various neurotransmitter-associated metabolites, which are highly associated with anxiety disorders, on the causal effects of identified bacterial genera on anxiety disorders might enable us to target metabolic pathways connecting the gut microbiota to anxiety disorders. Our study design allowed us to gain a deeper understanding of the complex interactions and gut-brain communication mechanisms mediating the gut microbiota and anxiety disorders. Building on this, we conducted additional MVMR analyses to investigate the impact of relevant dietary patterns, drug or alcohol use, and lifestyle factors on the causal effects of specific bacteria genera on anxiety disorders. By modifying dietary patterns and adjusting lifestyle factors such as smoking and physical activity, it may be possible to develop more precise and effective strategies for targeting specific gut microbiota to manage anxiety disorders.

However, our study has some limitations. First, our study only focused on individuals of European ancestry and did not consider the impact of sex. Thus, caution is needed when generalizing our findings to other populations, and future

research should include diverse populations and conduct sex-specific analyses. Second, it is essential to study the gut microbiome as a comprehensive ecosystem, rather than focusing solely on individual species or strains. Longitudinal studies would be valuable in revealing the dynamic changes in gut microbiota associated with the development of anxiety disorders. Third, in our study, we analyzed metabolites in multivariable MR that were detected in human serum. While we acknowledge that fecal samples may provide more appropriate and direct information, unfortunately, such data are currently unavailable. Additionally, other neurotransmitters, such as norepinephrine, and more crucial SCFAs like propionate and butyrate, might be important to consider, but summary data on these compounds or their related metabolites were also lacking. In summary, although our research provides valuable insights into the relationship between gut microbiota and anxiety disorders, it is crucial to acknowledge and address these limitations through further high-quality experimental and clinical studies. This should involve exploring higher taxonomic levels, incorporating the broader microbial diversity within the gut ecosystem, and considering the broader confounding factors within dietary and lifestyle variables.

5 Conclusion

Overall, we detected three causal associations through MR analysis on the impacts of 119 bacterial taxa on anxiety disorders. Among them, the genus *Eubacterium nodatum group* and genus *Ruminococcaceae UCG011* protected against anxiety disorders, while genus *Ruminococcaceae UCG011* was associated with an increased risk of anxiety disorders. A metabolite-dependent mechanism, primarily driven by tyrosine, phenylalanine, glycine, and cortisol, played a crucial role in genus *Eubacterium nodatum group* and genus *Ruminococcaceae UCG011* on anxiety disorders, while tryptophan was found to have a potential impact on genus *Ruminococcaceae UCG011*. In addition, excessive consumption of processed meat products and sugar, improper salt intake, smoking status, and excessive and prolonged physical activity could increase the risk of developing anxiety by regulating the gut microbiota. Further research is necessary to advance our understanding of the biological mechanisms responsible for the causal relationship between the microbiota-gut-brain axis and onset and development of anxiety disorders. This could help in the discovery of valuable biomarkers and potential therapy targets.

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.

Author contributions

M-MX: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. W-HQ: Supervision, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft. Q-YM: Resources, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft. Z-YY: Project administration, Validation, Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision. W-MY: Writing – review & editing, Conceptualization, Data curation, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization. T-NH: Supervision, Writing – review & editing, Data curation, Formal analysis, Methodology. YG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing, Writing – original draft. X-YC: Writing – original draft, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1380912/full#supplementary-material

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Anorexia nervosa and bulimia nervosa: a Mendelian randomization study of gut microbiota

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Background: Anorexia nervosa (AN) and bulimia nervosa (BN) poses a significant challenge to global public health. Despite extensive research, conclusive evidence regarding the association between gut microbes and the risk of AN and BN remains elusive. Mendelian randomization (MR) methods offer a promising avenue for elucidating potential causal relationships.

Materials and methods: Genome-wide association studies (GWAS) datasets of AN and BN were retrieved from the OpenGWAS database for analysis. Independent single nucleotide polymorphisms closely associated with 196 gut bacterial taxa from the MiBioGen consortium were identified as instrumental variables. MR analysis was conducted utilizing R software, with outlier exclusion performed using the MR-PRESSO method. Causal effect estimation was undertaken employing four methods, including Inverse variance weighted. Sensitivity analysis, heterogeneity analysis, horizontal multivariate analysis, and assessment of causal directionality were carried out to assess the robustness of the findings.

Results: A total of 196 bacterial taxa spanning six taxonomic levels were subjected to analysis. Nine taxa demonstrating potential causal relationships with AN were identified. Among these, five taxa, including *Peptostreptococcaceae*, were implicated as exerting a causal effect on AN risk, while four taxa, including *Gammaproteobacteria*, were associated with a reduced risk of AN. Similarly, nine taxa exhibiting potential causal relationships with BN were identified. Of these, six taxa, including *Clostridiales*, were identified as risk factors for increased BN risk, while three taxa, including *Oxalobacteraceae*, were deemed protective factors. *Lachnospiraceae* emerged as a common influence on both AN and BN, albeit with opposing effects. No evidence of heterogeneity or horizontal pleiotropy was detected for significant estimates.

Conclusion: Through MR analysis, we revealed the potential causal role of 18 intestinal bacterial taxa in AN and BN, including *Lachnospiraceae*. It provides new insights into the mechanistic basis and intervention targets of gut microbiota-mediated AN and BN.

KEYWORDS

anorexia nervosa, bulimia nervosa, gut microbiota, Mendelian randomization analysis, eating disorders

1 Introduction

Recent epidemiological studies indicate that 16% of global mortality can be attributed to mental disorders, rendering them among the leading causes of disability and premature death worldwide (Murray et al., 2020; Arias et al., 2022). Eating disorders are one of the common types of mental disorders, exhibiting an age-standardized prevalence of 174.0 per 100,000 population. The prevalence of eating disorders in high-income countries is about three times the global average (GBD 2019 Mental Disorders Collaborators, 2022). Anorexia nervosa (AN) and bulimia nervosa (BN) represent the most prevalent types of eating disorders and are uniquely identified as psychiatric conditions with elevated mortality risk in the Global Burden of Disease Study 2019 (GBD 2019 Demographics Collaborators, 2020). A common feature of patients with both disorders is an excessive focus on weight and body shape and attempts to control weight. Patients with AN often adopt excessive behaviors to avoid weight gain, while patients with BN experience multiple overeating followed by inappropriate weight-compensating behaviors (Timko et al., 2019). Studies have suggested that AN and BN may share the same psychopathological features and have reciprocal transformations, manifesting as similar behaviors, such as impulsivity and compulsion (Howard et al., 2020).

Cognitive behavioral therapy is the first-line treatment for adults with AN and BN (Treasure et al., 2020). Nevertheless, a meta-analysis found that over 60% of patients failed to fully abstain from core symptoms even after receiving the best available treatments (Slade et al., 2018). Maudsley model and focal psychodynamic psychotherapy are also first-line treatments for adults with AN. For adults with BN, third-wave behavioral therapies are feasible attempts, such as dialectical behavior therapy, acceptance and commitment therapy. However, previous evaluations have shown little difference in efficacy between these therapies and cognitive behavioral therapy (Byrne et al., 2017; Linardon et al., 2017). For adolescents with AN and BN, family-based interventions are recommended as first-line treatments by international evidence-based guidelines (Hilbert et al., 2017). However, a Cochrane review suggests that the evidence favoring family-based interventions over standard treatment or other psychological approaches is not very solid (Fisher et al., 2019). Therefore, new treatments for AN and BN, such as the novel ghrelin receptor agonist, and transcranial direct-current stimulation are still worthy of in-depth research and exploration (van Passel et al., 2020; Solmi et al., 2021).

The gut microbiota intricately participates in various physiological processes crucial for human well-being, including metabolic regulation and immune homeostasis (Fujisaka et al., 2018; Chen et al., 2022). Emerging evidence suggests that the microbiota and the central nervous system communicate bidirectionally via the microbiota-gut-brain axis, thereby influencing the pathophysiology of psychiatric disorders (Agirman et al., 2021; Cryan and Mazmanian, 2022; Fan et al., 2023). A recent study found that, multiple bacterial taxa, such as *Clostridium*, were altered in AN and correlated with estimates of eating behavior and mental health (Fan et al., 2023). A systematic review provided a clearer picture of the gut microbiota characteristics of AN patients. Preserved alpha-diversity and decreased beta-diversity were found in the qualitative synthesis. Three gut microbes (*Alistipes, Parabacterioides and Roseburia*), are able to effectively differentiate patients from controls (Di Lodovico et al., 2021). Analogously, investigations into BN have corroborated a potential association between gut microbiota and BN development. Different pathological behaviors may be associated with a reduction in microbial diversity and typical microbiota-derived metabolites (Castellini et al., 2023). These findings underscore the intertwined relationship between gut microbiota and the progression of AN and BN. However, the causal link between specific bacterial taxa and AN and BN necessitates further elucidation.

Conventionally, randomized controlled trials represent the gold standard for establishing causality. However, ethical dilemmas, compliance issues and difficulties in family coordination add additional challenges to the study of AN and BN, especially for adolescents (Frostad and Bentz, 2022; Tsiandoulas et al., 2023). Mendelian randomization (MR) offers a pragmatic alternative approach for assessing causality by utilizing single nucleotide polymorphisms (SNPs) as genetic instrumental variables (IVs) to statistically infer the causal impact of exposures on outcomes (Kurilshikov et al., 2021). This method emulates the random allocation process, thereby mitigating the influence of confounding variables (Emdin et al., 2017). Moreover, since the microbiome does not induce alterations in an individual's DNA sequence, analyzing the causal relationship between gut microbiota and AN and BN through MR studies holds practical clinical relevance (Thomas, 2019). In this investigation, we employed two-sample MR analysis to scrutinize the potential causal role of gut microbiota in AN and BN, delineated specific pathogenic bacterial taxa, and elucidated their similarities, disparities, and associated mechanisms.

2 Materials and methods

2.1 Study overview

According to the law of independent assortment, genetic variants will be randomly assorted to gametes during meiosis. MR analysis uses this law as a principle to simulate randomized controlled trials using SNPs as genetic IVs. Because of this, MR study is seen as an appropriate method for analyzing the causal effects of exposure on clinical outcomes.

In this study, each bacterial taxon contained in the gut microbiota was categorized as a separate exposure. Of all, 196 gut microbiota taxa were selected as exposures (Kurilshikov et al., 2021), AN and BN were defined as the outcome variable (Wade et al., 2013; Duncan et al., 2017). Two-sample MR analysis was conducted using summary statistics from genome-wide association studies (GWAS) to discern which bacterial taxa were causally associated with AN and BN, respectively. The overall design of the study is shown in Figure 1.

The analysis adhered to three pivotal assumptions inherent to MR studies (Emdin et al., 2017; Skrivankova et al., 2021): (1) genetic variation in exposed populations correlates with the exposure of interest, (2) genetic variation remains independent of confounding variables, and (3) genetic variation influences outcomes solely through the exposure of interest. The causal relationship between exposure factors and outcome factors was confirmed by testing 3 hypotheses that confirmed an indirect relationship between IV and outcome variables and was achieved only by exposure factors. A detailed information chart is provided in Supplementary material (Skrivankova et al., 2021).

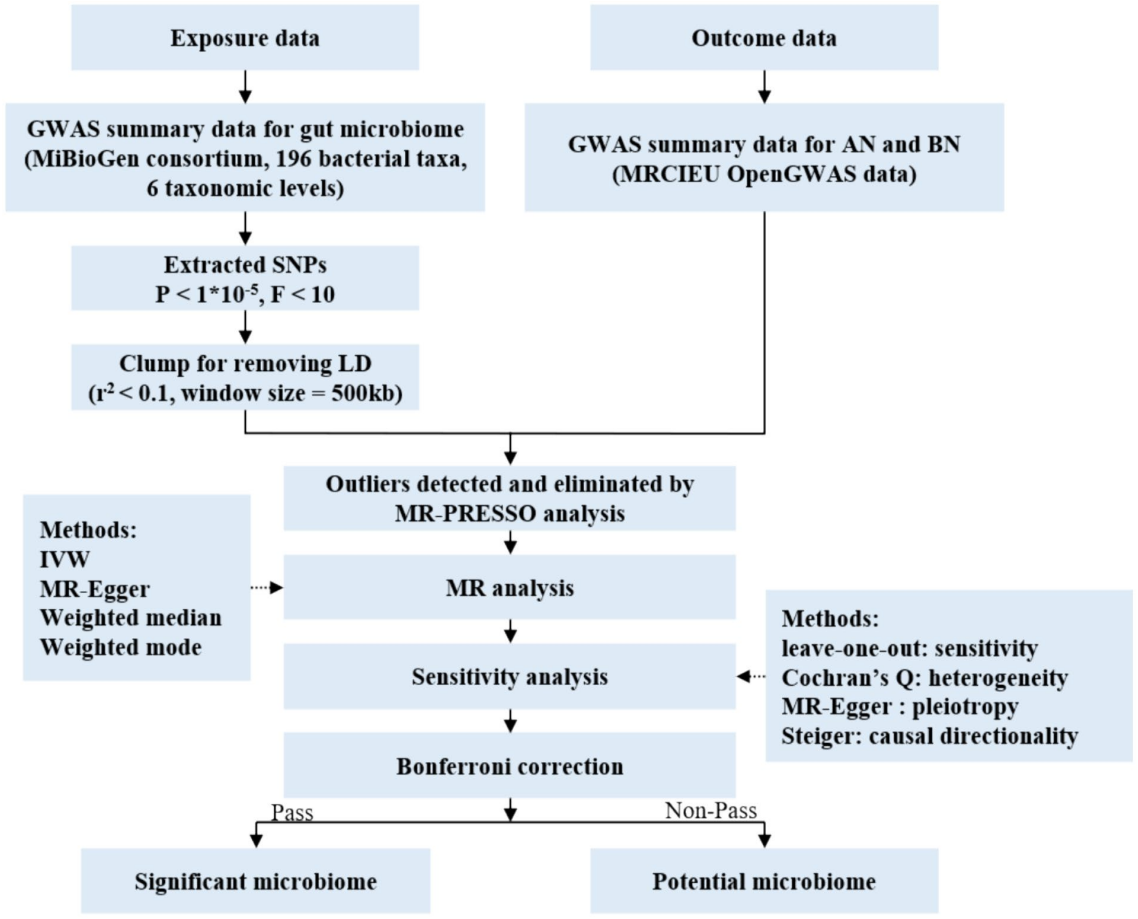

FIGURE 1
Flow chart of the study. GWAS, genome wide association study; SNPs, single nucleotide polymorphisms; MR, Mendelian randomization; LD, linkage disequilibrium; IVW, inverse variance weighted.

2.2 Genetic association data screening for AN and BN

All SNP data related to AN and BN were sourced from the MRC IEU OpenGWAS data infrastructure (Lyon et al., 2021), developed by the MRC Integrated Epidemiology Unit at the University of Bristol. This repository aggregates genetic associations from 50,037 GWAS datasets, totaling 346,312,366,530 genetic associations. Considering sample size, sequencing depth, ethnicity, and data recency, GWAS datasets for AN and BN authored by Duncan et al. (2017) and Wade et al. (2013), respectively, were selected. These studies encompassed 13 study cohorts with a combined sample size of 16,919 individuals, drawn from diverse pedigrees to mitigate bias (Supplementary Tables S1, S2).

2.3 The selection of IVs

The genetic IVs of each bacterial taxon were obtained from the MiBioGen consortium, which comprised 18,340 individuals from 24 cohorts. The consortium utilized standardized analytical pipelines for both microbiota phenotype and genotype, ensuring uniform data processing methods. This approach was employed to mitigate potential variations introduced by technical differences in generating microbiota data (Kurilshikov et al., 2021). This study used three different regions (V4: 10,413 samples, 13 cohorts, V3–V4: 4,211 samples, 6 cohorts and V1–V2: 3,716 samples, 5 cohorts) (V: hypervariable region sequencing for identifying bacterial taxa) of the 16S rRNA gene to analysis the composition of gut microbiota and identified genetic variants that influent the relative abundance of microbial taxa by use of microbiota Quantitative Trait loci mapping (Kurilshikov et al., 2021). Following the removal of 15 unknown bacterial taxa, the final dataset encompassed 196 taxa (Supplementary Table S3).

SNP screening entailed the following criteria (Sanna et al., 2019): (1) genome-wide SNPs with significance ($p < 1 \times 10^{-5}$), (2) exclusion of weak IVs with an F-statistic <10, (3) linkage disequilibrium (LD) testing to ensure independence of selected IVs ($r^2 < 0.1$ within a 500 kb range), and (4) removal of SNPs with incompatible or palindromic allele frequencies.

2.4 MR analysis and sensitivity assessment

MR analysis was conducted using R software (version 4.3.2) to evaluate the potential causal effect of gut microbes on AN and BN risk.

MR estimates for each SNP were derived using the Wald ratio method, with meta-analysis performed using four methods: Inverse variance weighted (IVW), MR Egger, weighted median, and weighted mode (Hartwig et al., 2017). The IVW method, recognized for its superior accuracy under practical conditions (Bowden et al., 2016), served as the primary analytical approach, supplemented by the other three methods. Bonferroni correction was applied at each taxonomic level (phylum, class, order, family, and genus) to account for multiple comparisons. MR estimates with $p < 0.05$ were considered statistically significant, while false discovery rate (FDR) p-values <0.05 denoted unequivocal significance. All estimates were expressed as odds ratio (OR) with a 95% confidence interval (CI) per standard deviation increase in the corresponding exposure.

The MR-PRESSO test was employed to identify outliers and address heterogeneity. In instances of detected heterogeneity among genetic IVs, outliers were eliminated, and MR analysis was re-executed. Sensitivity analyses were conducted via the leave-one-out method, Cochran's Q statistic was utilized to assess potential heterogeneity, and the MR Egger intercept test was employed to estimate horizontal pleiotropy. Finally, the Steiger method facilitated causal directionality analysis to mitigate potential effects of reverse causation.

3 Results

3.1 Overview of genetic IVs

Multiple SNPs were considered for each of the 196 bacterial taxa, following stringent screening based on genome-wide significance thresholds, LD testing, and validation of the F statistic. Any SNPs identified as outliers by MR-PRESSO (global test: $p < 0.05$) were excluded. All retained SNPs exhibited F-statistics exceeding 10, indicating a robust correlation between the genetic IVs and the corresponding bacterial taxa. The final roster of retained SNPs and pertinent statistics are detailed in Supplementary Tables S4, S5.

3.2 Relationship of intestinal bacterial taxa to AN and BN

Among the 196 taxon phenotypes examined, stringent Bonferroni correction did not reveal any unequivocal and significant causal relationships between gut microbiome and the risk of AN or BN (FDR $p < 0.05$). Nevertheless, based on analyses employing the IVW method and three additional methods, we ascertained potential causal relationships between certain intestinal taxa and the risk of AN or BN ($p < 0.05$).

As shown in Table 1, we identified nine taxa with potential causal relationships with AN. Among these nine taxa, *Peptostreptococcaceae* (IVW OR = 1.341, 95% CI 1.039–1.733, $p = 0.024$), *Coprococcus3* (IVW OR = 1.563, 95% CI 1.084–2.252, $p = 0.017$), *Escherichia Shigella* (IVW OR = 1.490, 95% CI 1.109–2.003, $p = 0.008$), *Lachnospiraceae NC2004 group* (IVW OR = 1.287, 95% CI 1.019–1.626, $p = 0.034$), *Lachnospiraceae UCG010* (IVW OR = 1.363, 95% CI 1.018–1.825, $p = 0.038$) were determined to have a causal effect on AN risk. And *Cyanobacteria* (IVW OR = 0.724, 95% CI 0.563–0.930, $p = 0.012$), *Gammaproteobacteria* (IVW OR = 0.619, 95% CI 0.406–0.943, $p = 0.026$), *Mollicutes RF9* (IVW OR = 0.740, 95% CI 0.584–0.939, $p = 0.013$), and *Eubacterium brachy group* (IVW OR = 0.778, 95% CI 0.643–0.942, $p = 0.010$) trended toward a lower risk of AN. Among the other three MR analysis methods (MR Egger, weighted median, weighted mode), only the weighted median method for the *Coprococcus3* and *Lachnospiraceae NC2004 group* and the MR Egger calculations for *Escherichia Shigella* were statistically significant. It is noteworthy that all these results are in agreement with the IVW calculation method for this group, which to some extent reflects the stability of the results.

The scatter plot (Figure 2) visualized the causal relationship between gut bacterial taxa and AN. Each point in the graph represents a SNP, and the short lines of the cross at each point reflect its 95% CI. The abscissa is the effect of the SNP on the exposure (gut microbe), and the ordinate is the effect of the SNP on the outcome (AN). The slash lines of different colors represent the MR fitting results of different calculation methods. A slope greater than 0 indicates that the exposure factor (gut microbe) is a disadvantage of AN. For the fitting results of different methods, the results of IVW are generally the main ones. As shown in Figure 2, except for *Cyanobacteria* and *Lachnospiraceae NC2004 group*, the results of the other MR analysis methods for the remaining seven taxa were in agreement with their respective IVW results. However, despite the inconsistencies between the MR Egger method results of these two gut microbes and the IVW method, the results of the IVW method remained robust due to their wide CIs and loss of statistical significance.

As shown in Table 2, we identified nine taxa with potential causal relationships with BN. Among these nine taxa, *Clostridiales* (IVW OR = 1.128, 95% CI 1.016–1.252, $p = 0.024$), *Bilophila* (IVW OR = 1.114, 95% CI 1.021–1.214, $p = 0.015$), *Coprobacter* (IVW OR = 1.085, 95% CI 1.005–1.171, $p = 0.037$), *Holdemania* (IVW OR = 1.096, 95% CI 1.016–1.182, $p = 0.018$), *Ruminococcaceae UCG009* (IVW OR = 1.082, 95% CI 1.004–1.166, $p = 0.038$), and *Slackia* (IVW OR = 1.101, 95% CI 1.004–1.208, $p = 0.041$) were determined to be the increased risk of BN. While *Rhodospirillales* (IVW OR = 0.921, 95% CI 0.851–0.996, p = 0.038), *Oxalobacteraceae* (IVW OR = 0.937, 95% CI 0.890–0.985, $p = 0.011$), *Lachnospiraceae UCG008* (IVW OR = 0.927, 95% CI 0.871–0.987, $p = 0.018$) were identified as protective factors. Of the other three MR analysis methods, only the weighted median method for the *Rhodospirillales*, *Oxalobacteraceae*, *Bilophila*, and *Holdemania* were statistically significant. All of these results are consistent with the IVW calculations for this group, reflecting the stability of the study results. As shown in Figure 3, except for *Rhodospirillales*, *Ruminococcaceae UCG009*, and *Slackia*, the results of other MR analysis methods for the remaining six taxa were in agreement with their respective IVW results. However, the results of the IVW method remained robust due to the wide CIs and loss of statistical significance of the MR Egger method. It is worth noting that due to the relative shortage of SNPs in *Slackia*, the MR Egger method calculates too wide CIs, which to some extent reflects the disadvantage of the method being overly conservative.

3.3 Sensitivity analysis of MR results

Prior to conducting MR analysis, MR-PRESSO analysis was performed to exclude potential abnormal SNPs. Subsequent tests were undertaken to ensure the sensitivity of the findings and mitigate

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TABLE 1 Significant MR results of potential causal effect of gut microbiota on AN.

Level	Exposure	*N* SNPs	Method	OR	95% CI	*p*-value
Phylum	*Cyanobacteria*	8	IVW	0.724	0.563–0.930	0.012*
			MR Egger	1.142	0.479–2.719	0.775
			Weighted median	0.767	0.541–1.088	0.137
			Weighted mode	0.545	0.299–0.994	0.088
Class	*Gammaproteobacteria*	6	IVW	0.619	0.406–0.943	0.026*
			MR Egger	0.533	0.141–2.011	0.406
			Weighted median	0.645	0.376–1.108	0.113
			Weighted mode	0.626	0.310–1.265	0.249
Order	*Mollicutes RF9*	13	IVW	0.740	0.584–0.939	0.013*
			MR Egger	0.776	0.381–1.580	0.498
			Weighted median	0.760	0.549–1.053	0.099
			Weighted mode	0.773	0.481–1.242	0.308
Family	*Peptostreptococcaceae*	14	IVW	1.341	1.039–1.733	0.024*
			MR Egger	1.436	0.805–2.561	0.244
			Weighted median	1.288	0.914–1.814	0.148
			Weighted mode	1.212	0.730–2.011	0.47
Genus	*Coprococcus3*	9	IVW	1.563	1.084–2.252	0.017*
			MR Egger	3.927	1.072–14.381	0.078
			Weighted median	1.704	1.036–2.804	0.036*
			Weighted mode	1.902	0.868–4.168	0.147
	Escherichia Shigella	10	IVW	1.490	1.109–2.003	0.008*
			MR Egger	2.772	1.184–6.491	0.047*
			Weighted median	1.384	0.924–2.072	0.115
			Weighted mode	1.158	0.559–2.401	0.702
	Eubacterium brachy group	9	IVW	0.778	0.643–0.942	0.010*
			MR Egger	0.829	0.346–1.985	0.686
			Weighted median	0.827	0.642–1.065	0.141
			Weighted mode	0.861	0.599–1.239	0.444
	Lachnospiraceae NC2004 group	9	IVW	1.287	1.019–1.626	0.034*
			MR Egger	0.956	0.359–2.547	0.931
			Weighted median	1.461	1.057–2.019	0.022*
			Weighted mode	1.598	0.965–2.644	0.106
	Lachnospiraceae UCG010	10	IVW	1.363	1.018–1.825	0.038*
			MR Egger	1.879	0.881–4.005	0.141
			Weighted median	1.256	0.858–1.839	0.240
			Weighted mode	1.180	0.697–1.996	0.553

*Represents that *p* meets the significance threshold. *N* SNPs represents the number of SNPs being used as IVs; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted, MR, Mendelian randomization.

potential biases. The list of SNPs retained after the exclusion of aberrant SNPs by the MR-PRESSO method, along with relevant statistics, are presented in Supplementary Tables S4, S5. Further MR-PRESSO global test analysis revealed no outliers among IVs with significant causal relationships with AN or BN, as detailed in Table 3.

However, there might be heterogeneity in IVs from different analysis platforms, experiments, and populations, which can affect the results of MR analysis (Cui et al., 2023). To assess potential heterogeneity, Cochran's *Q* statistic was computed, with all *p*-values exceeding 0.05, indicating the absence of heterogeneity in the study. This finding was corroborated by the results of funnel plot analyses (Supplementary Figures S1, S3). When IVs affect outcomes through factors other than exposure factors, they indicate that IVs are pleioty. Pleiotropy leads to the failure of the independence and exclusivity assumptions (Bowden et al., 2015; Verbanck et al., 2018). MR Egger intercept test results yielded *p*-values greater than 0.05, signifying the absence of pleiotropy in the study. Similarly, Steiger method analyses affirmed that all significant findings implicated gut microbiota in AN

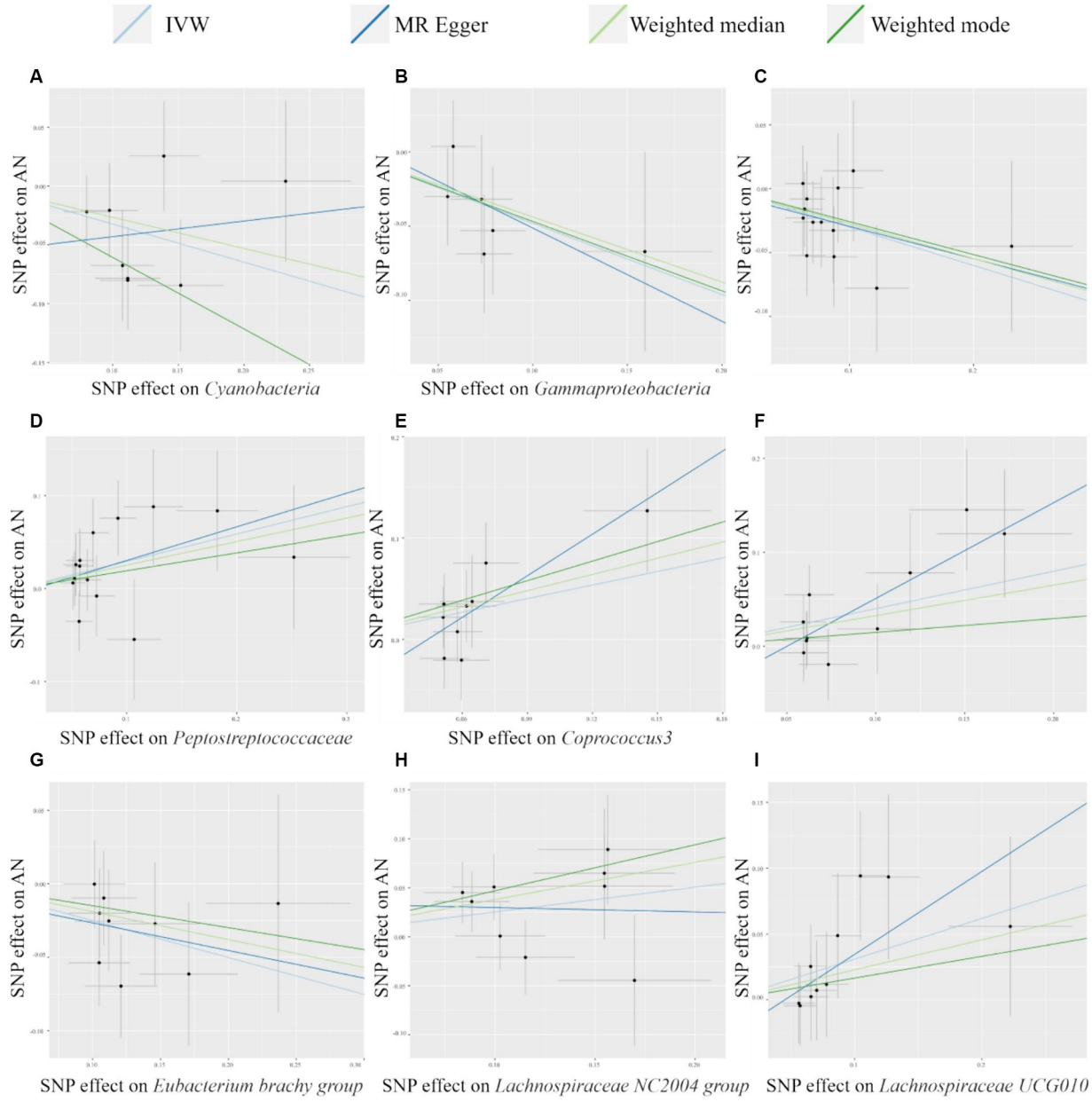

FIGURE 2
Scatter plots of MR analysis of potential causal effect of 9 gut microbiota on AN. **(A)** *Cyanobacteria*, **(B)** *Gammaproteobacteria*, **(C)** *Mollicutes RF9*, **(D)** *Peptostreptococcaceae*, **(E)** *Coprococcus3*, **(F)** *Escherichia Shigella*, **(G)** *Eubacterium brachy group*, **(H)** *Lachnospiraceae NC2004 group*, **(I)** *Lachnospiraceae UCG010*. IVW, inverse variance weighted; AN, anorexia nervosa; MR, Mendelian randomization; SNP, single nucleotide polymorphism.

or BN, with no evidence of reverse causality interference. Finally, sensitivity analysis employing the leave-one-out method demonstrated that the exclusion of any individual SNP did not substantially alter the results (Supplementary Figures S2, S4). These comprehensive analyses bolster the sensitivity and validity of the MR findings regarding the causal relationship between intestinal bacterial taxa and AN or BN.

4 Discussion

The escalating health and social burden attributed to eating disorders underscores the urgent need for effective treatments (Murray et al., 2020; Arias et al., 2022). It has been well-documented that eating disorders such as AN and BN interact with a range of mental and organic disorders (AlHadi et al., 2022). Blocking the progression of AN and BN by modulating the gut microbiota and its metabolites has received widespread attention from clinical researchers (Jiao et al., 2023; Himmerich and Treasure, 2024). However, the ethical dilemma and compliance problems in adolescent treatment add additional challenges to clinical research (Frostad and Bentz, 2022; Tsiandoulas et al., 2023). By leveraging genetic IVs, MR analysis facilitates a deeper understanding of the intricate interplay between gut microbiota and eating disorders, thereby offering promising avenues for targeted therapeutic interventions (Thomas, 2019).

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TABLE 2 Significant MR results of potential causal effect of gut microbiota on BN.

Level	Exposure	*N* SNPs	Method	OR	95% CI	*p*-value
Order	*Clostridiales*	10	IVW	1.128	1.016–1.252	0.024*
			MR Egger	1.003	0.784–1.282	0.984
			Weighted median	1.089	0.941–1.259	0.252
			Weighted mode	1.077	0.900–1.288	0.438
	Rhodospirillales	9	IVW	0.921	0.851–0.996	0.038*
			MR Egger	1.055	0.687–1.620	0.813
			Weighted median	0.908	0.825–1.000	0.049*
			Weighted mode	0.906	0.806–1.019	0.137
Family	*Oxalobacteraceae*	12	IVW	0.937	0.890–0.985	0.011*
			MR Egger	0.970	0.757–1.244	0.817
			Weighted median	0.906	0.844–0.972	0.006*
			Weighted mode	0.891	0.788–1.007	0.092
Genus	*Bilophila*	10	IVW	1.114	1.021–1.214	0.015*
			MR Egger	1.341	0.735–2.447	0.367
			Weighted median	1.131	1.005–1.272	0.041*
			Weighted mode	1.178	0.989–1.403	0.100
	Coprobacter	7	IVW	1.085	1.005–1.171	0.037*
			MR Egger	1.514	0.891–2.573	0.185
			Weighted median	1.119	1.017–1.233	0.022*
			Weighted mode	1.123	0.986–1.279	0.130
	Holdemania	9	IVW	1.096	1.016–1.182	0.018*
			MR Egger	1.094	0.699–1.712	0.705
			Weighted median	1.103	1.002–1.215	0.045*
			Weighted mode	1.104	0.986–1.236	0.125
	Lachnospiraceae UCG008	9	IVW	0.927	0.871–0.987	0.018*
			MR Egger	0.738	0.491–1.108	0.186
			Weighted median	0.949	0.874–1.032	0.220
			Weighted mode	0.957	0.845–1.083	0.505
	Ruminococcaceae UCG009	9	IVW	1.082	1.004–1.166	0.038*
			MR Egger	0.960	0.602–1.531	0.867
			Weighted median	1.062	0.962–1.172	0.232
			Weighted mode	1.043	0.925–1.175	0.513
	Slackia	4	IVW	1.101	1.004–1.208	0.041*
			MR Egger	0.451	0.001–163.645	0.816
			Weighted median	1.094	0.973–1.231	0.135
			Weighted mode	1.079	0.942–1.234	0.353

*Represents that *p* meets the significance threshold. *N* SNPs represents the number of SNPs being used as IVs; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted, MR, Mendelian randomization.

Employing a two-sample MR analysis framework with GWAS datasets, we identified nine bacterial taxa with potential causal associations with AN and nine bacterial taxa with potential causal associations with BN. A previous study found that core microbiota depletion signs were observed in patients with AN. Overrepresented taxa in patients taxonomically belonged to *Alistipes*, *Clostridiales*, *Christensenellaceae*, and *Ruminococcaceae*. And underrepresented taxa were *Faecalibacterium*, *Agathobacter*, *Bacteroides*, *Blautia*, and *Lachnospira* (Prochazkova et al., 2021). Similar studies have found that gut microbiota-associated metabolites, such as trimethylamine N-oxide (Wagner-Skacel et al., 2022), choline (Doose et al., 2023), serotonin (Hata et al., 2019), and p-cresyl sulfate (Miyata et al., 2021), in the onset and progression of AN and BN.

In this investigation, we identified five bacterial taxa, including *Peptostreptococcaceae*, *Coprococcus3*, *Escherichia Shigella*, *Lachnospiraceae NC2004 group*, and *Lachnospiraceae UCG010*, as

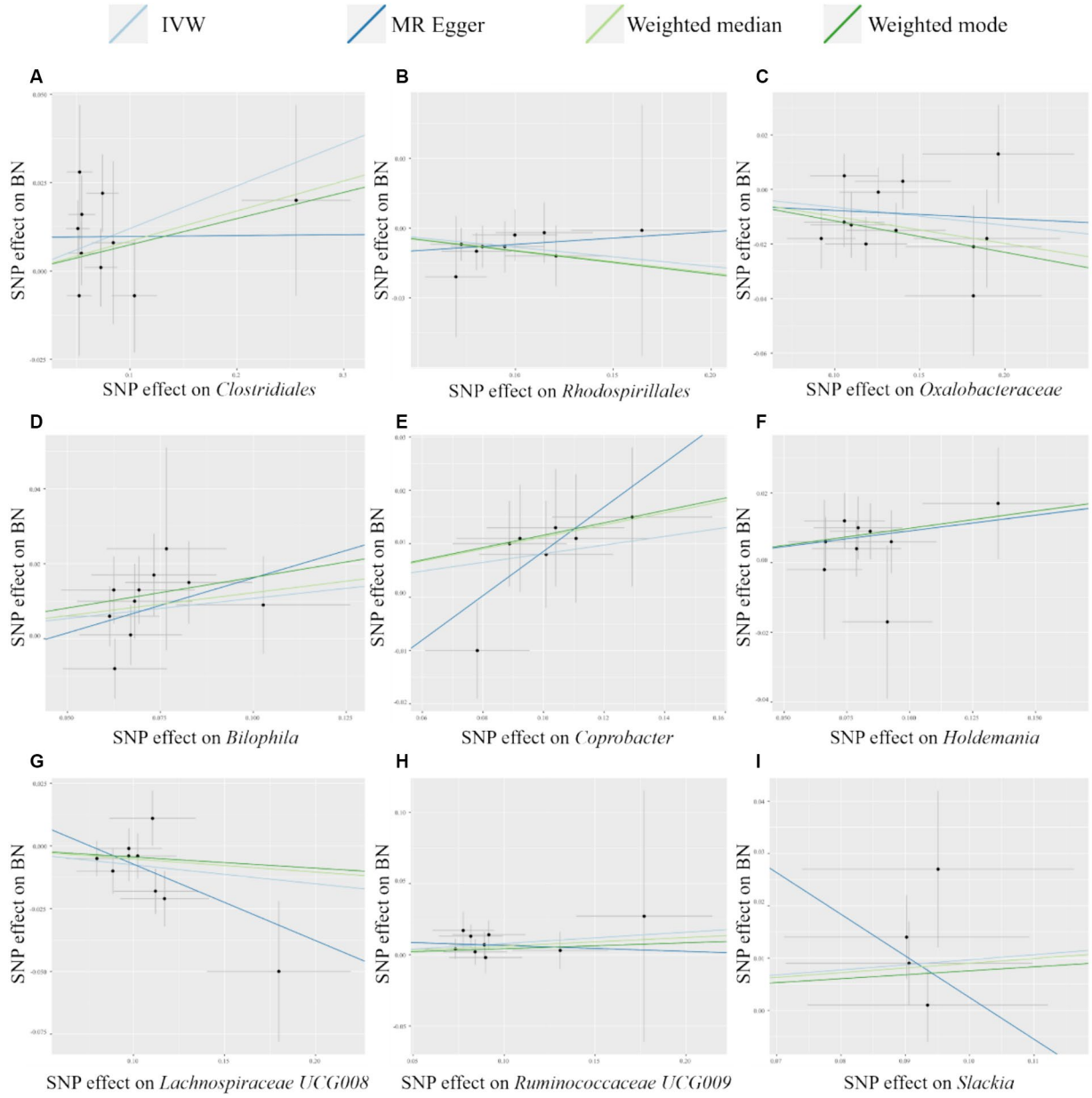

FIGURE 3
Scatter plots of MR analysis of potential causal effect of 9 gut microbiota on BN. **(A)** *Clostridiales*, **(B)** *Rhodospirillales*, **(C)** *Oxalobacteraceae*, **(D)** *Bilophila*, **(E)** *Coprobacter*, **(F)** *Holdemania*, **(G)** *Lachnospiraceae UCG008*, **(H)** *Ruminococcaceae UCG009*, **(I)** *Slackia*. IVW, inverse variance weighted; BN, bulimia nervosa; MR, Mendelian randomization; SNP, single nucleotide polymorphism.

potential causes for the increased risk of AN. The findings concerning *Coprococcus* and *Escherichia Shigella* align with those reported in a previous meta-analysis study (Zang et al., 2023). Hanachi et al. (2019) reported a decrease in the richness and diversity of gut microbiota in severely malnourished AN patients who received enteral nutrition. This study further identified a negative correlation between the severity of functional intestinal disorders in AN patients and *Peptostreptococcaceae* by 16S rRNA analysis. This seems to contradict our conclusion that *Peptostreptococcaceae* is a potential cause of AN. However, the abundance of *Peptostreptococcaceae* is influenced by a variety of factors. The abundance of *Peptostreptococcaceae* is increased in stool samples from patients with ulcerative colitis and colorectal cancer (Cheng et al., 2020). And it tends to decrease after probiotic consumption, suggesting that the abundance of *Peptostreptococcaceae* is easily influenced by food intake and probiotics (Hibberd et al., 2017; Zaccaria et al., 2023). This appears to explain the decrease in the abundance of *Peptostreptococcaceae* in patients with functional intestinal disorders of AN after receiving enteral nutrition, which is not related to the onset of AN. This finding underscores the intricate role of the intestinal microbiota in the interplay between AN pathogenesis and its accompanying symptoms.

Our investigation also revealed that four bacterial taxa, namely *Cyanobacteria*, *Gammaproteobacteria*, *Mollicutes RF9*, and *Eubacterium brachy group*, exhibited the potential to reduce the risk of AN. Previous 16S rRNA investigations have highlighted correlations between *Eubacterium* and AN (Hanachi et al., 2019; Yuan et al., 2022),

TABLE 3 Significance levels of different tests for MR results.

Outcome	Expose	Egger	Q	Steiger	MR-PRESSO global test
AN	*Cyanobacteria*	0.324	0.445	0.000	0.452
AN	*Gammaproteobacteria*	0.829	0.765	0.000	0.875
AN	*Mollicutes RF9*	0.894	0.959	0.000	0.980
AN	*Peptostreptococcaceae*	0.802	0.694	0.000	0.778
AN	*Coprococcus3*	0.190	0.764	0.000	0.651
AN	*Escherichia Shigella*	0.166	0.791	0.000	0.666
AN	*Eubacterium brachy group*	0.889	0.809	0.000	0.883
AN	*Lachnospiraceae NC2004 group*	0.559	0.541	0.000	0.629
AN	*Lachnospiraceae UCG010*	0.394	0.914	0.000	0.917
BN	*Clostridiales*	0.330	0.682	0.000	0.698
BN	*Rhodospirillales*	0.546	0.997	0.000	0.924
BN	*Oxalobacteraceae*	0.780	0.292	0.000	0.395
BN	*Bilophila*	0.558	0.655	0.000	0.088
BN	*Coprobacter*	0.268	0.805	0.000	0.745
BN	*Holdemania*	0.996	0.938	0.000	0.981
BN	*Lachnospiraceae UCG008*	0.301	0.434	0.000	0.473
BN	*Ruminococcaceae UCG009*	0.625	0.892	0.000	0.916
BN	*Slackia*	0.794	0.220	0.000	0.322

AN, anorexia nervosa; BN, bulimia nervosa; MR, Mendelian randomization.

but directional causality remains elusive, a view that is confirmed by our findings. These microbes are one of the major producers of butyrate and play an important role in immunomodulatory processes at the intestinal mucosal level, which may be one reason for the reduced risk in AN patients (Yuan et al., 2022). *Cyanobacteria* are single-celled prokaryotes capable of oxygen-producing photosynthesis, which are mainly used in fields such as fertilizers and fuels (Arias et al., 2021). Recent studies have found that *Cyanobacteria* contains a variety of bioactive components that can induce autophagy and apoptosis, regulate epigenetic modifications, and exert antitumor effects (Bouyahya et al., 2024). Xu et al. (2019) found that *Cyanobacteria* were associated with neuropsychological behavior induced by long-term alcohol exposure, and the mechanism may be related to the secretion of brain-derived neurotrophic factor. Depression-like behavior induced by a high-fat diet in mice is also associated with increased abundance of *Cyanobacteria* (Hassan et al., 2019). Similarly, *Gammaproteobacteria* have been shown to be abundant in young adults with major depressive disorder, regardless of psychotropic medication (Liu et al., 2020). Mice exposed to chronic social defeat stress showed mild depressive-like behavior and an increase in the abundance of *Mollicutes* (Kosuge et al., 2021). And a study on anti-anxiety drugs found that (R)-ketamine might work by downregulating *Mollicutes* (Yang et al., 2017). Based on the available evidence, these gut microbes (*Cyanobacteria, Gammaproteobacteria, Mollicutes*) are all positively associated with depression, and their potential role in reducing AN risk remains to be confirmed by clinical studies. These three types of gut microbes seem to help distinguish between AN and depression, which have similar clinical manifestations.

In a previously published MR analysis, *Actinobacteria*, *Bilophila*, *Holdemania*, *Lactobacillus*, *Ruminococcaceae UCG009*, and two unknown gut microbes were identified as risk factors for the development of AN, which is very different from our conclusions (Xia et al., 2023). Interestingly, the results are highly similar to those of our BN study. Reviewing the original published GWAS datasets, we found that the main reason for this was because the earlier GWAS datasets could not be distinguished from the two disease subtypes at a technical level due to the crossover of AN and BN diagnoses (Boraska et al., 2014). At the same time, the limitations of detection techniques have resulted in relatively small numbers of SNPs captured from earlier datasets, limiting the exploration of more critical causal associations (Duncan et al., 2017). Therefore, our study can be regarded as an update of this study, which provides cross-validation of the findings of BN in this study.

In our results, six taxa, including *Clostridiales*, *Bilophila*, *Coprobacter*, *Holdemania*, *Ruminococcaceae UCG009*, and *Slackia*, are potential contributors to the increased risk of BN. *Ruminococcaceae* are positively associated with autism, depression, and are abundant in patients with AN (Prochazkova et al., 2021). Similar studies showed that the abundance of *Clostridiales* in feces of activity-based anorexia mice with food restriction increased significantly (Breton et al., 2019). Given the crossover nature and common pathological basis of AN and BN diagnoses, their potential risks to BN are promising. *Bilophila* is an opportunistic pathogen, and the close association of increased abundance with intestinal inflammation has been confirmed (Alexander et al., 2023; Zahavi et al., 2023). Recent studies have found that a ketogenic diet can exacerbate cognitive impairment caused by intermittent hypoxia, the mechanism of which is associated with impairment of hippocampal function due to the enrichment of gut microbes such as *Bilophila* (Olson et al., 2021). Changes in *Bilophila* abundance have been observed in patients with mental disorders such

as autism spectrum disorders. As for *Coprobacter*, its abundance is currently thought to be associated with chronic insomnia and cognitive function (Feng et al., 2021). Similarly, *Holdemania* exhibits a high abundance in patients with depression (Barandouzi et al., 2020) and is closely associated with anxiety and Parkinson's disease (Jang et al., 2020). *Slackia* stands out as a typical causative agent, however, recent studies suggest that it may be involved in the development of Alzheimer's disease (Nagpal et al., 2019). Therefore, although there are no reports about *Coprobacter*, *Holdemania* and *Slackia* in patients with AN or BN, their research is still worthy of attention.

Our study identified three taxa—*Rhodospirillales*, *Oxalobacteraceae*, and *Lachnospiraceae UCG008*—as protective factors for BN. *Rhodospirillales* is mainly used in water purification and new energy sources (Chun et al., 2018). Zhang et al. (2023) found that sleep deprivation led to a significant increase in the abundance of *Rhodospirillales* and enhanced pro-inflammatory cytokine responses as well as learning and memory impairments in mice. Another study found a significant increase in the abundance of *Rhodospirillales* in socially isolated mice (Siddi et al., 2024). Studies of *Oxalobacteraceae* have found that it reduces the risk of delirium, attention deficit hyperactivity disorder (Wang et al., 2023; Yu et al., 2023). Therefore, these gut microbes may have a close relationship with mental disorders, and further clinical research on AN and BN is worth exploring.

It is notable that AN and BN share similar clinical mechanisms, yet previous studies have not consistently identified a specific group of gut microorganisms commonly associated with both disorders. In our current investigation, we observed that while the *Lachnospiraceae NC2004 group* and *Lachnospiraceae UCG010* were identified as risk factors for AN, *Lachnospiraceae UCG008* emerged as a protective factor for BN. Previous studies have found that *Lachnospiraceae NC2004 group* was able to reduce the risk of gastroduodenal ulcers, and was able to reduce circulating inflammatory cytokine levels (Xue et al., 2023). *Lachnospiraceae UCG010* may reduce the risk of cholelithiasis and narcolepsy (Liu et al., 2023; Sheng et al., 2024). In contrast, *Lachnospiraceae UCG008* has been shown to be associated with a higher risk of periodontitis (Ye et al., 2023), and is a potential risk factor for hemorrhagic stroke (Shen et al., 2023). Based on current evidence, we cannot definitively explain the contradiction between the different genera of *Lachnospiraceae*, but it appears to be related to inflammation and immunity (Sun et al., 2021; Zeng et al., 2023). However, despite belonging to distinct strains, *Lachnospiraceae* appears to be a common influencing factor for both AN and BN. Previous research has linked reductions in *Lachnospiraceae* to an increased risk of developing depression (Liu et al., 2022), autism (Li et al., 2023), and Alzheimer's disease (Hung et al., 2022). The elevated risk of AN observed in our study further underscores the widespread association of *Lachnospiraceae* with mental disorders (Wang et al., 2023). Notably, although *Lachnospiraceae* have exhibited favorable organismal protective effects across a spectrum of diseases (McCulloch et al., 2022; Yan et al., 2023), their contrasting effects on AN and BN suggest a complex relationship between gut microbiota and disease that warrants further elucidation. The exact mechanism underlying these observations remains to be clarified through additional clinical intervention studies.

The therapeutic feasibility of microbial supplements for the treatment of psychiatric disorders has gained considerable recognition (Sanada et al., 2020; Góralczyk-Bińkowska et al., 2022). Liu et al. confirmed that probiotic supplementation mitigated intestinal damage induced by dietary restriction in AN patients (Liu et al., 2021). Subsequent clinical investigations substantiated that administering a probiotic complex reduces inflammation levels and gastrointestinal distress symptoms in AN patients (Gröbner et al., 2022). There is a potential causal relationship between the gut microbiota and AN and BN identified in this study, and the regulation of these microbiota by probiotics may be a promising therapeutic target for AN and BN.

In this study, we have, for the first time, elucidated the causal relationship of certain intestinal taxa with AN and BN, offering novel perspectives for subsequent mechanistic exploration and drug research. Leveraging causal inference through MR design effectively circumvented the influences of confounding bias and reverse causation. Furthermore, we conducted rigorous tests to ensure the sensitivity of our findings. However, this study does entail some limitations. Firstly, the sample size and the number of relevant loci in the current gut microbiota GWAS data are constrained. To mitigate the risk of inadequate IVs at the genus and species levels, potentially leading to imprecise bacterial characterization, we conducted summary analyses of bacterial features at higher taxonomic levels. It is anticipated that with advancements in microbiome GWAS and the accumulation of larger sample sizes, more specific bacterial features will be discerned and complemented. Secondly, to enhance statistical power, the gut microbiota and disease GWAS datasets analyzed in this study were amalgamated from multi-source samples. While this approach facilitates broader extrapolation of conclusions, it may introduce some heterogeneity in the results. Therefore, despite employing a meticulous methodology to exclude heterogeneous data, the results should be interpreted cautiously.

5 Conclusion

In summary, our study identified the potential causal involvement of 18 intestinal bacterial taxa, notably including *Lachnospiraceae*, in the pathogenesis of AN and BN through MR analysis. These potentially valuable gut microbiota may indicate the risk of disease development and provide feasible targets for the pathogenesis of AN and BN and probiotic therapy. Further clinical intervention and intestinal microbial testing have broad prospects for the research and treatment of AN and BN.

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.

Author contributions

ZYu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. MG: Data curation, Formal analysis, Investigation, Writing – original draft. BY: Data curation, Formal analysis, Investigation, Writing – original draft. YW: Data curation, Investigation, Writing – original draft. ZYa: Investigation, Software, Writing – original draft. RG: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

MG was employed by Yabao Pharmaceutical Group Co., Ltd.

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1396932/full#supplementary-material

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Gut microbiota and serum metabolomic alterations in modulating the impact of fecal microbiota transplantation on ciprofloxacin-induced seizure susceptibility

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Introduction: The gut microbiota and the microbiota-gut-brain axis have gained considerable attention in recent years, emerging as key players in the mechanisms that mediate the occurrence and progression of many central nervous system-related diseases, including epilepsy. In clinical practice, one of the side effects of quinolone antibiotics is a lower seizure threshold or aggravation. However, the underlying mechanism remains unclear.

Methods: We aimed to unravel the intrinsic mechanisms through *16S* rRNA sequencing and serum untargeted metabolomic analysis to shed light on the effects of gut microbiota in ciprofloxacin-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models.

Results: We observed that ciprofloxacin treatment increased seizure susceptibility and caused gut dysbiosis. We also found similar changes in the gut microbiota of rats with lithium pilocarpine-induced epilepsy. Notably, the levels of *Akkermansia* and *Bacteroides* significantly increased in both the ciprofloxacin-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models. However, *Marvinbryantia, Oscillibacter,* and *Ruminococcaceae_NK4A214_group* showed a coincidental reduction. Additionally, the serum untargeted metabolomic analysis revealed decreased levels of indole-3-propionic acid, a product of tryptophan-indole metabolism, after ciprofloxacin treatment, similar to those in the plasma of lithium pilocarpine-induced epilepsy in rats. Importantly, alterations in the gut microbiota, seizure susceptibility, and indole-3-propionic acid levels can be restored by fecal microbiota transplantation.

Conclusion: In summary, our findings provide evidence that ciprofloxacin-induced seizure susceptibility is partially mediated by the gut microbiota and tryptophan-indole metabolism. These associations may play a role in

epileptogenesis, and impacting the development progression and treatment outcomes of epilepsy.

KEYWORDS

gut microbiota, ciprofloxacin, seizure susceptibility, microbiota-gut-brain axis, fecal microbiota transplantation, untargeted metabolism, lithium pilocarpine

1 Introduction

Epilepsy is a chronic neurological disease characterized by recurrent and transient brain dysfunction. It affects approximately 65 million patients worldwide and poses a substantial global health challenge (Fisher et al., 2014). Despite extensive research into the pathogenic mechanisms of epilepsy, its etiology remains unclear. Approximately one-third of patients with epilepsy who do not respond to currently available antiseizure medications develop drug-resistant epilepsy (DRE). Moreover, some daily factors, such as fatigue, hunger, alcohol or stimulant beverage consumption, insomnia, or infections can aggravate seizures. These observations underscore the complexity of epilepsy and the need for further exploration to understand its etiology and management.

Studies of the proposed concept of the microbiota-gut-brain axis (MGBA) have demonstrated its vital role in the pathological process of epilepsy (Yue et al., 2022). The gut microbiota (GM) is a complex group of symbiotic microorganisms (bacteria, viruses, and fungi) dwelling in the gastrointestinal tract, and is considered the second genome in humans (Ursell et al., 2012). MGBA facilitates communication between the brain and GM through various pathways. These include the autonomic and enteric nervous systems, immune system and neuroimmunity, neurotransmitters, short-chain fatty acids (SCFAs), spinal mechanisms, hypothalamic-pituitary-adrenal axis, and peptidoglycans, etc. (Cryan et al., 2019). Studies on the relationship between GM and epilepsy initially emerged with a ketogenic diet (Olson et al., 2018; Gong et al., 2021). Several clinical studies have shown substantial differences in the GM profiles among epilepsy patients, healthy individuals, and those with DRE (Arulsamy et al., 2020; Gong et al., 2020). The role of the GM in epileptogenesis and the development of epilepsy has been gradually confirmed in different epileptic animal models (Citraro et al., 2021; Mengoni et al., 2021; Mu et al., 2022b). The aforementioned evidence underscores the important role of the GM in epilepsy.

In clinical practice, it has been observed that the use of certain antibiotics may increase susceptibility to seizures, particularly within the β-lactam, fluoroquinolone families, fourth-generation cephalosporins, and carbapenems (Wanleenuwat et al., 2020). Ciprofloxacin (CPF) is an antibiotic belonging to the fluoroquinolone class that is effective against a wide range of bacteria. Previous studies have shown that quinolone antibiotics, including CPF, affect seizure susceptibility in various animal models and patients with epilepsy (Abdel-Zaher et al., 2012; Arafa et al., 2013; Cheraghmakani et al., 2021; Sivarajan and Ramachandran, 2023). When CPF is applied, fecal concentrations are high and CPF exerts a long-lasting effect on the GM (Zimmermann and Curtis, 2019). Previous studies on the mechanism of quinolone antibiotic-induced seizures have primarily focused on their potential to change neurotransmitter levels, disrupt neurotransmitter-receptor binding, have a chemical structure similar to that of epileptogens, and increase oxidative stress due to drug interactions (Ilgin et al., 2015; Wanleenuwat et al., 2020). However, the vital role of the GM has been neglected.

Therefore, the above-mentioned evidence predominantly examines the variations in GM between individuals with epileptic status and healthy controls, or among those undergoing different drugs or diet treatments. There is a notable lack of investigation into the therapeutic effects of GM modulation and the underlying mechanisms of the MGBA. Additionally, there has been a lack of recognition regarding the significant impact of CPF on the GM. Our study aims to combine the two aspects and fill this gap by focusing on elucidating the pathway of CPF induce seizure susceptibility through the GM.

To date, no studies have examined whether CPF increases seizure susceptibility from the perspective of GM and metabolite changes in animals. Recent studies have revealed that the GM is a major determinant of plasma metabolome, potentially playing a more dominant role than genetics (Loh et al., 2024). Therefore, based on the aforementioned research progress, we hypothesized that the GM plays a pivotal role in the mechanism by which CPF enhances seizure susceptibility, potentially via metabolic pathways in the MGBA. Our investigation is the first to delve into the GM and its relationship with seizure susceptibility induced by CPF using *16S* ribosomal RNA amplicon sequencing. Furthermore, we explored the potential therapeutic effects of transplanting fecal samples from healthy controls. And we aimed to identify the core genera through joint analysis with lithium pilocarpine-induced epilepsy. Finally, we aimed to unravel the intrinsic mechanisms through serum untargeted metabolomic analysis to shed light on how the GM influences epilepsy. Our study contributes to a better understanding of how the GM functions in epilepsy via MGBA. These insights may pave the way for developing novel therapeutic approaches from a new perspective.

Abbreviations: AD, Alzheimer's disease; AHR, aryl hydrocarbon receptor; BBB, blood-brain barrier; CPF, ciprofloxacin; CNS, central nervous system; DRE, drug-resistant epilepsy; EEG, electroencephalogram; GM, gut microbiota; IPA, 3-indolepropionic acid; IAA, 3-Indoleacrylic_acid; LEfSe, linear discriminant analysis effect size; MGBA, microbiota-gut-brain axis; PTZ, pentylenetetrazole; TRP, tryptophan; SCFAs, short-chain fatty acids; SPF, specified pathogen free; VEEG, video-electroencephalogram.

2 Materials and methods

2.1 Animals and experimental design

Adult male Sprague–Dawley (SD) rats weighing 180 ± 20 g with specified pathogen free (SPF) grade were used in this experiment (Vital River Laboratory Animal Technology Co., China). After one week of quarantine, the animals were individually housed in sterilized acrylic cages. The temperature and humidity of the room were kept at 21 ± 1°C and 55%, respectively. The rats were maintained under a 12:12-h light/dark cycle (lights on at 6 am) with free access to standard rodent food (SPF Biotechnology Co., China) and pure water. All animal care and experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Institutional Ethical Committee for Animal Welfare of the Seventh Affiliated Hospital of Sun Yat-sen University and in strict accordance with the National Institutes of Health guidelines for animal use in research.

2.1.1 Ciprofloxacin-induced seizure susceptibility in rats

After 1 week adaption, all rats were randomly numbered and divided into two groups (Figure 1A).

Antibiotic Group (CPF $n = 33$): All the rats received ciprofloxacin monohydrochloride (TargetMol, USA) by gavage at a dose of 100 mg/kg body weight (dissolved in normal saline at 10 mg/mL) per day for 14 days. After CPF gavage, 19 rats were tested for seizure susceptibility ($n = 6$ at 45 mg/kg; $n = 13$ at 60 mg/kg), and the remaining 14 rats received fecal microbiota transplantation (FMT) three times a week for 14 days according to FMT procedure (Bokoliya et al., 2021).

Control Group (Ctrl $n = 30$): All rats were administered the same volume of normal saline (NS) by gavage for 14 days ($n = 30$). Subsequently, 16 rats were tested for seizure susceptibility ($n = 6$ at 45 mg/kg; $n = 10$ at 60 mg/kg). The remaining 14 rats received phosphate-buffered saline (PBS) at the same frequency as the CPF group, and served as fecal donors for FMT.

Seizure susceptibility testing was divided into two dose gradients (45 mg/kg and 60 mg/kg). The CPF dose used was calculated based on the daily dosage of the patient (1–1.5 g/d) and then adjusted according to the body surface area of the human and rat.

2.1.2 Lithium pilocarpine-induced epilepsy rat model

Animals were divided into two groups: control (Normal) and epilepsy (Pilo). In the Pilo group, rats were injected intraperitoneally (i.p.) with lithium chloride (127.2 mg/kg). After 18 h, the rats were administered scopolamine (1 mg/kg, intraperitoneally). Thirty min later, pilocarpine hydrochloride (30 mg/kg, i. p.) was administered to induce status epilepticus. Seizure onset was identified visually and scored according to the Racine Scale (Racine, 1972). Rats that did not exhibit a score of 4 or above within 30 min of pilocarpine injection were repeatedly injected (10 mg/kg, i.p.) every 30 min until a scale 4 or above seizure was induced. We used 60 mg/kg as the maximum dose; otherwise, animals were not included in this study. Ninety min after the first grade 4 or higher seizure, diazepam (10 mg/kg, i.p.; Jinyao medicine, China) was administered to terminate the seizures. The control group ($n = 3$) was injected with 0.9% NS at each time point as the Pilo group. After 2 months, the rats were transferred to monitoring cages and monitored using an infrared night vision camera for 1 week. The monitored video results were analyzed in a double-blind manner by two senior investigators to identify seizures. Animals that experience spontaneous seizures are believed to develop epilepsy. Four rats were confirmed to have spontaneous seizures. Finally, fecal and serum samples from the two groups were collected for *16S* rRNA sequencing and LC-MS/MS analysis.

2.2 Electrode implantation

After CPF or FMT gavage, the animals were surgically implanted with epidural recording electrodes for seizure susceptibility testing and video-electroencephalogram (VEEG) monitoring. Rats were anesthetized with isoflurane (5% anesthesia induction) for 2 min, and anesthesia was maintained at an isoflurane concentration of 2%. After anesthesia, the rats were mounted on a stereotaxic apparatus (RWD, Shenzhen). According to the brain coordinates identified in the rat stereotaxic atlas by Paxinos and Watson (2007), four burr holes were drilled through the skull. For electroencephalogram (EEG) recording, two stainless skull screw electrodes (Selectaplus, Dentsply, DeTrey GmbH, Dreieich, Germany; 1.0 mm in diameter) were advanced into the bilateral motor cortex (anteroposterior + 3.0 mm, mediolateral ± 2.5 mm), and two into the skull bilaterally over the cerebellum to serve as a reference and ground electrodes (Figure 1B). The reference and ground skull screw electrodes were placed extra-axially, overlying the cerebellum. All electrodes were secured using dental cement.

2.3 VEEG recording and seizure susceptibility test

Two days after surgery, the rats were placed in custom-made transparent cages and allowed to move freely (one rat/cage). EEG signals were recorded and digitized using a PowerLab8/35 system (Analog-to-Digital Converter Instruments, Colorado Springs, CO, USA) at a sampling rate of 2 kHz using synchronized video recording. EEG signals were digitally filtered (high-pass at 0.5 Hz, low-pass at 70 Hz, 50 Hz notch filter) and examined for the presence of spikes. The behaviors of the animals were recorded using a camera. We first recorded 10-min baseline EEG signals. After baseline recording, all animals were injected intraperitoneally with a single dose of pentylenetetrazole (PTZ) to induce acute seizures, which was dissolved in NS (18 mg/mL or 24 mg/mL). VEEG was recorded for 90 min (Figure 1C). The onset and termination of a seizure are readily recognizable as abrupt changes in EEG frequency, amplitude, and epileptic behavior. The seizure behavioral scale criteria based on the Racine scale were as follows: Score 0: no response; Score 1: staring and

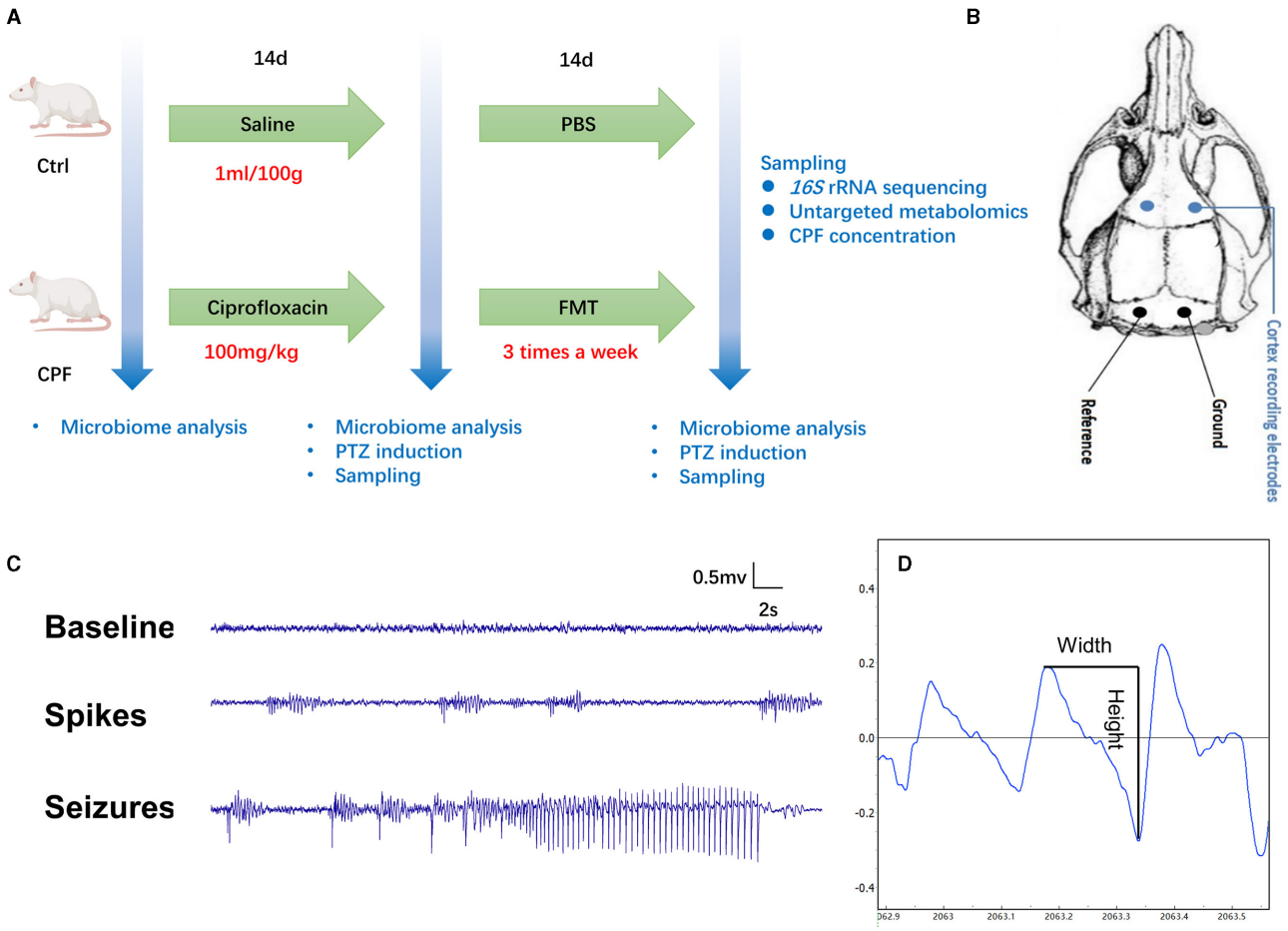

FIGURE 1
Flowchart depicting the study methodology and the assessment of seizure susceptibility. **(A)** The experimental design comprised rats receiving either CPF ($n = 33$) or NS ($n = 30$) gavage. On the 14th day, 19 rats in the CPF group (CPF-14 d) and 16 rats in the Ctrl group (CPF-14 d) underwent PTZ injection to assess seizure susceptibility and were subsequently sampled. The remaining rats continued FMT treatment ($n = 14$ in each group), and on the 28th day, 14 rats from both the CPF (CPF-FMT) and Ctrl groups (Ctrl-PBS) were tested for seizure susceptibility and sampled. **(B)** Epidural recording electrodes implantation. **(C)** An example of baseline, spikes and PTZ-induced seizure detected during the epilepsy susceptibility test with video monitoring. **(D)** The width and height in the spike mode pattern.

mouth clonus; Score 2: head nodding; Score 3: unilateral forelimb clonus; Score 4: rearing and bilateral forelimb clonus; and Score 5: rearing and losing the balance (Racine, 1972). The number of seizures, average and accumulated duration of seizures, latency to the first spike cluster and first seizure, total power change (0–500 Hz), and number of single spikes were calculated as indicator parameters for epilepsy susceptibility. A Fast Fourier Transformation was used to calculate the total power in the 0–500 Hz frequency band. EEG spikes were defined as high-voltage (>4 SD above background) positive or negative single deflections that lasted <50 ms in 90-min recording screened using the above-mentioned software PowerLab8/35 system (Figure 1D) (Mengoni et al., 2021). Each putative spike detected and electrographic seizures were verified in a blinded manner by two independent observers who were not aware of the treatment of the animals.

2.4 Weight record and samples collection

Body weight was recorded every 3d throughout the duration of gavage to adjust the volume of CPF or fecal slurry. Fecal samples were collected at specific time points during the study: before CPF treatment initiation (day 0), 14 d after CPF/NS gavage, and 2 weeks after FMT. The collection time was standardized between 9:00 and 11:00 AM to minimize variations due to circadian effects. Two to three fecal boluses were collected aseptically from each animal. To ensure that the collected feces were not polluted by the environment, the samples were directly deposited into 1.5 mL sterile Eppendorf (EP) microtubes. Then, the samples were promptly transferred to a freezer set at −80°C for long-term storage until further analysis. The day after the susceptibility test, the rats were euthanized, and the brain tissue was quickly removed and frozen at −80°C for subsequent experiments.

2.5 Normal fecal microbiota transplantation

For normal fecal suspension preparation, fresh fecal samples were obtained from control rats. Approximately 1 g of fecal samples (equivalent to approximately five fresh fecal samples) was immediately steeped and homogenized in 5 mL of PBS. The mixture was then centrifuged at 2,500 rpm (500 × g) for 10 min at 4°C to pellet insolubilized material. The supernatant underwent further processing by passing it through a nylon filter with a pore size of 40 μm to effectively eliminate particulate and fibrous matter, generating the microbiome suspension (Gheorghe et al., 2021). After the 2-day antibiotics washout period, each rat in the CPF group received 1 mL/100 g body weight fecal slurry from the control group via oral gavage three times a week for 2 weeks.

2.6 Fecal DNA extraction, *16S* rRNA sequencing and bioinformatics analysis

Fecal DNA was extracted using the ALFA-SEQ Advanced Stool DNA Kit (Findrop, Guangzhou, China) according to the manufacturer's instructions. The concentration and purity were assessed using a NanoDrop One (Thermo Fisher Scientific, MA, USA). The V3-V4 hypervariable region of the *16S* ribosomal RNA genes was amplified using specific primer carrying Illumina overhang adapter sequences (forward: 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′; reverse 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) with a 12 bp barcode. The primers were synthesized by Invitrogen (Carlsbad, CA, USA). The length and concentration of the PCR products were determined using 1% agarose gel electrophoresis. Samples with a bright strip between 290 and 310 bp were used for further experiments. The library was sequenced on an Illumina Nova 6000 platform and 250 bp paired-end reads were generated (Guangdong Magigene Biotechnology Co. Ltd. Guangzhou, China). Paired-end clean reads were merged using usearch fastq_mergepairs. Fastp (version 0.14.1, https://github.com/OpenGene/fastp) was used to control the quality of the raw data using a sliding window (-W4-M20) to obtain paired-end clean tags. The raw sequences were processed using the UPARSE pipeline in R to generate operational taxonomic units (OTUs), Taxonomy was assigned using Greengenes (http://greengenes.lbl.gov/) database, and taxonomic information was annotated using usearch-sintax (by setting the confidence threshold to default to ≥0.8).

Alpha- and beta-diversity calculations were performed using the QIIME2 and R software (v3.6.1). The Chao1 and Shannon_2 indices in our samples were calculated using alpha_div (V10, http://www.drive5.com/usearch/). Principal coordinate analysis (PCoA) was based on the unweighted UniFrac distance, and the *p*-value of the analysis of similarities (ANOSIM) was obtained using a permutation test. We then conducted a linear discriminant analysis effect size (LEfSe) to determine the differential taxa based on the homogeneous OTUs table. Phylogenetic investigation of communities by reconstruction of unobserved state 2 (PICRUST2, https://huttenhower.sph.harvard.edu/picrust/) was used to predict the function and metabolic enzymes of *16S* rDNA.

2.7 Serum untargeted metabolomic analysis

Blood samples were collected from SD rats (Ctrl and CPF) at three specific time point (0, 14, and 28 d), and from rats in the Normal and Pilo groups after model validation. For serum extraction, blood samples collected from the tail and portal veins were stored at 22 ± 2°C for 60 min, centrifuged at 3,500 rpm for 15 min at 25°C, and the supernatant was stored at −80°C. Fifty microliters of rat serum were transferred to an EP tube. After the addition of 200 μL of extract solution (acetonitrile: methanol = 1: 1, containing isotopically-labeled internal standard mixture), the samples were vortexed for 30 s, sonicated for 10 min in ice-water bath, and incubated for 1 h at −40°C to precipitate proteins and centrifuged at 12,000 rpm (RCF = 13,800 ×g, R = 8.6 cm) at 4°C for 15 min. The resulting supernatants were transferred to fresh glass vials for further analysis. A quality control sample was prepared by mixing equal aliquots of the supernatant from each sample. Untargeted metabolite data analysis was performed LC-MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo Fisher Scientific).

2.8 Determination of ciprofloxacin concentration in rat cortex

Brain tissue samples were thawed at 4°C and 30 mg of tissue was added to 400 μL 75% methanol-water, swirled for 60 s, fully ground with small steel balls, placed in liquid nitrogen for 1 min, thawed at room temperature, and ultrasound on ice for 10 min (repeated three times). Finally, the mixture was centrifuged at 17,000 × g at 4°C for 15 min, and filtration was done using a 0.22-μm organic membrane filter head.

The internal standard is diluted with 50% methanol-water to achieve a specific concentration of the internal standard diluent. A diluent containing the internal standard was used to dilute the mixed standard and sample 1:1 by volume. The final internal standard concentration was 100 ng/mL. Parameters: Column temperature: 40°C, loading volume: 1 μL, positive ion mode A: 0.1% formic acid aqueous solution. B: Acetonitrile. Mass spectrum conditions: based on the Multiple Reaction Monitoring mode, the AB4500 mass spectrometer collected the primary and secondary mass spectrum data in negative mode. Electrospray Ionization ion source parameters are set as follows: Gas temp: 500°C, Curtain gas: 25 Psi, Collision gas: 10 Psi, ionspray voltage: 4500 V, atomization temperature: 500°C.

2.9 Statistical analysis

For two-group comparisons, the unpaired Student's *t*-test or Mann–Whitney U test was performed. The Cox proportional hazards model was used to compare seizure onset rates. GraphPad

Prism 8.02 and SPSS 25.0 were used for statistical analysis and figure production. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Modulation of rat seizure susceptibility via CPF-induced alterations in GM

3.1.1 CPF gavage increases the seizure susceptibility in SD rats

To determine whether a daily dose of CPF via gavage could enhance seizure susceptibility in SD rats, we induced acute seizure attacks by administering a single i.p. injection of PTZ after 14-day period of CPF or NS gavage (Figure 2A).

Initially, we evaluated seizure susceptibility using 45 mg/kg (18 mg/ml) PTZ ($n = 6$ each in CPF and Ctrl groups). A significant increase in the number of seizures ≥ scale 3 was observed in the CPF-14 d group compared with that in the Ctrl-14d ($p< 0.05$, Figure 2B). Furthermore, there was a substantial increase in the accumulated time of seizures ($p < 0.01$, Figure 2C). To confirm these results, we conducted a seizure susceptibility test with 60 mg/kg (24 mg/mL) PTZ ($n = 13$ in CPF, $n = 10$ in Ctrl). While the number of seizures ≥ scale 3 did not exhibit a significant difference, the CPF-14 d demonstrated a significant augmentation in the average duration of each seizure compared with that in Ctrl-14d ($p < 0.05$, Figure 2E). In addition, the occurrence of single spikes during the 90-min recording significantly increased ($p < 0.01$, Figure 2F). The power can also reflect the EEG discharge (Figure 2K), and the total power in the 0–500 Hz range during the 30 to 60-min interval demonstrated a significant increase in the CPF-14 d, indicating a prolonged period of excitation following CPF gavage ($p < 0.05$, Figure 2H). The death rate during seizures in the CPF-14 d group was 23.1%, whereas none of the rats in the control group died during seizures.

To avoid CPF from entering the brain and causing seizures, we measured CPF concentrations in the cortex. To eliminate this possibility, our results showed that there was no significant difference in CPF concentration in the cortex between the CPF and control group after the 14-day gavage and washout period (Figure 2J).

3.1.2 FMT can reduced seizure susceptibility caused by CPF

To examine whether restoring the GM could reduce the increased seizure susceptibility caused by CPF, we also detected seizure susceptibility after FMT between the CPF-FMT and Ctrl-PBS groups.

After 45 mg/kg PTZ injection ($n = 6$), no significant differences were observed in the number of seizures and accumulative seizure duration following FMT treatment compared to Ctrl-PBS group (Figures 2B, C). The seizure onset rates were 50%, 100%, and 83% in Ctrl-14 d, CPF-14 d, and CPF-FMT, respectively (Figure 2D).

Next, we used 60 mg/kg PTZ to detect seizure susceptibility. FMT treatment resulted in a significant decrease in the average duration of seizures (CPF-14 d vs. CPF-FMT, $p < 0.01$, Figure 2E) and the occurrence of single spikes decreased (CPF-14 d vs. CPF-FMT, $p < 0.01$, Figure 2F), approaching the levels seen in Ctrl-PBS. There was no significant difference in the total power between the two groups in the total power in 0–500 Hz range after FMT (Figure 2I). Moreover, the percentage of seizure onset between CPF-14 d, Ctrl-14 d, and CPF-FMT also exhibited a trend similar to that of 45 mg/kg PTZ ($p = 0.0407$, Figure 2G).

3.2 Gut dysbiosis caused by CPF gavage is similar to that in the lithium pilocarpine-induced epilepsy rat model and can be relieved by FMT

3.2.1 GM diversity in the two rat models

Results from *16S* rRNA sequencing of the GM in different groups showed that CPF gavage markedly disturbed GM diversity, exhibiting a pattern similar to that of the lithium pilocarpine-induced epilepsy rat model (Figure 3).

First, in the CPF-induced seizure susceptibility model, OTUs decreased after CPF treatment. Following Illumina sequencing and application of the 97% similarity threshold, the detected OTUs were similar between the rats in the Ctrl and CPF groups at the start of the experiment, with similar OTUs numbers (CPF-0 d = 509, Ctrl-0 d = 514). However, the number of OTUs significantly decreased to 374 in the CPF-14 d group, while the Ctrl-14 d group contained 613 OTUs.

Second, alpha diversity analysis revealed a reduction in richness and diversity during CPF gavage. As measured using Chao1 and Shannon_2, alpha-diversity significantly decreased in CPF-14 d group compared with that in the Ctrl-14 d group ($p < 0.01$ Chao1, $p < 0.001$, Shannon_2, Figures 3A, C). Moreover, the PCoA analysis of the beta-diversity of the GM quantified by the Bray–Cuitis distance indicated a clear distinction between CPF-14 d and Ctrl-14 d groups (Anosim R = 0.946, $p < 0.001$) shown in Non-metric Multidimensional Scaling (NMDS) (Figure 3B, stress = 0.082) and PCoA (Figure 3D).

Notably, we observed a similar trend in GM diversity in a lithium pilocarpine-induced epilepsy model. Alpha diversity was also significantly decreased in the Pilo group in terms of richness ($p = 0.026$; Figure 3E). PCoA for beta diversity revealed a distinction between the Pilo and Normal groups, although the difference was not statistically significant ($p = 0.054$, Figure 3F).

3.2.2 Composition and differential microbiota in the two rat models

Having identified clear changes in the GM diversity, we aimed to obtain more detailed information, including microbial community composition and species differences. We considered all detailed data regarding different taxa at the phylum and genus levels, with a relative abundance of over 0.01% and the top 15 genera.

In the CPF-induced seizure susceptibility model, Firmicutes and Bacteroidetes were the predominant bacterial phyla. Prior to gavage, the percentages of Firmicutes (65.0% in Ctrl-0 d, 57.3% in CPF-0 d) and Bacteroidetes (30.2% in Ctrl-0 d, 34.4% in CPF-0

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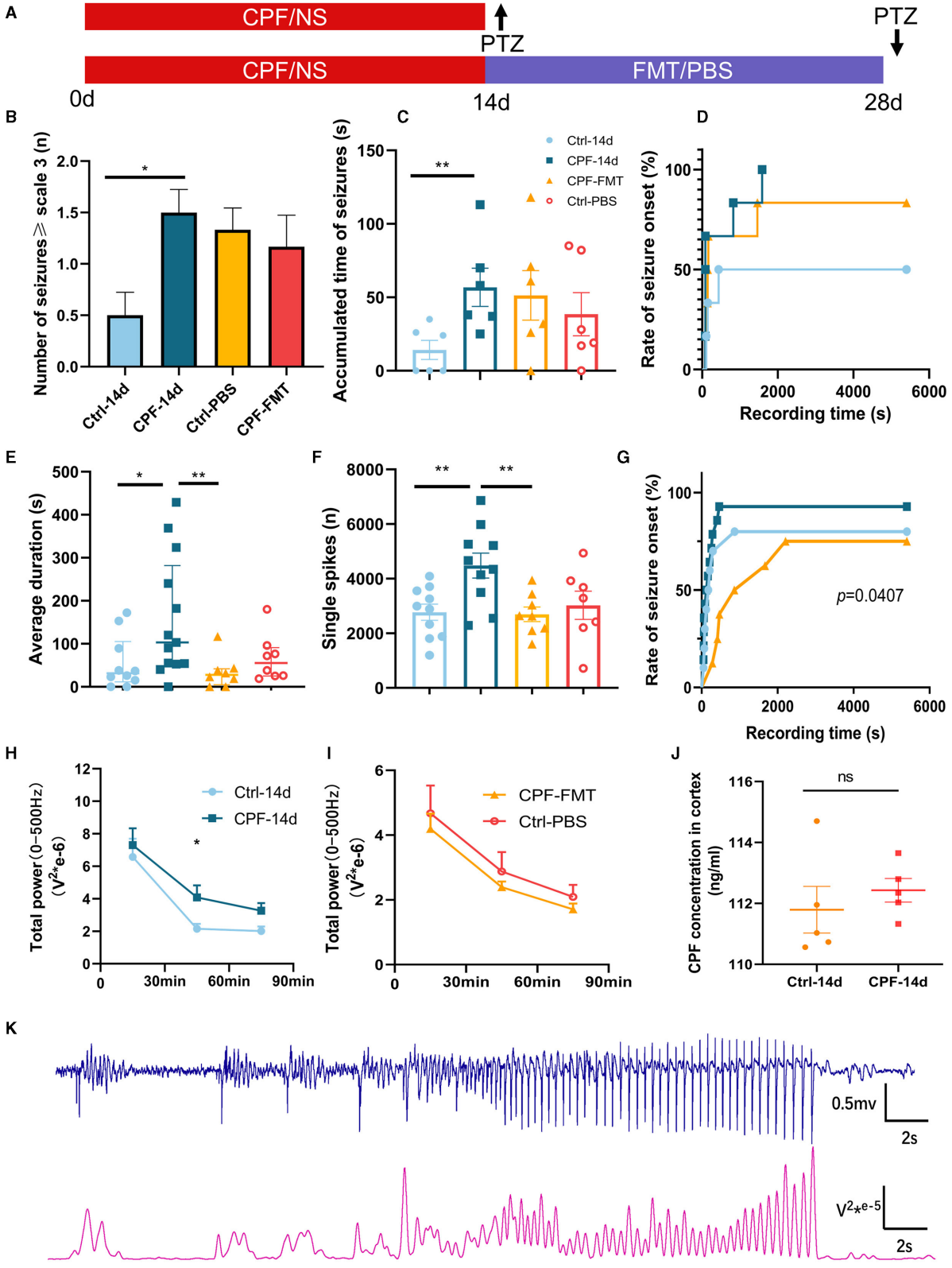

FIGURE 2
Modulation of seizure susceptibility via CPF-induced alterations in GM. **(A)** Schematic diagram of experimental process. **(B)** The number of seizures ≥ scale 3 (PTZ 45 mg/kg). **(C)** The accumulated time of seizures (PTZ 45 mg/kg). **(D)** The rate of seizure onset (PTZ 45 mg/kg). **(E)** The average duration of each seizure (PTZ 60 mg/kg). **(F)** The number of single spikes during recording 90 min (PTZ 60 mg/kg). **(G)** The rate of seizure onset (PTZ 60 mg/kg). **(H)** Total power of 0–500 Hz after 14-day CPF gavage (PTZ 60 mg/kg). **(I)** Total power of 0–500 Hz after FMT (PTZ 60 mg/kg). **(J)** CPF concentration in cortex after CPF gavage ($n = 5$ in each group). **(K)** A part of the correspondence between EEG and 0–500 Hz power curves. Statistics: normally distributed data are given as the mean ± SEM, others are presented as median with interquartile range. *$p < 0.05$, **$p < 0.01$.

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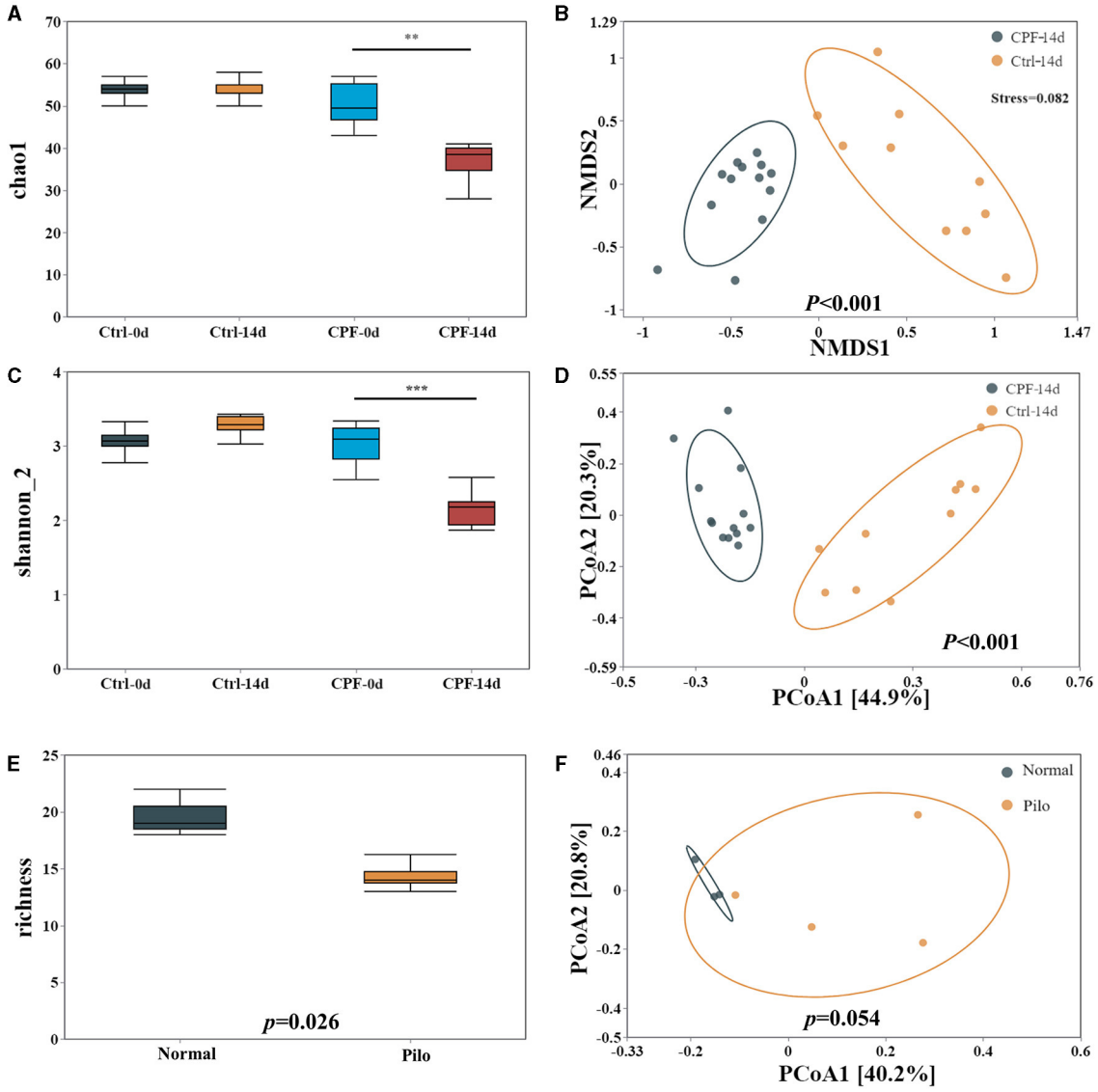

FIGURE 3
Alterations in GM diversity in CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models. **(A)** Alpha diversity in the model of CPF-induced seizure susceptibility in Chao1. **(B)** Beta diversity in the model of CPF-induced seizure susceptibility in NMDS2. **(C)** Alpha diversity in the model of CPF-induced seizure susceptibility Shannon_2. **(D)** Beta diversity in the model of CPF-induced seizure susceptibility in PCoA. **(E)** Alpha diversity in the model of lithium pilocarpine-induced epilepsy. **(F)** Beta diversity in the model of lithium pilocarpine-induced epilepsy. Statistics: Student *t* test was used for two-group comparison and Kruskal-Wallis test was used for multiple groups. $^{**}p < 0.01$, $^{***}p < 0.001$.

d) were similar between the two groups. However, Firmicutes decreased to 21.9% after CPF administration. Notably, the relative abundance of Verrucomicrobia, which ranked third, increased substantially after CPF gavage, increasing from 7.1% to 38.1% (Figure 4D). At the genus level, *Lactobacillus, Ruminococcaceae, Parabacteroides, Ruminococcaceae_NK4A214_group*, and *Butyricicoccus* exhibited reduced relative abundance following CPF gavage. Conversely, certain bacterial taxa experienced a relative increase, including *Bacteroides, Akkermansia, Eisenbergiella,* and *Phascolarctobacterium* (Figure 4C). Furthermore, research on presumed distinctive microbial differences between the two groups was performed using LEfSe analysis. As illustrated in Figure 4H, *Akkermansia, Bacteroides, Phascolarctobacterium, Escherichia, Hungatella,* and *Ruminiclostridium_5* were identified as characteristic bacteria in CPF-14 d. In contrast, *Lactobacillus, Ruminococcus_2, Romboutsia, Parabacteroides*, etc., were found to be the characteristic bacteria in Ctrl-14 d. A heat map of the relative abundances is shown in Figure 4F.

In the lithium pilocarpine-induced epilepsy model, the GM composition was entirely different from that of the normal group. Firmicutes (74.5% in Normal and 65.1% in Pilo) and Bacteroidetes (24.1% in Normal and 20.9% in Pilo) were the two most abundant phyla. Similar to the increased relative abundance of CPF-induced seizure susceptibility, the relative abundance of Verrucomicrobia was much higher in the Pilo group (11.6% in Pilo and 0.04% in Normal

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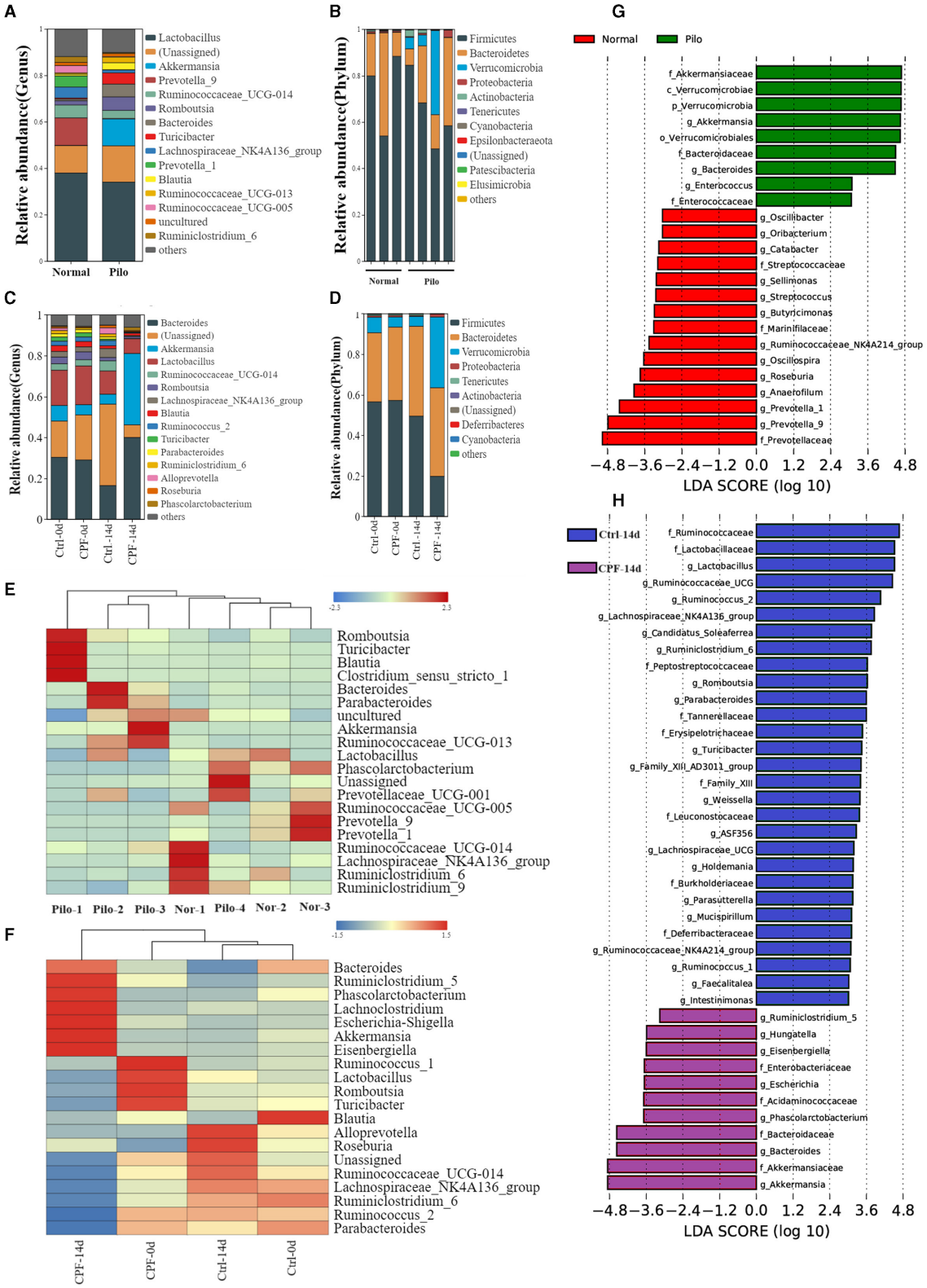

FIGURE 4
Analysis of the differential GM in CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models. **(A, B)** Microbial composition at the genus and phylum levels between the Pilo and Normal group (top 15 microbiota with relative abundance >0.01%). **(C, D)** Microbial composition at the genus and phylum levels between the CPF and Ctrl group at different timepoints (top 15 microbiota with relative abundance >0.01%). **(E)** Heatmap of relative abundance at the genus level between Pilo and Normal group. **(F)** Heatmap of relative abundance at the genus level between CPF and Ctrl group. **(G)** Discriminant taxa between Pilo and Normal group ranked by their linear discriminant analysis (LDA) effect size (LEfSe) of microbiota, LDA score >3. **(H)** Discriminant taxa between CPF and Ctrl group at 14th day (CPF-14 d vs. Ctrl-14 d) ranked by LEfSe, LDA score >3.

groups; Figure 4B). At the genus level, *Lactobacillus, Prevotella-9,* and *Lachnospiraceae_NK4A136_group* exhibited reduced relative abundance in the Pilo group. However, the abundances of *Akkermansia, Romboutsia,* and *Bacteroides* increased (Figure 4A). LEfSe analysis revealed that *Akkermansia, Bacteroides,* and *Enterococcus* were the characteristic bacteria in Pilo. In contrast, *Prevotella, Anaerofilum, Roseburia, Oscillospira, Butyricicoccus,* and *Ruminococcaceae_NK4A214_group* were the characteristic bacteria under normal conditions (Figure 4G). A heat map of the relative abundances is shown in Figure 4E. Most importantly, we found some commonalities in GM diversity and compositional changes in both models.

3.2.3 FMT treatment reconstituted GM dysbiosis and reduction after CPF gavage

Following FMT, we performed *16S* rRNA sequencing and subsequent analysis to verify the restoration of the GM profile. Post-FMT, the number of OTUs increased to 699 in the CPF-FMT group compared with 609 in the Ctrl-PBS group. Furthermore, the alpha-diversity metrics, as indicated by the Chao1 and Shannon_2 indices, reverted to levels comparable to those of the Ctrl-PBS (Figures 5A, B). Beta-diversity analysis revealed an overlap between the CPF-FMT and Ctrl-PBS samples (Figure 5D), in contrast to the distinct separation observed between the CPF-14 d and Ctrl-14 d (Figure 5C). However, while recovery trended toward Ctrl-PBS, it did not completely overlap with Ctrl-0 d and Ctrl-14 d (Figure 5F), underscoring the dynamic shifts in the GM throughout growth and development. Consistent alterations were also evident in the phylum and genus compositions (Figures 5G, H), along with their relative abundances (Figure 5E), showing that FMT helped gut dysbiosis recover to that of the Ctrl group.

3.2.4 The common alteration of genera and the change of seizure susceptibility

There were some similar changes in the GM of the CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy models. To explore the core GM that might have shared potential to influence susceptibility to epilepsy, we conducted a comparative and correlated analysis of the GM profiles between CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models.

The results of LEfSe analysis in both models were compared to identify common changes in the GM. In total, we observed 11 common taxa that exhibited the same significant change in direction in both models (Figures 6A, B). At the genus level, *Akkermansia* and *Bacteroides* increased in both models, while *Marvinbryantia*, *Oscillibacter*, and *Ruminococcaceae_NK4A214_group* decreased. The relative abundances of the above five genera after CPF and FMT treatments are shown in Figures 6C–G, and the relative abundances in Pilo compared to Normal are shown in Figures 6H–L. Notably, we discovered that *Akkermansia* increased in both the CPF-14 d and Pilo groups, with a noticeable increase spanning the entire phylum. From the phylum to genus level, there was a notable increase in Verrucomicrobia, *Verrucomicrobiae, Verrucomicrobiales, Akkermansiaceae,* and *Akkermansia*. Correlation analysis revealed that *Bacteroides, Eisenbergiella, Escherichia_Shigella, Phascolarctobacterium*, and *Akkermansia* were positively associated with heightened seizure susceptibility. In contrast, *Lachnospiraceae_NK4A136_group, Ruminiclostridium_6, Ruminococcus_2*, and *Ruminococcaceae_UCG-014* showed negative correlations (Figure 6M).

3.3 Untargeted metabolomics profile changed in serum after CPF gavage

To further investigate how GM influences epilepsy and its susceptibility mechanisms, we explored how GM affects host metabolism in serum. Based on the differential GM, the analysis of microbial functional prediction using PICRUSt2 revealed that the metabolic patterns in serum were similar between Ctrl-14 d and CPF-FMT but dissimilar to CPF-14 d in KEGG Orthology. Some processes, including amino acid transport and metabolism, extracellular structure, and lipid metabolism, exhibited similarities (Figure 7A). The heatmap shows the correlation between different metabolites and groups (Figure 7B). Among the discriminant metabolites in CPF-14 d, we identified a decrease in Adrenochrome, Acetylpterosin C, and 3-indolepropionic acid (IPA) compared to those in Ctrl-14 d. D-glucose, the main energy source for the brain, also decreased. However, 2-piperidone and nicotinic acid mononucleotides increased (variable importance of projection >1, $p < 0.05$, Figures 7C–H).

3.4 Dynamic changes in IPA serum levels in CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models

In a cross-analysis of the similarities and differences in serum metabolomics between the two rat models, we observed a consistent decrease in the common metabolite, IPA, a key metabolic product of tryptophan. The heatmap illustrated clear associations between certain GM taxa and altered metabolites (Figure 8A). The GM taxa that were significantly related to these changes included *Parabacteroides, Ruminococcus_1, Ruminococcus_2, Ruminiclostridium_6, Eisenbergiella, Escherichia_shigella, Bacteroides, Phascolarctobacterium,* and *Akkermansia*. We found that some indole-associated bacteria, including *Lactobacillus, Parabacteroides,* and *Clostridiaceae_1*, were consistent with the changes in IPA during CPF gavage and FMT (Figure 8B). In the CPF group, there was a significant decrease in IPA ($p < 0.01$). Although 3-indoleacrylic acid levels also decreased, this decrease did not reach statistical significance (Figures 8C, D). Notably, the levels of corresponding indole derivatives showed a more pronounced decrease in the Pilo group, along with reduction in the levels of other products associated with tryptophan, including L-tryptophan, and L-kynurenine (Figures 8E–H).

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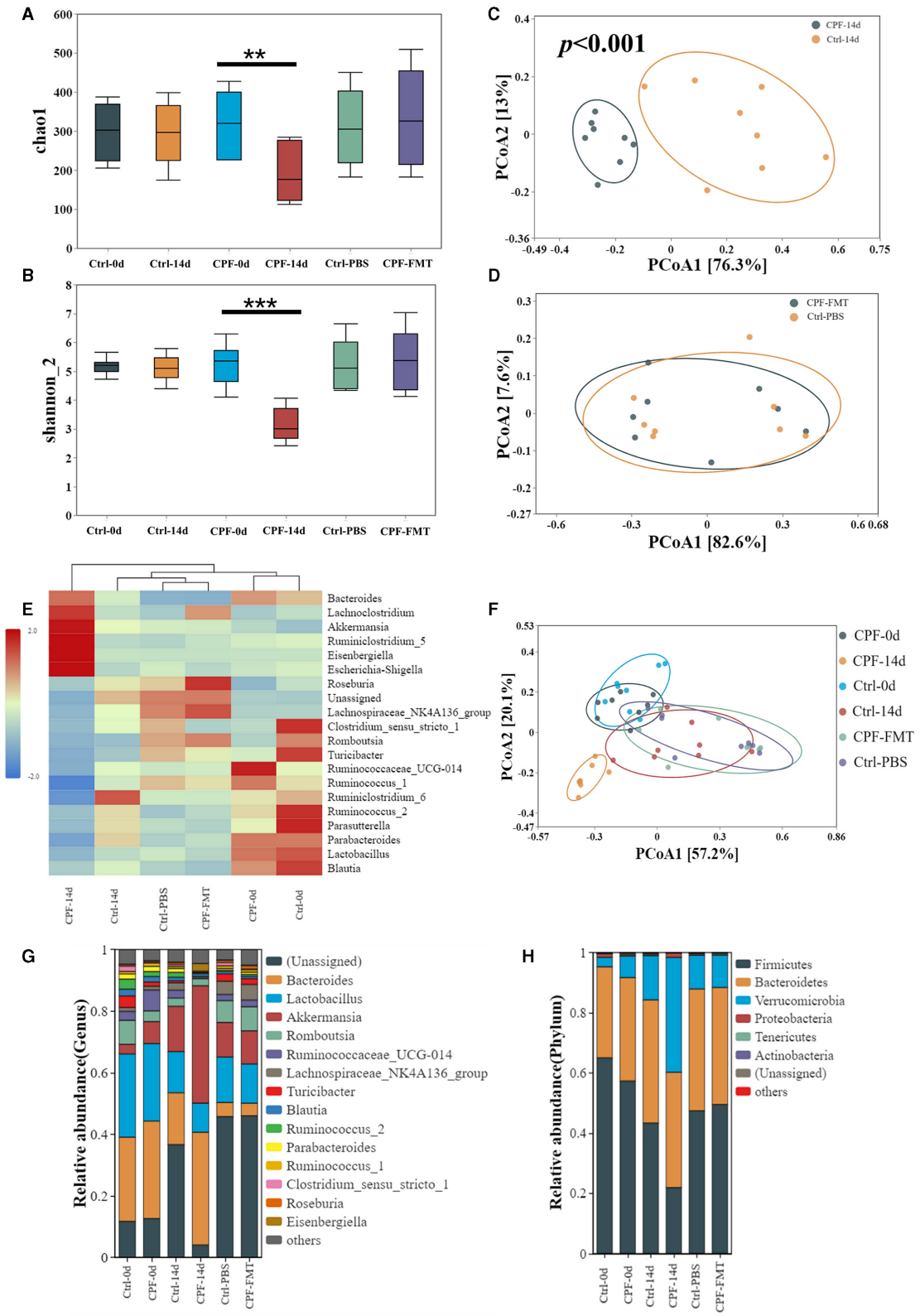

FIGURE 5
FMT treatment reconstituted GM dysbiosis after CPF gavage. **(A, B)** Alpha diversity of the GM between groups at different timepoints (0 d, 14 d, 28 d) in Chao1 and Shannon_2. **(C)** Beta diversity of the GM beween CPF-14 d and Ctrl-14 d in PCoA (Anosim R = 0.946, $p < 0.001$). **(D)** Beta diversity of the GM beween CPF-FMT and Ctrl-PBS in PCoA. **(E)** Heatmap of relative abundance at the genus level at different timepoints (0 d, 14 d, 28 d). **(F)** Beta diversity of the GM beween CPF and Ctrl group at different timepoints (0 d, 14 d, 28 d). **(G, H)** Microbial composition at the phylum and genus levels between the CPF and Ctrl groups at different timepoints (0 d, 14 d, 28 d) (top 15 microbiota with relative abundance >0.01%). Statistics: Student *t* test was used for two-group comparison and Kruskal-Wallis test was used for multiple groups. $^{**}p < 0.01$, $^{***}p < 0.001$.

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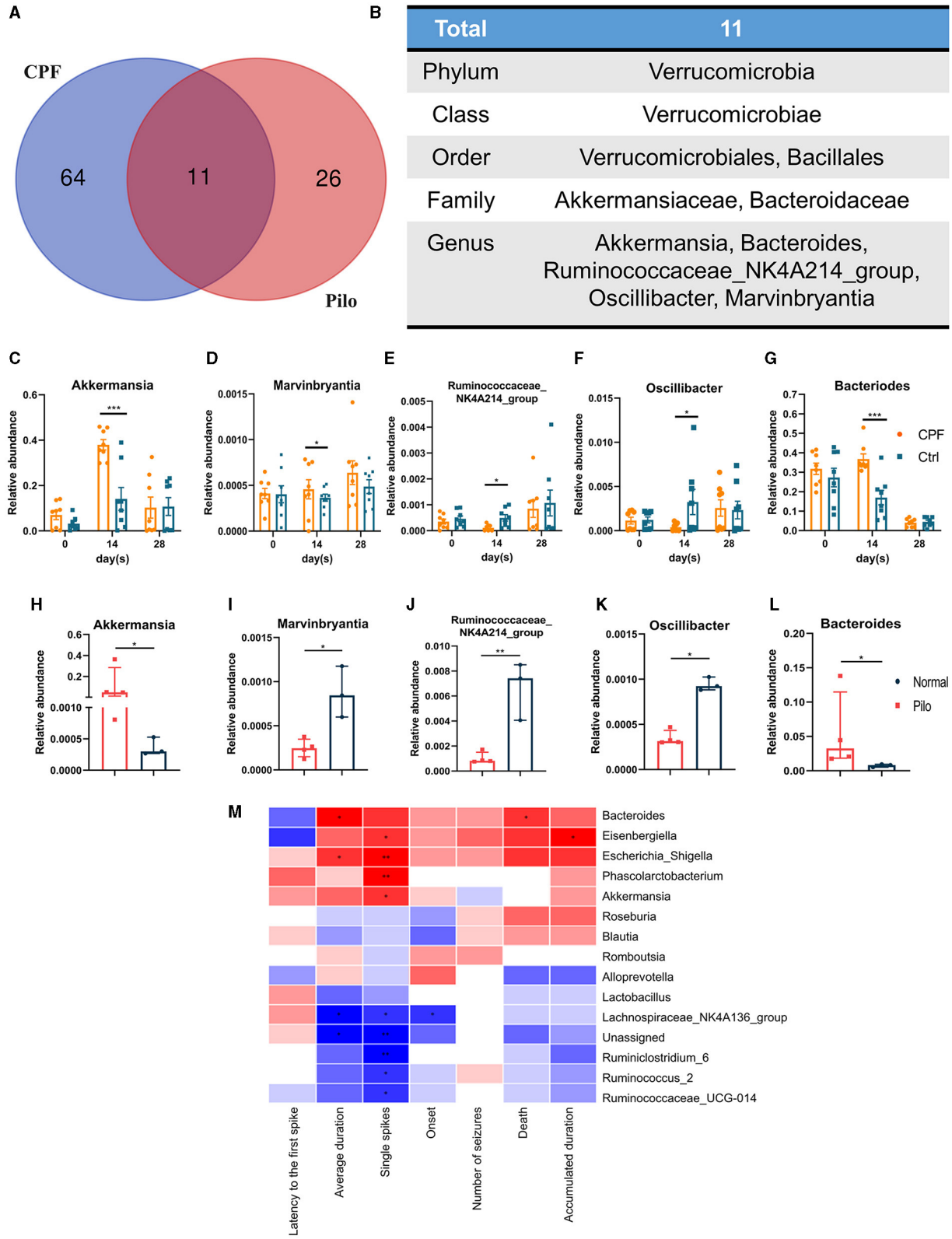

FIGURE 6
Common alterations observed in some genera in CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models. **(A)** Venn diagram of GM of CPF-induced seizure susceptibility and lithium pilocarpine-induced rat models. **(B)** The total 11 differential GM in all classification levels. **(C–G)** Relative abundances of five significantly altered bacterial genera in CPF compared to Ctrl. **(H–L)** Relative abundances of five significantly altered bacterial genera in Pilo compared to Normal. **(M)** Correlation heatmap of seizure susceptibility and GM abundances. Statistics: normally distributed data are given as the mean ± SEM, others are presented as median with interquartile range. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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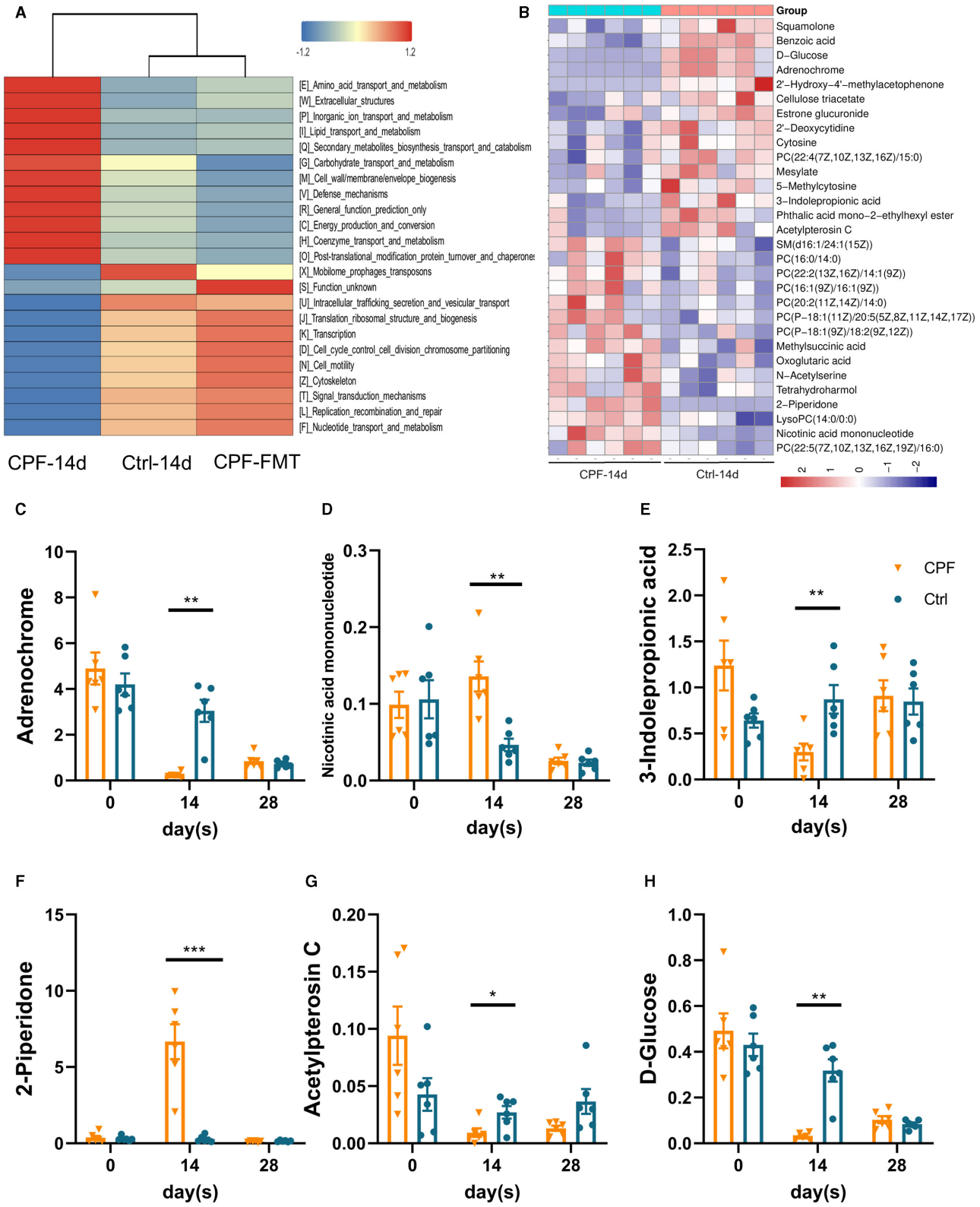

FIGURE 7
Untargeted metabolomics shifted in the serum after CPF gavage and FMT. **(A)** Comparison of PICRUSt-based metagenomics prediction among Ctrl-14 d, CPF-14 d and CPF-FMT. **(B)** Heatmap of hierarchical clustering analysis between CPF-14 d and Ctrl-14 d of the serum untargeted metabolism. **(C–H)** The significant different metabolites affected by CPF and FMT treatment (3-indolepropionic acid, acetylpterosin C, 2-piperidone, D-glucose, Nicotinic acid mononucleotide, and Adrenochrome). Statistics: normally distributed data are given as the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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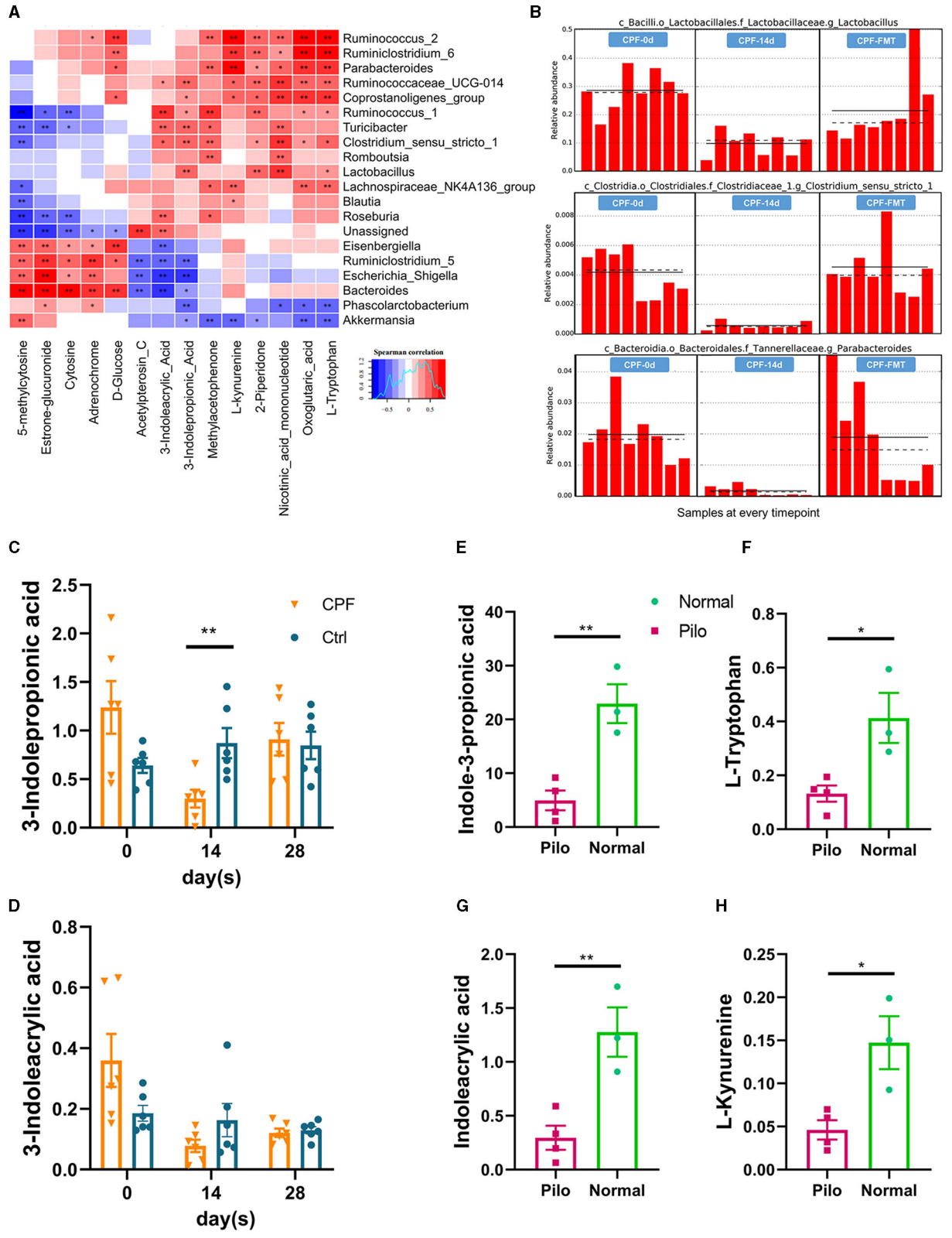

FIGURE 8
Dynamic modulation of 3-indolepropionic acid in CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models. **(A)** Microbial community and untargeted serum metabolomics correlation heatmap analysis. **(B)** The relative abundances of tryptophan-indole metabolism related GM in different timepoints by lefSE analysis (*Lactobacillus* $p = 0.00041$, *Parabacteroides* $p = 0.00043$, and *Clostridiaceae_1* $p = 0.00046$). **(C, D)** Changes of serum metabolites related to tryptophan-indole metabolism in CPF group. **(E–H)** Changes of serum metabolites related to tryptophan-indole metabolism in lithium pilocarpine-induced epilepsy model. Statistics: normally distributed data are given as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.

4 Discussion

In this study, we demonstrated for the first time that the therapeutic dose of oral CPF increases susceptibility to epilepsy by directly altering the GM and its metabolites, without increasing CPF concentrations in the brain tissue. Moreover, in a more detailed analysis, we observed the diversity, composition and identical changes in some core genera in CPF-induced seizure susceptibility and lithium pilocarpine-induced epilepsy rat models. These changes may play a crucial role in epilepsy. Finally, we identified one of the metabolites of the tryptophan-indole metabolic process, IPA, as potentially significant in the pathological process of epilepsy. This study provides valuable insights into the intricate interplay between the GM and epilepsy, opening avenues for refined treatment strategies for epilepsy.

Numerous clinical studies have demonstrated that some antibiotics can induce epilepsy or increase susceptibility to epilepsy, including β-lactam, fluoroquinolone families, fourth-generation cephalosporins, and carbapenems (Wanleenuwat et al., 2020). In our study, we focused on CPF primarily because clinical observations have demonstrated that quinolone antibiotics can induce seizures (Slavich et al., 1989; Springuel, 1998; Kisa et al., 2005; Striano et al., 2007; Sutter et al., 2015). This phenomenon has also been corroborated in animal experiments, such as mice and zebrafish (Abdel-Zaher et al., 2012; Arafa et al., 2013; Sivarajan and Ramachandran, 2023). In fact, the research on the mechanism of antibiotic-induced epilepsy has been investigated for decades, which can be traced back to the 1990s. Here is a summary of some previous findings. First, some antibiotics have been found to inhibit GABA synthesis or block GABA receptors. β-lactams (i.e., penicillins, cephalosporins, and carbapenems) and quinolones all have been shown to act on these process (Segev et al., 1988; Hantson et al., 1999). Second, activation of excitatory transmission through activation of N-methyl-D-aspartate (NMDA) receptors, as may occur with carbapenems and quinolones (Akahane et al., 1993; Schmuck et al., 1998; Lode, 1999). Additionally, the structural similarities between certain antibiotics and epileptogenic compounds have been explored (De Sarro et al., 1989), with specific attention given to substituents at the C7 position that enhance binding to GABA receptors (Lode, 1999). Furthermore, interactions between older generation ASMs and certain antibiotics are known to influence the pharmacokinetics (Patsalos and Perucca, 2003). Coadministration of valproic acid and carbapenem will decrease the plasma valproic acid concentration by 58%, and increase the valproic acid clearance (Tobin et al., 2009). Finally, some antibiotics increase oxidative stress, potentially compromising the efficacy of epilepsy treatment (Ilgin et al., 2015; Wanleenuwat et al., 2020). However, many of these studies often induced acute and subacute seizures using large doses of CPF administered intraperitoneally, intravenously, or even intrathecally. Consequently, they may have overlooked the significant impact of CPF on the GM. Our study is the first to reveal that prolonged, clinically relevant oral administration of CPF promotes seizure susceptibility through the GM. Additionally, despite its lower BBB permeability than that of levofloxacin, CPF exhibits a higher proconvulsive potential, suggesting that its increased susceptibility to epilepsy may involve other pathways (Akahane et al., 1993). CPF is known to have a high concentration in the gastrointestinal tract (Zimmermann and Curtis, 2019), and induces gut dysbiosis (Stewardson et al., 2015; Zhu et al., 2020). Finally, considering the widespread use of CPF in the treatment of urinary tract and reproductive system infections (Thai et al., 2023), complete avoidance is challenging in certain clinical scenarios. Therefore, CPF is an appropriate instrument for studying the relationship between epilepsy and MGBA, and our study provides valuable insights for practical applications in a clinical setting.

Analysis of GM revealed a decrease in α-diversity, an estimate of species richness in CPF-14 d and Pilo group. This result is corresponding with previous animal and clinical studies (Gong et al., 2020; Oliveira et al., 2022). Decreased diversity and evenness have often been considered to reflect disease status. At the genus level, we found that some probiotic strains decreased. *Lactobacillus* can produce tryptophan and indole derivatives that regulate the immune process (Xia et al., 2021; Sun et al., 2022; Yan et al., 2022). In addition, it can modulate several neurotransmitters (Wu and Shah, 2017) and enhance the production of non-volatile acids and SCFAs (Dalile et al., 2019). Administration of probiotics, including *Lactobacillus* and *Bifidobacterium,* can reduce the seizure severity in the chronic epilepsy model induced by PTZ (Bagheri et al., 2019; Kilinc et al., 2021). Additionally, *Butyricicoccus, Parabacteroides* (Liao et al., 2024), and *Ruminococcaceae* (Peled et al., 2024) can produce SCFAs to promote health. Specifically, we found consistent changes in five genera across the two rat models: *Akkermansia, Bacteroides, Marvinbryantia, Oscillibacter,* and *Ruminococcaceae_NK4A214_group*. We observed similar findings in previous studies we observed similar findings on the GM and epilepsy (Russo, 2022; Yue et al., 2022). Among these, our particular focus was on *Akkermansia* because it ranked at the front position in the LEfSe analysis in the two rat models, which has been discussed in many previous studies. *Bacteroides* can produce SCFAs and are beneficial to the host; however, they can be opportunistic pathogens and are related to some pathological status (Shin et al., 2024). It could also be related to regulate the secretion of IL-6 and IL-17 in dendritic cells to associated with seizure severity (Xie et al., 2017).The functions of *Marvinbryantia* and *Oscillibacter* remain unclear, but they are often reported to be associated with health status or serve as protective factors (Fang et al., 2016; Xu et al., 2020; Crossland et al., 2023; Guo et al., 2023). This may be related to the production of SCFAs, which contribute to the maintenance of GM homeostasis. This suggests that these genera could be core contributors, play a substantial role in epileptogenesis, and serve as potential target genera for epilepsy treatment from the perspective of GM.

In our study, *Akkermansia* increased significantly and was ranked first in the LEfSe analysis of the two rat models. In fact, *Akkermansia* stands out as a known member of the GM and has been extensively investigated, particularly in the context of metabolic disorders and inflammatory processes (Cani et al., 2022). In studies of metabolic disorders, including obesity (Everard et al., 2014), diabetes (Plovier et al., 2017), and liver steatosis (Kim et al., 2020), *Akkermansia* has been found to be positively associated with improved metabolic health and better clinical outcomes. The same result was also observed in some inflammatory bowel diseases (Liu et al., 2021) and in response to cancer

checkpoint immunotherapies (Santoni et al., 2018). Therefore, it is regarded as a paradigm for next-generation probiotics (Abuqwider et al., 2021; Cani et al., 2022). However, the "success story" of *Akkermansia* has not been without its share of controversies. In our study, the relative abundance of *Akkermansia* spanning the entire phylum significantly increased in CPF-14 d and Pilo groups. From the phylum to genus level, Verrucomicrobia, *Verrucomicrobiae, Verrucomicrobiales, Akkermansiaceae,* and *Akkermansia* displayed increased levels across all strata in the context of epilepsy. This observation is consistent with numerous studies on epilepsy (Huang et al., 2019, 2022; Arulsamy et al., 2020; Gong et al., 2021; Yue et al., 2022). Furthermore, *Akkermansia* increases have been noted in other neurological conditions, such as anxiety and depression induced by chronic stress (Li et al., 2019), multiple sclerosis (Cox et al., 2021), and Parkinson's disease (Zhao et al., 2021).

Akkermansia muciniphila, as the name suggests, is an exclusive mucin-degrading specialist present in the human intestine from early life (Derrien et al., 2008). *A. muciniphila* produces oligosaccharides, acetate, propionate, 1,2-propylene glycol, and ethanol via the breakdown of mucus and mucin (Derrien et al., 2004). Elevated levels of *A. muciniphila* are associated with reduced intestinal mucosal thickness, increased gut permeability, and inflammation. These factors may contribute to systemic inflammation and subsequent brain damage (Citraro et al., 2021). Previous findings suggest a potentially positive association with inflammatory status (Everard et al., 2013). The increase in *Akkermansia* in the lithium pilocarpine-induced epilepsy rat model suggests that inflammation and metabolic changes may play a role within the GM in the traditional model of chronic epilepsy. Overall, we believe that *Akkermansia* may play a complex role in the development of epilepsy, depending on the different populations and treatment choices. The relative abundance of *Akkermansia* may need to be maintained at a specific level to strike a balance between excess and deficiency.

Similar to the shared alterations observed in the GM, we conducted a comparative analysis of common changes in metabolites between the CPF and Pilo groups. Tryptophan-indole metabolism has emerged as a common phenomenon. In particular, one of the products, IPA, exhibited a consistent decreasing trend in both rat models. This change was more pronounced in the Pilo group. This discrepancy may be attributed to the fact that the lithium pilocarpine-induced epilepsy model manifests evident spontaneous seizures, whereas CPF primarily lowers the seizure threshold, representing a condition that is not strictly synonymous with an epilepsy model.

Tryptophan (TRP) metabolism pathways leading to serotonin, kynurenine, and indole derivatives are under the direct or indirect control of the GM (Agus et al., 2018). GM encodes enzymes that metabolize TRP to 5-HT, KYN, and indole metabolites, which affect TRP availability (O'Mahony et al., 2015). TRP is the only amino acid that contains an indole. In contrast to other TRP metabolites, indoles result exclusively from microbial metabolism (Kennedy et al., 2017). Indoles and their derivatives can be used as ligands to regulate inflammation and autoimmune responses *in vivo*, and play important roles in MGBA (Zhou et al., 2023b). In our studies, we have found some genera significantly related to this process (Figure 8A). These genera have been reported to be associated with metabolites involved in tryptophan metabolism, such as IPA, indoleacrylic acid (IAA), L-kynurenine, and L-tryptophan (Wikoff et al., 2009; Zelante et al., 2013; Roager and Licht, 2018; Su et al., 2022).

TRP metabolism has been explored in the context of epilepsy, with some studies focusing on kynurenic acid metabolism. We found that the concentration of kynurenic acid decreased significantly in the serum of the Pilo group, which corresponds to the findings of (Dey et al., 2022), who discovered that in a lithium-pilocarpine rat model, there was a reduction in kynurenic acid levels in the hippocampus and anterior temporal lobe. Furthermore, the exogenous application of kynurenic acid inhibits glutamatergic activity in slice preparations from rats with temporal lobe epilepsy. Promoting kynurenic acid production has been suggested as a strategy to mitigate spasms in animal models of infantile spasms (Mu et al., 2022a). In a pilot study of patients with epilepsy, tryptophan and kynurenine were shown to have the potential as diagnostic markers for epilepsy (Zhou et al., 2023a).

While evidence linking epilepsy to indole and its derivatives is currently limited, a growing body of research is shedding light on their relationship with the central nervous system (CNS), which serves as a neuroprotective factor. In 1980, Young et al. (1980) conducted an experiment on rats, indicating that IPA in the CSF is not derived from the CNS, but from bacterial metabolism in the gut. It has a high permeability to the BBB and does not exhibit a gradient. IPA is a small molecule of indoles that can passing through the lipid bilayer via free diffusion (Piñero-Fernandez et al., 2011). IPA was detected in the gut, serum, and brain of SPF mice compared to germ-free mice, thereby its production was interpreted to be fully mediated by the GM (Sarkar et al., 2016; Loh et al., 2024). The GM affects the diversity of indole compounds in serum, and GM induction is sufficient to introduce IPA into the host (Wikoff et al., 2009). Probiotics increase the level of IPA, reduce the inflammatory process, lower the HPA axis regulation factors, and reduce depressive-like behavior (Abildgaard et al., 2017). All of these showed a close correlation between the GM and IPA. In CNS studies, plasma IPA levels were significantly lower in patients with Huntington's disease (Rosas et al., 2015). Liu et al. (2020) found that IPA and 5-HT treatment significantly attenuated cognitive deficits in diabetic mice. In Alzheimer's disease (AD), IPA can protect against Aβ-induced neuronal death as a scavenger of free radicals (Chyan et al., 1999). Sun et al. (2022) found that the indoles derivates inhibited the activation of the NF-κB signal pathway as well as the formation of the NLRP3 inflammasome, reduced the release of inflammatory cytokines, and then reduced the neuroinflammation in AD transgenic mice. Xie et al. (2022) found that the administration of IPA attenuated the activity of neurotoxic reactive A1 astrocytes in a mouse model of ischemic stroke. And IPA protects neurons from ischemia-induced damage by reducing DNA damage and lipid peroxidation (Hwang et al., 2009). In recent years, it has become evident that indole derivates, including IAA and IPA, are acting as ligands of the aryl hydrocarbon receptor (AHR). AHR is a transcription factor extensively expressed in astrocytes and microglia (Gutiérrez-Vázquez and Quintana, 2018). Its activation leads to alterations in both the innate and adaptive immune responses, subsequently

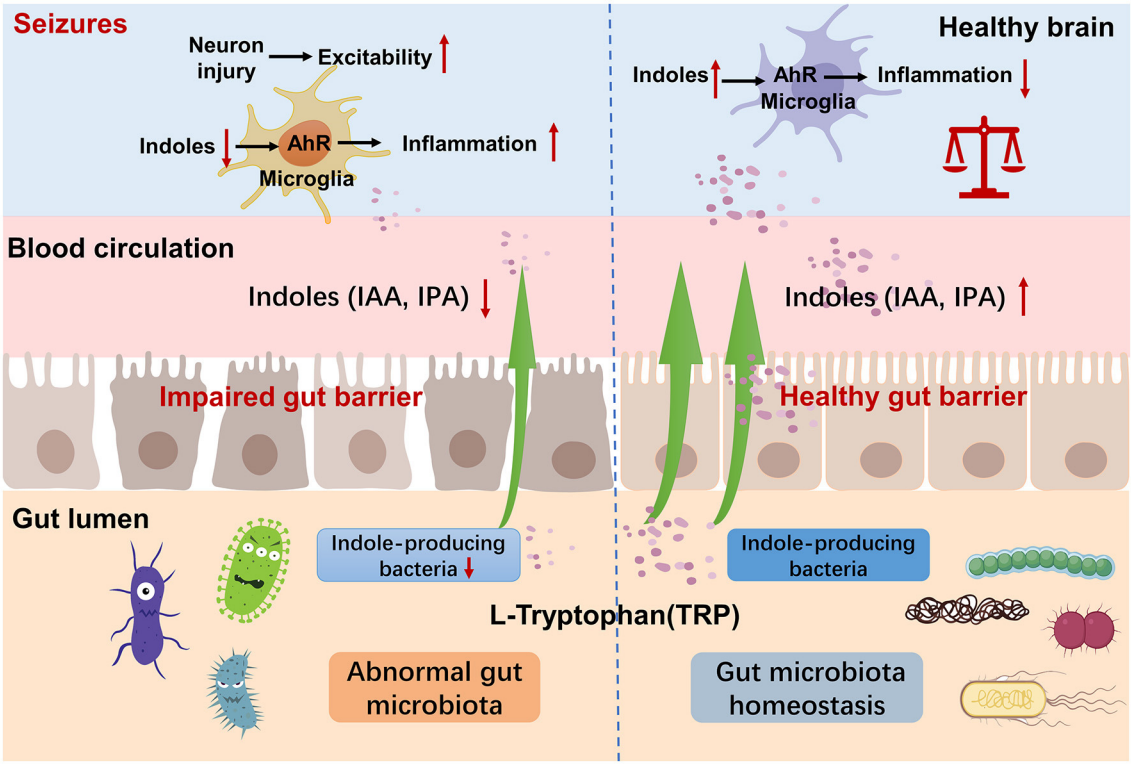

FIGURE 9
Mechanism of tryptophan-indole metabolism in neuroinflammation and epilepsy. Indole-producing bacteria regulate the AhR signaling pathway in microglia via indoles and mediated neuroinflammation.

regulating intestinal epithelial function, gut barrier integrity, and GM composition (Dong et al., 2020; Scott et al., 2020; Li et al., 2021). Previous study on multiple sclerosis mouse model showed that AHR acts as a negative regulator of NF-κB activation (Rothhammer et al., 2016). In recent years, more and more researchers have confirmed that neuroimmunity and inflammation also plays a crucial role in epilepsy (Vezzani et al., 2011, 2015). The changes in GM and tryptophan-indole metabolism mentioned above may collectively contribute to the development of epilepsy (Figure 9).

4.1 Limitations

The utilization of animal models in this study poses limitations when extrapolating the findings to human conditions. Due to the differences of species, the translatability of results from animal models to humans, especially concerning complex biological processes such as GM interactions, demands cautious interpretation and may not fully encompass the intricacies of human physiology.

5 Conclusions and future directions

In this study, we presented new evidence for the first time that CPF can directly alter GM, leading to increased epilepsy susceptibility in rats. The reversal of FMT on the susceptibility to epilepsy confirmed that GM and MGBA may be pivotal mechanism underlying the increased susceptibility to epilepsy induced by CPF. Changes in the genera *Akkermansia, Bacteroides, Marvinbryantia, Oscillibacter*, and *Ruminococcaceae_NK4A214_group* have been shown to be associated with epileptic activity in two epilepsy related animal models. In addition, GM-produced amino acid metabolites may be critical to the mechanisms of MGBA in relation to seizures and epilepsy. IPA, a product of tryptophan-indoles metabolism, may play a crucial role in this process from the perspective of neuroimmune regulation.

In future research on the mechanisms or clinical translation potentials referring the GM and epilepsy, a starting point could be the core GM genera or the tryptophan-indole metabolic pathway mentioned above. For instance, modulation of the relative abundance of core GM genera through probiotics or targeted drugs, or supplementation of tryptophan metabolites such as kynurenic acid and IPA, could be investigated. Further molecular mechanism studies are necessary to elucidate how GM influence seizure susceptibility or the development of epilepsy, and to identify molecular-level treatment targets.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI BioProject, accession PRJNA1056293, PRJNA1056516.

Ethics statement

The animal study was approved by the Institutional Ethical Committee for Animal Welfare of the Seventh Affiliated Hospital of Sun Yat-sen University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – original draft, Writing – review & editing. YL: Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. QZ: Data curation, Investigation, Methodology, Writing – review & editing. MY: Data curation, Methodology, Writing – review & editing. HLi: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. RN: Formal analysis, Methodology, Writing – review & editing. HLa: Formal analysis, Supervision, Writing – review & editing. JW: Methodology, Writing – review & editing. XY: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Visualization, Writing – review & editing. LZ: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Validation, Writing – review & editing.

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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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Baseline gut microbiota profiles affect treatment response in patients with depression

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The role of the gut microbiota in the pathophysiology of depression has been explored in numerous studies, which have confirmed that the baseline gut microbial profiles of patients with depression differ from those of healthy individuals. The gut microbiome affects metabolic activity in the immune and central nervous systems and regulates intestinal ecology through the neuroendocrine system. Additionally, baseline changes in the gut microbiota differed among patients with depression who demonstrated varying treatment response. Currently, probiotics are an emerging treatment for depression; however, the efficacy of modulating the gut microbiota in the treatment of depression remains uncertain. Additionally, the mechanisms by which changes in the gut microbiota affect treatment response in patients with depression remain unclear. In this review, we aimed to summarize the differences in the baseline gut microbiota between the remission and non-remission groups after antidepressant therapy. Additionally, we summarized the possible mechanisms that may contribute to antidepressant resistance through the effects of the gut microbiome on the immune and nervous systems, various enzymes, bioaccumulation, and blood–brain barrier, and provide a basis for treating depression by targeting the gut microbiota.

KEYWORDS

major depressive disorder, gut microbiota, gut-brain axis, psychobiotic, treatment response

1 Introduction

Depression is a psychiatric disorder characterized by various symptoms, including depressed mood, anhedonia, appetite changes, sleep disturbances, psychomotor retardation and/or agitation, fatigue, feelings of guilt, poor concentration, suicidal ideation, and cognitive impairment (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018; Burrows et al., 2020). The etiology of depression involves abnormal neuroendocrine (Farzi et al., 2018), neuroimmune (Birmann et al., 2021), metabolic (Lukić et al., 2022), and neurotransmitter (Cammisuli et al., 2022) functioning. Epidemiological studies have estimated the global prevalence of depression to be 4.4% (Ferrari et al., 2013), affecting approximately 350 million people worldwide (Joca et al., 2015). According to the World Health Organization World Mental Health survey, the estimated lifetime prevalence of depression is 14.6% in

high-income countries and 11.1% in low- and middle-income countries (Kessler and Bromet, 2013). In China, the prevalence of depression is 6.87%, affecting approximately 90 million people (Liang et al., 2018). Epidemiological surveys have shown that a substantial proportion of patients with major depressive disorder (MDD) fail to respond to current first-line antidepressants, imposing a substantial healthcare burden on society (Zhdanava et al., 2021). According to a meta-analysis, only 46% of individuals achieved remission by the end of treatment, even when a combination of psychotherapy and pharmacotherapy was used (de Maat et al., 2007). In addition, the adverse effects of antidepressants cause many patients to avoid these treatment options. The clinical treatment of depression includes drug therapy, cognitive behavioral therapy, physical therapy, exercise therapy, and acupuncture (Guideline Development Panel for the Treatment of Depressive Disorders, 2022; Qaseem et al., 2023), with antidepressants being the most common treatment method (Cipriani et al., 2018).

Patients with depression may not respond to treatment due to the inherent environmental and biological aspects of the disease (Minelli et al., 2022), including genetic predisposition (Pettai et al., 2016; Fabbri et al., 2018; Minelli et al., 2022), inflammatory factors (Schmidt et al., 2016; Szałach et al., 2019), thyroid autoimmunity (Dwyer et al., 2020), neurotrophic factors (Pettai et al., 2016), and dietary influences (Molendijk et al., 2018; Chakraborti et al., 2021; Marx et al., 2021; Herselman et al., 2022; Wang et al., 2022). In recent years, numerous studies have shown an association between the gut microbiota and patient response to depression treatment. Some studies have analyzed the fecal microbiota of patients undergoing treatment for depression, and found that the baseline composition of the gut microbiota differed between the two groups (Liskiewicz et al., 2021; Lee et al., 2022). Here, the findings of studies investigating the relationship between the gut microbiota and prognosis of depression are summarized to provide a basis for the treatment of depression by targeting the gut microbiota.

2 The gut–brain axis

The gut microbiota in adult humans includes bacteria, viruses, fungi, archaea, and protozoa (Thursby and Juge, 2017). The gut microbiota is predominantly composed of six phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia), with Firmicutes and Bacteroidetes constituting the major phyla (Laterza et al., 2016; Sherwin et al., 2016). Individuals have unique gut microbiome profiles which depend on various factors, including the intestinal environment, hormonal changes, immunity, lifestyle, dietary habits, and drug use (Wang and Liu, 2020; Perler et al., 2023).

Some studies have suggested that the gut microbiota interacts with the central nervous system (CNS) through the gut–brain axis (Góralczyk-Bińkowska et al., 2022; Kim et al., 2023; Borrego-Ruiz and Borrego, 2024). Evidence supports the idea that the gut microbiota may regulate higher-level CNS functions, such as behavior and mood, through bidirectional signal transmission (Sherwin et al., 2016).

Numerous studies have observed two-way communication between the microbiota and the brain (Kunugi, 2021; Sharma et al., 2021; Trzeciak and Herbet, 2021; Ye et al., 2021; Hou et al., 2022; Dziedzic et al., 2024) through the gut–brain axis, which encompasses multiple components, including the CNS, spinal cord, autonomic nervous system, enteric nervous system, immune system, and hypothalamic–pituitary–adrenal axis (Carabotti et al., 2015). The gut microbiota can produce various molecules that act at distal sites to imitate the function of endocrine organs (Tsigos et al., 2000; Clarke et al., 2014), such as short-chain fatty acids (SCFAs), neurotransmitters (including serotonin, dopamine, norepinephrine, and γ-aminobutyric acid), cholic acids, tryptophan, L-dopa, adipokines, and hormones (Clarke et al., 2014). The mechanisms of action of the gut–brain axis are shown in Figure 1.

Genes, socioeconomic status, diet, medications, and environmental factors can influence the gut–brain axis. Lifestyle factors, especially diet, are crucial in regulating the gut–brain axis. Additionally, drugs, especially antibiotics, can directly affect the gut microbiota, which, in turn, can impact the gut–brain axis (Hou et al., 2022).

3 Baseline changes in gut microbiota during different responses to depression

Several studies have reported the differences in the gut microbiota profiles of patients with depression and healthy individuals (Jiang et al., 2015; Trzeciak and Herbet, 2021). Recent studies have shown that, after treatment, the gut microbiota differs between patients who achieve clinical remission and those who do not (Liskiewicz et al., 2021; Ye et al., 2021; Lee et al., 2022). Individuals who achieved remission had a higher baseline abundance of their gut microbiota compared to non-remitters (Wang et al., 2023). The baseline gut microbiota was defined as the first gut microbiota measurement after enrollment and before initiation of antidepressant treatment.

3.1 Methods for evaluating response to depression treatment

The treatment response to depression is primarily evaluated using clinical scales, with treatment effectiveness evaluated by changes in the scores after treatment. Therefore, reliable and valid instruments are necessary for diagnosing and treating depression.

Over the past few decades, various instruments have been developed to assess the severity of depressive symptoms, including the Hamilton Depression Rating Scale (HAMD) (Hamilton, 1960), Montgomery–Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979), and Beck Depression Inventory (Beck et al., 1961). The first two are observer rating instruments, while the latter is a self-rating measure.

The HAMD is the most commonly used scale for clinically assessing depression (Hamilton, 1960). The original version included 17 questions related to depression; however, the number of items gradually increased to 24 as the understanding of the disease improved; currently, there are three versions of the HAMD, with 17 (HAMD-17), 21 (HAMD-21), and 24 (HAMD-24) questions. The HAMD-24 comprises 24 questions related to depression, with most items scored on a five-point scale ranging from zero to four points. A total score of >35 indicates MDD. Remission from depression is defined as HAMD-24 score ≤ 8 at the end-point (Schramm et al., 2020). The MADRS consists of 10 questions, each scored from zero to

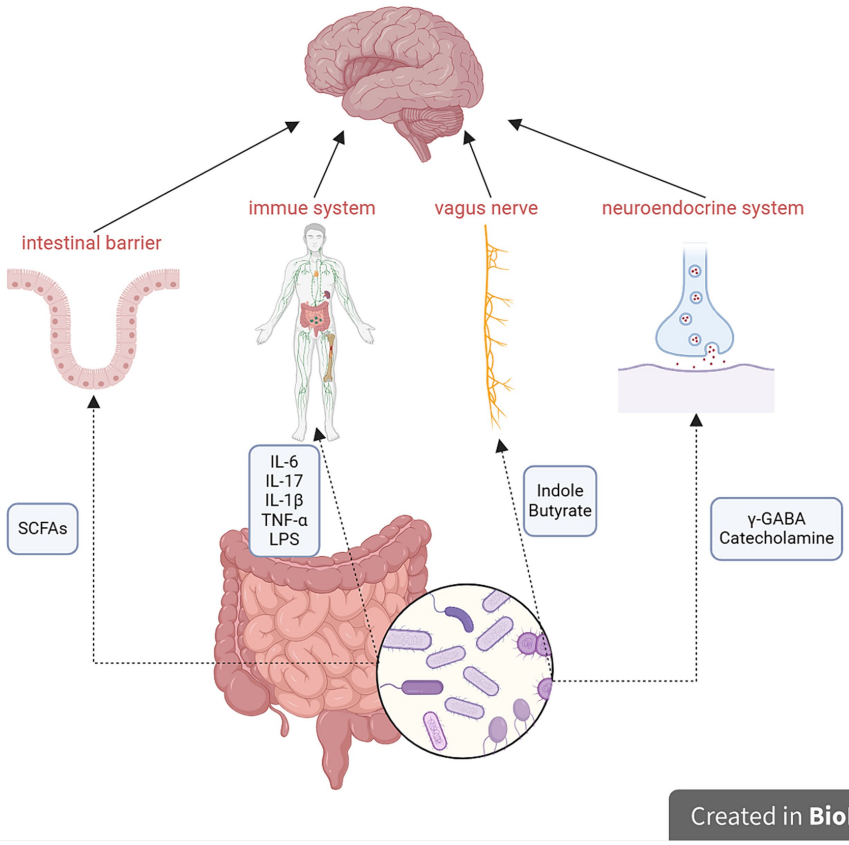

FIGURE 1
Mechanisms of the gut–brain axis. Metabolites of the gut microbiota can affect the intestinal barrier, immune system, vagus nerve, and neuroendocrine system to regulate the function of the brain via the gut–brain axis. Conversely, the brain can also influence the gut microbiota. SCFAs, short-chain fatty acids; γ-GABA, γ-aminobutyric acid.

six (Montgomery and Asberg, 1979). A total score of ≥30 indicates MDD, and a total score of ≥12 suggests remission (Bharwani et al., 2020).

Treatment response can also be defined as a reduction of 50% or more from the baseline score on standardized scales, such as HAMD and MADRS, after treatment for depression (Keller, 2003). Some studies have evaluated treatment response by observing animal behavior before and after treatment as well as through specific experiments, such as open-field, tail suspension, and forced swimming tests (Ding et al., 2021; Song et al., 2022).

3.2 Comparison of baseline changes in the gut microbiota between patients with varying response to treatment

3.2.1 Changes in the level of alpha and beta diversity

Alpha diversity, which considers richness and the relative abundances of species, can be used to compare sample groups. Specifically, low alpha diversity is a sign of dysbiosis. Gut microbiota can be divided into different operational taxonomic units (OTUs) using alpha diversity (Knight et al., 2018). Beta diversity calculates the mean value of species divergence between the focal and neighboring samples. It captures the dissimilarity between a pair of samples or communities, thereby generating a distance matrix based on the presence or absence of species abundance data (Nguyen et al., 2021). Some studies have revealed no significant community-level alpha or beta diversity changes between remission and non-remission groups (Sanada et al., 2020; Liskiewicz et al., 2021; Lee et al., 2022). However, other clinical studies have found a negative association between microbial alpha diversity and depression severity (Madan et al., 2020; Shen et al., 2021).

Some studies have revealed that remitters exhibit greater baseline diversity than non-remitters (Jiang et al., 2015; Bharwani et al., 2020; Duan et al., 2021). In one study, patients with MDD were divided into the remitter and non-remitter groups based on their MADRS scores after treatment with escitalopram. OTU levels were examined after 3 and 6 months of treatment, and it was found that OTUs were not altered in non-remitters. Overall, 35 OTUs differed between remitters and non-remitters at 3 months; however, 16 OTUs were no longer different after 6 months. At 6 months, 42 OTUs differed, of which 23 were unique, and 19 were the remaining OTUs at 3 months. The results indicated that antidepressants could affect the gut microbiota of patients with MDD at the OTU level (Bharwani et al., 2020). Another study revealed that 125 and 87 OTUs were uniquely present in non-remission and remission groups, respectively (Duan et al., 2021).

3.2.2 Changes in the phylum Firmicutes

Another study revealed that 125 and 87 OTUs were uniquely present in non-remission and remission groups, respectively (Duan et al., 2021). Clostridiales was strongly negatively correlated with the severity of depression (Liskiewicz et al., 2021; Xiong et al., 2022; Wang et al., 2023). An increase in the abundance of the order Clostridiales was found in the remission group after 6 months of escitalopram treatment (Bharwani et al., 2020). The abundance of *Faecalibacterium* negatively correlated with the severity of depressive symptoms (Jiang et al., 2015), and lower levels of *Faecalibacterium* were associated with MDD development (Kelly et al., 2016; Zheng et al., 2016; Cheung et al., 2019). Animal studies showed that the ingestion of *Faecalibacterium prausnitzii* improved anxiety- and depression-like behaviors in chronic unpredictable mild stress (CUMS) mice (Hao et al., 2019). In one study, the enrichment of baseline *Faecalibacterium* levels in levomilnacipran-treated patients with MDD aged >60 years was associated with remission outcomes (Lee et al., 2022). The abundance of *Faecalibacterium* was lower in MDD patients than in healthy controls, but was restored after sertraline treatment (Zhou et al., 2023). Higher abundances of *Faecalibacterium* were also associated with higher quality of life indicators (Valles-Colomer et al., 2019).

In addition, enrichment of *Agathobacter* (Lee et al., 2022), *Coprococcus* (Valles-Colomer et al., 2019; Gao et al., 2023), *Roseburia* (Zhou et al., 2023), and *Eubacterium* (Xiong et al., 2022; Wang et al., 2023) was associated with the treatment outcome of remission. The increased relative abundances of the family Christensenellaceae (Dong et al., 2022) and decreased relative abundances of the families Ruminococcaceae, Lactobacillaceae, and Peptostreptococcaceae and the genera *Coprococcus* (Sanada et al., 2020), *Oscillibacter* (Xiong et al., 2022), *Tyzzerella*, and *Butyricicoccus* (Shen et al., 2023) were related to non-remission (Fontana et al., 2020; Duan et al., 2021).

Firmicutes are important butyrate-producing bacteria (Kim et al., 2020). Butyrate was reported to reduce depression-like behaviors, produce antidepressant effects, and relieve symptoms of depression (Zhou et al., 2018).

3.2.3 Changes in the phylum Bacteroidetes

The severity and treatment response of depression are strongly correlated with Paraprevotella abundance (Lin et al., 2017; Liskiewicz et al., 2021; Hu et al., 2023). An experiment using CUMS mice showed an increase in the relative abundance of *Prevotellaceae_UCG-003* in the remission group but not in the non-remission group (Duan et al., 2021). Clinical studies showed that the abundance of Bacteroidota, especially *Odoribacter*, was higher in responders (Matsuzaki et al., 2024). By contrast, another study found that the baseline abundance of Bacteroidetes gradually decreased after treatment with vortioxetine hydrobromide, consistent with the remission of depressive symptoms (Ye et al., 2021).

Parabacteroides protect the nervous system by altering the levels of neurotransmitters, including glutamate and γ-aminobutyric acid, in the hippocampus(Liskiewicz et al., 2021). *Odoribacter* might generate butyrate by accelerating L-lysine degradation, which can promote the barrier function of gut epithelium (Matsuzaki et al., 2024; Vasileva et al., 2024). The level of L-lysine was proved to be lower in responders after antidepressive treatment(Matsuzaki et al., 2024).

3.2.4 Changes in the phylum Actinobacteria

The relative abundances of Actinobacteria and Eggerthellaceae are higher in non-responders than in responders (Fontana et al., 2020; Alexander et al., 2022; Dong et al., 2022). A significant increase in the proportion of *Bifidobacterium* was found after treatment with vortioxetine hydrobromide (Ye et al., 2021; Gao et al., 2023). In another study, the abundance of *Enterorhabdus* was higher in CUMS mice than in the control group. However, this increase was reversed after venlafaxine treatment (Shen et al., 2023).

3.2.5 Changes in the phylum Proteobacteria

Studies demonstrated that with the remission of depressive symptoms, the abundance of Proteobacteria significantly decreases after treatment with vortioxetine hydrobromide (Ye et al., 2021). Responders demonstrated reduced Proteobacteria abundance when compared with non-responders (Fontana et al., 2020). Another study showed that puerarin relieved CUMS-induced depression-like behavior in rats and reduced the abundance of *Desulfovibrio* (Song et al., 2022). Changes in the gut microbiota of patient with depression with varying responses to treatment are shown in Table 1.

3.3 Possible mechanisms of how the gut microbiota affect treatment response in depression

Although the interactions between drugs and gut microbiota exist, the underlying mechanisms remain unclear.

3.3.1 Gut microbiota affects drug pharmacokinetics

Hepatic metabolites of drugs are secreted into the gut for further metabolism. Bacterial metabolites of a drug can, in turn, be absorbed and transported to the liver. During this process, drugs interact with the gut microbiota. However, drugs can alter the intestinal microenvironment and/or directly affect the growth, composition, and function of bacteria (Weersma et al., 2020). Some antidepressant drugs, such as sertraline, fluoxetine, and escitalopram, exert antibacterial effects, disrupting the integrity and stability of the gut microbiota (Macedo et al., 2017; Karine de Sousa et al., 2018). Moreover, changes in the gut microbiota can affect drug metabolism and influence the efficacy of drugs.

A systematic analysis tested the ability of 76 gut microbial strains to metabolize 271 drugs administered orally and found that 176 drugs (66%) were metabolized by at least one microbial strain (Zimmermann et al., 2019b). The gut microbiota can alter the structure, bioavailability, biological activity, and toxicity of drugs via enzymes, directly affecting individual responses to a particular drug (Maini Rekdal et al., 2019; Zimmermann et al., 2019a,b; Weersma et al., 2020). For example, the gut microbiota can transform the chemical structure of drugs and modulate xenobiotic metabolism, including drug metabolic pathways (Wilson and Nicholson, 2017). The gut microbiota can enhance the activity of indoleamine 2,3-dioxygenase 1, a rate-limiting enzyme which transforms tryptophan into kynurenine and its derivatives, thereby affecting the bioavailability of antidepressants (Agus et al., 2018; Wu et al., 2022). Changes in the gut microbiota can also impact intestinal permeability and the function of the intestinal barrier, which affects drug absorption (Kelly et al., 2015). Firmicutes break down

TABLE 1 Summary of the articles on changes in the baseline of gut microbiota associated with the response to treatment.

Sample size	Assessment	Treatment	Changes in the gut microbiota	Reference
LVM ($N = 17$) Placebo ($N = 12$)	HAMD Remission was defined as having a HAMD score ≤ 6	Randomized to receive either levomilnacipran or placebo for 12 weeks	No community-level alpha or beta diversity differences between groups. *Flavonifractor* abundance increased while *Roseburia* abundance decreased in the remission group.	Lee et al. (2022)
$N = 15$	MADRS Remission was defined as MADRS ≤12	Received citalopram or escitalopram for 6 months	*Clostridiales* abundance elevated in remitters.	Bharwani et al. (2020)
$N = 16$	HAMD-24 Early improvement: 20% reduction in HAMD-24 scored at 2 weeks; Response: 50% reduction in HAMD-24 score at 6 weeks; Remission: HAMD-24 score ≤ 8 at 6 weeks	Treated with escitalopram under standardized conditions	Treatment response was positively correlated with Paraprevotella and strongly negatively correlated with *Clostridiales*, *Clostridia*, *Firmicutes*, and RF32.	Liskiewicz et al. (2021)
Patients ($N = 26$) HCs ($N = 28$)	HAMD-17 MDD group: HAMD-17 score ≥ 24	Received vortioxetine hydrobromide for 4 and 8 weeks	Bacteroidetes and Proteobacteria abundances decreased while Firmicutes abundance increased, with the alleviation of depressive symptoms.	Ye et al. (2021)
$N = 1,054$	RAND-36 health-related quality of life (QoL) survey	–	*Faecalibacterium* and *Coprococcus* were associated with a higher QoL.	Valles-Colomer et al. (2019)
CUMS mice	–	Received escitalopram for 4 weeks	Escitalopram can increase the alpha diversity of the gut microbiota. Prevotellaceae_UCG-003 abundance increased in the remission group while that of Ruminococcaceae and Lactobacillaceae decreased relative to that in the non-remission group.	Duan et al. (2021)
P ($N = 63$) HC ($N = 30$)	HAMD-24 The responder group was defined as a 50% reduction of the score	All patients received one type of SSRI or venlafaxine for 8 weeks	Baseline abundances of Actinobacteria, Christensenellaceae, Eggerthellaceae, *Adlercreutzia*, and Christensenellaceae R7 groups were significantly lower in the remission group.	Dong et al. (2022)
CUMS mice Control: 10 CUMS: 10 Venlafaxine: 10	–	–	CUMS mice had decreased *Blautia*, *Oscillibacter*, *Tyzzerella*, and *Butyricicoccus* abundances and increased *Enterorhabdus* abundance, and all changes could be reversed by venlafaxine treatment.	Shen et al. (2023)
CUMS model	Open-field test; sucrose preference test	XYS high-dose group, XYS medium-dose group, XYS low-dose group, model group, fluoxetine group	Improvement of depression-like behavior in CUMS was related to increased levels of *Roseburia* sp. and *Eubacterium* sp.	Xiong et al. (2022)
CUMS model	Open-field test; sucrose preference test; forced swimming test	($N = 6$ per group) Control group; CUMS group; fluoxetine group; puerarin low-dose group; puerarin high-dose group.	Puerarin reduced CUMS-induced depressive-like behavior and the abundances of *Desulfovibrio*, Verrucomicrobiae, and Verrucomicrobia.	Song et al. (2022)

(Continued)

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TABLE 1 (Continued)

Sample size	Assessment	Treatment	Changes in the gut microbiota	Reference
29 patients with schizophrenia	HAMD 25% or more reduction of HAMD score at 4 weeks from baseline was considered to indicate a responder	*Bifidobacterium* each day for the first 4 weeks	Responders had higher lipid and energy metabolism at baseline than did non-responders.	Yamamura et al. (2021)
Male C57BL/6 mice with chronic restraint stress (CRS)	Open-field test; tail suspension test; forced swimming test	(6 per group) Control group; CRS group; CRS + AKK; AKK group; CRS + *Lactobacillus* L; CRS + *Lactobacillus* H	*Akkermansia muciniphila* reduced depressive-like behaviors of CRS mice and regulated the structure and metabolism of the gut microbiota under chronic stress.	Ding et al. (2021)
SSRI with the probiotic *Lactobacillus plantarum* 299v ($n = 40$) SSRI with placebo ($n = 39$)	HAMD-17	Participants received either an SSRI with the probiotic *Lactobacillus plantarum* 299v for 8 weeks or an SSRI with the placebo of the probiotic for the same period.	*Lactobacillus plantarum* 299v improved cognitive performance and decreased kynurenine levels in patients with MDD. No significant changes were observed in the treatment effect in either probiotic or placebo groups.	Rudzki et al. (2019)
Patients ($N = 34$) HCs ($N = 20$)	Assessment of the response was based on a retrospective assessment of longitudinal clinical course and evaluation of treatment response patterns	–	Proteobacteria, Tenericutes, and Peptostreptococcaceae levels increased in non-responders, whereas Actinobacteria levels increased in responders.	Fontana et al. (2020)
Patients ($N = 110$) HCs ($N = 166$)	HAMD-17 Remission was defined as having a HAMD score of seven or lower	Patients were treated with escitalopram for 12 weeks	Clostridiaceae decreased in the remission group when compared with that in the non-remission group. *Eubacterium hallii* increased in the remission group and decreased in the non-remission group.	Wang et al. (2023)
$N = 34$	HAMD; HAMA Responders were defined as HAMA or HAMD <8 at time point 3; remitters were defined as HAMA or HAMD <8 at time point 1	Patients were treated with antidepressants and antipsychotics	*N*-ε-acetyllysine, cysteate, glycyl-L-leucine, taurine, guanosine, L-lysine, and malonate levels were lower in responders. Bacteroidota abundances, especially that of *Odoribacter* were higher in responders.	Matsuzaki et al. (2024)
$N = 104$	HAMD-17 Responders were defined as demonstrating a minimum 50% reduction in their HAMD-17 scores after treatment	Patients received paroxetine treatment for 8 weeks	Baseline IL-6 levels were negatively correlated the reductions in HAMD-17 scores.	Dong et al. (2021)
$N = 62$	HAMD-17 Responders were defined as exhibiting a ≥ 50% reduction in their HAMD-17 scores after antidepressant treatment	Patients were treated with fluoxetine treatment for 8 weeks	Relative abundances of *Blautia*, *Coprococcus*, and *Bifidobacterium* were positively correlated with the efficacy of SSRI antidepressants.	Gao et al. (2023)
CUMS model	Sucrose preference test; forced swimming test	Negative control group; CUMS model group; paroxetine group (paroxetine 5 mg/kg)	TGF-β, IL-22, IL-10, and IL-17 levels were significantly lower in rats who responded to paroxetine than those in non-responders. Propionate, lactate, isoleucine, and citrulline levels significantly increased in responders to paroxetine therapy.	Liu et al. (2024)

MDD, major depressive disorder; HAMD, Hamilton Depression Rating Scale; HAMA, Hamilton Anxiety Rating Scale; MADRS, Montgomery–Asberg Depression Rating Scale; CUMS, chronic unpredictable mild stress; HC, healthy control; P, patients; SSRI, selective serotonin reuptake inhibitor; XYS, Xiaoyao San.

10.3389/fmicb.2024.1429116

carbohydrates into SCFAs (Stilling et al., 2016; Deleu et al., 2021), which have been shown to enhance the integrity of the blood–brain and intestinal barriers (Unger et al., 2016). Reduced levels of Firmicutes results in decreased SCFA production (Huang et al., 2018). Additionally, gut microbiota possess tryptophanase, which can produce indole to regulate intestinal barrier permeability (Trzeciak and Herbet, 2021). Significant increases in lactate levels were observed in CUMS rats that responded to treatment (Liu et al., 2024). Supplementation with either *Bifidobacterium* or *Lactobacillus* can enhance the integrity of the intestinal barrier and alleviate the symptoms of stress-induced intestinal leakage (Couto et al., 2020; Lenoir et al., 2020).

3.3.2 Gut microbiota influences immune regulation

Evidence suggests that systemic inflammation, mediated by intestinal dysbiosis, could play a crucial role in the development of therapy resistance in patients with depression (Vitetta et al., 2014). TGF-β, IL-22, IL-10, and IL-17 levels were significantly higher in CUMS rats who responded to paroxetine therapy than those in non-responders (Liu et al., 2024). Baseline IL-6 levels were negatively correlated with reduced HAMD-17 scores in clinical studies (Dong et al., 2021). *Faecalibacterium prausnitzii* produces butyrate in the human colon, and appropriate levels of butyrate production can improve mucin secretion, prevent intestinal leakage, and suppress inflammation (Furusawa et al., 2013; Samara et al., 2022). It can also regulate intestinal epithelial cells to decrease pro-inflammatory cytokine levels and increase anti-inflammatory factor levels (Couto et al., 2020; Lenoir et al., 2020). Increased *F. prausnitzii* levels can lead to increased SCFA production and higher levels of inflammatory factors, such as IL-10, in the plasma (Hao et al., 2019). Microbiota metabolites can also modulate the proportions of T helper 17 and regulatory T cells to promote resilience to stress-induced depressive-like behaviors (Westfall et al., 2021). High levels of Prevotellaceae UCG-003 may regulate intestinal inflammation by producing succinate, which, in turn, activates dendritic cells (Koh et al., 2016). Bifidobacteria increase butyrate levels by altering the relative abundances of other microbiota involved in lipid metabolism, therefore possessing anti-inflammatory properties (Sugahara et al., 2015). Moreover, Bifidobacteria regulate the levels of pro-inflammatory cytokines and anti-inflammatory factors (Couto et al., 2020; Lenoir et al., 2020; Samara et al., 2022). Furthermore, members of the genus *Eggerthella*, including *Eggarthella lenta*, induce intestinal inflammation by activating Th17 cells (Alexander et al., 2022).

3.3.3 Gut microbiota affects the nervous system

The gut microbiota is essential for nervous system communication via the vagus nerve. The vagus nerve plays an important role in behavioral abnormalities in antibiotic-treated mice after ingesting *Lactobacillus* (Wang et al., 2020). In animal models, dysbiosis impairs vagus signaling, affects brain structure, regulates brain-derived neurotrophic factors, and reduces hippocampal protein synthesis (Fond et al., 2015; Dehghani et al., 2022). The relative abundance of Proteobacteria is correlated with stress-induced behavioral changes (Wong et al., 2016; Werbner et al., 2019). Members of the genus Bifidobacteria play crucial roles in maintaining the balance of the gastrointestinal tract by reducing oxidative stress (Kant et al., 2015). Additionally, *Oscillibacter* is beneficial in protecting brain modulatory functions, ultimately leading to increased amygdala and hippocampal volume (Lee et al., 2022). *Parabacteroides* can alter neurotransmitter levels in the brain, including those of glutamate and aminobutyric acid (Olson et al., 2018). Furthermore, *Bifidobacterium* and *Lactobacillus* stimulate γ-aminobutyric acid production by metabolizing indigestible fibers (Couto et al., 2020; Lenoir et al., 2020).

3.3.4 Other effects of the gut microbiota

The gut microbiota may affect the therapeutic outcomes of antidepressants by altering the permeability of the blood–brain barrier permeability during treatment (Xu et al., 2023). Higher abundances of Firmicutes were associated with higher levels of SCFAs (Stilling et al., 2016; Deleu et al., 2021). SCFAs can enhance the integrity of the blood–brain barrier (Unger et al., 2016). The gut microbiota can modulate the availability of antidepressants through bioaccumulation (Doestzada et al., 2018; Klünemann et al., 2021), resulting in direct reductions in drug availability and changes in the secretion of metabolites (Wu et al., 2017; Klünemann et al., 2021). *Streptococcus salivarius*, *Bacteroides uniformis*, *Bacteroides thetaiotaomicron*, and *Escherichia coli* increase duloxetine bioaccumulation, thereby decreasing its bioavailability (Klünemann et al., 2021). Figure 2 summarizes the possible mechanisms by which the gut microbiota affects treatment response in patients with depression.

3.4 Current progress in microbiota-targeting therapies

Some studies using adjuvant microbial supplements for the treatment of depression have achieved preliminary success (Rieder et al., 2017); Current research findings indicate that probiotic therapy may have moderate efficacy in alleviating depressive symptoms (Nikolova et al., 2021; Tian et al., 2022). Most studies have utilized the genera *Lactobacilli* and *Bifidobacteria* as probiotics for treating depression. However, the effectiveness of probiotic monotherapy is limited (Nikolova et al., 2021). Since most antidepressants exhibit antimicrobial activity (Nikolova et al., 2021), probiotics can promote therapeutic benefits by restoring the balance of the gut microbiota and minimizing gastrointestinal discomfort. However, the complexity and variability of the gut microbiota, as well as its susceptibility to various influencing factors, lead to heterogenous trial results (Tian et al., 2022).

Current preclinical research findings indicate that fecal microbiota transplantation has significant potential in the treatment of MDD, but there are still limited number of studies on humans, and several issues remain, including resistance to microbiota colonization, potential pathogen transmission, and ethical considerations related to donor-recipient matching (Meng et al., 2024). Further studies on the treatment of MDD using gut microbiota are needed.

4 Conclusion

Multiple studies have confirmed that differences exist in the gut microbiota of patients with MDD and healthy subjects; however, they have not conclusively shown that these differences are correlated with disease severity. This review summarized the existing studies that compared the baseline gut microbiota between remission and

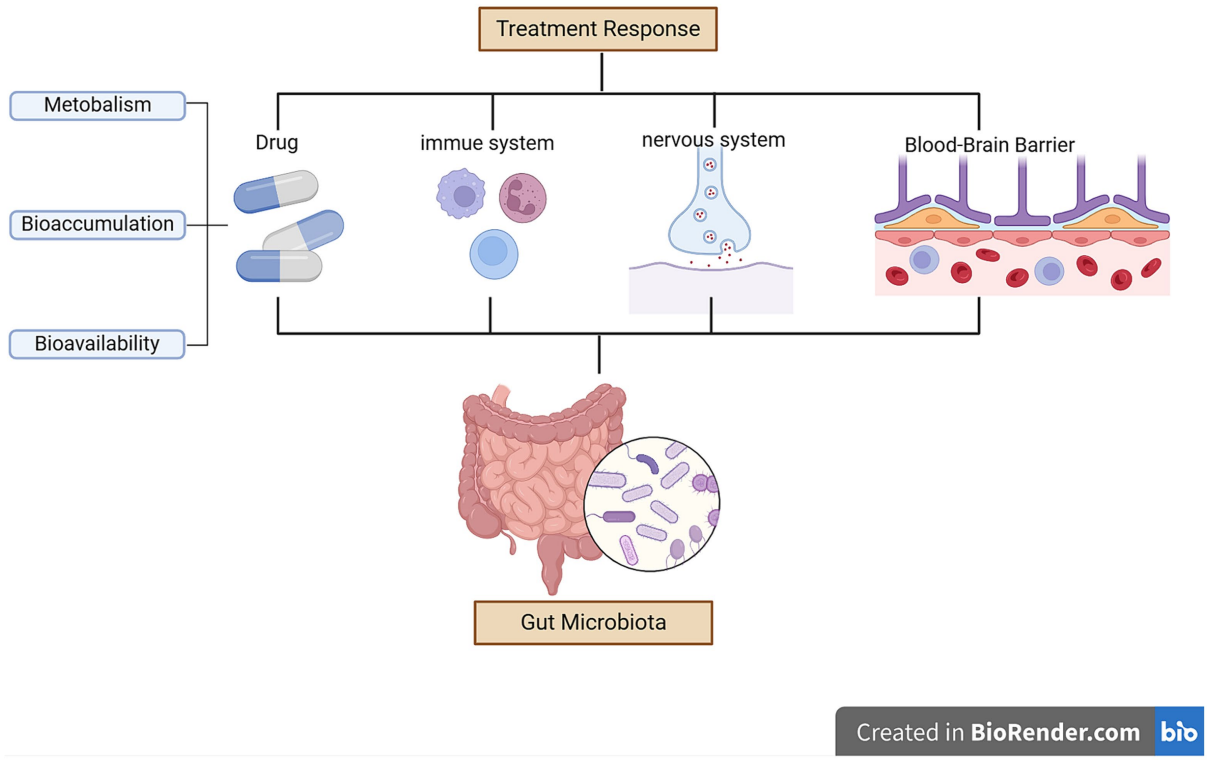

FIGURE 2
Mechanisms underlying how the gut microbiota can affect treatment response in depression.

non-remission groups and found that changes in the abundances of specific microbiota are associated with treatment response in MDD.

However, most of these studies have certain limitations. Most notably, the sample sizes are often too small to confirm significant differences between the two groups. Some trials have not included healthy controls, and some have lost a high proportion of participants to follow-up, leading to greater selection bias. Recent studies suggest that baseline changes in specific gut microbiota are related to MDD remission; however, the provided evidence is insufficient to confirm that these specific organisms can be used as predictors for the treatment response of depression. In addition, the detection of the gut microbiota is greatly affected by individual differences and environmental factors, which warrants further research.

This review provides supporting evidence for microbiota therapy and indicates novel research directions for using the gut microbiota as a target in prognosticating and treating depression. Continued research is crucial for understanding the relationship between the gut microbiota and depression, which can offer new prospects in treating this complex condition. Therapies targeting the gut microbiota are expected to be widely utilized as a treatment option for depression in the future.

Author contributions

YX: Writing – original draft, Writing – review & editing. HZ: Methodology, Writing – review & editing, Conceptualization. YY: Methodology, Writing – review & editing, Supervision. XG: Writing – review & editing, Methodology, Validation. QX: Funding acquisition, Writing – review & editing. ZD: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

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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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Gut microbiota from patients with Parkinson's disease causes motor deficits in honeybees

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Objective: Parkinson's disease (PD) is possibly caused by genetic factors, environmental factors, and gut microbiota dysbiosis. This study aims to explore whether the microbiota contributes to the behavior abnormalities of PD.

Methods: We transplanted gut microbiota from patients with PD or healthy controls (HC) into microbiota-free honeybees. We also established two more groups, namely the rotenone (ROT) group, in which PD-like symptoms of honeybees were induced by rotenone, and the conventional (CV) group, in which honeybees were colonized with conventional gut microbiota. The climbing assay was performed to assess the motor capabilities of honeybees. Histopathological examination was conducted to evaluate the integrity of gut mucosa. Tyrosine hydroxylase (TH) gene expression levels and dopamine (DA) concentrations in the brain were also examined. Additionally, metagenomics and full-length 16S rRNA analyses were performed to identify alterations in gut microbiota profiles, both in PD patients and honeybees.

Results: Honeybees in the PD and ROT groups exhibited slower climbing speeds, downregulated TH gene expression, and impaired gut barriers. Both the HC and PD groups of honeybees successfully harbored a portion of gut microbiota from corresponding human donors, and differences in microbial composition were identified. *Morganella morganii* and *Erysipelatoclostridium ramosum* exhibited significantly increased relative abundance in the HC group, while *Dorea longicatena*, *Collinsella aerofaciens*, *Lactococcus garvieae*, *Holdemanella biformis*, *Gemmiger formicilis*, and *Blautia obeum* showed significantly increased relative abundance in the PD group. Functional predictions of microbial communities in the PD group indicated an increased synthesis of hydrogen sulfide and methane.

Conclusion: A novel PD model was induced in honeybees with rotenone and gut microbiota from PD patients. This study linked PD-related behaviors to altered gut microbiota, highlighting a potential gut microbiota-brain axis involvement in PD pathogenesis. We identify previously unrecognized associations of *Dorea longicatena*, *Collinsella aerofaciens*, *Lactococcus garvieae*, *Holdemanella biformis*, *Gemmiger formicilis*, and *Blautia obeum* with PD. Additionally, pathways related to hydrogen sulfide and methane synthesis have been previously suggested as potential contributors to the development of PD, and our research further supports this hypothesis.

KEYWORDS

Parkinson's disease, gut microbiota, honeybee model, rotenone, microbiota-gut-brain axis

Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, is a progressive and degenerative disease characterized by motor and non-motor symptoms (Hou et al., 2021). The classic motor symptoms include resting tremors, bradykinesia, postural instability, and rigidity, whereas non-motor symptoms involve a wide range of features such as depression, anxiety, sleep disturbance, and gastrointestinal complaints including constipation, abdominal bloating, and dysphagia (Hayes, 2019). The onset of motor symptoms is primarily associated with the loss of dopaminergic neurons in the substantia nigra (Scheperjans et al., 2015). In dopaminergic neurons, tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis (Kostrzewa et al., 2005) and is used as a marker for the activity of the dopaminergic neurons (Hou et al., 2021).

Previously, PD was thought to be caused primarily by a combination of genetic and environmental factors (Kalia and Lang, 2015), but recent studies have revealed that gut microbiota might play an important role in the pathogenesis of PD. The composition of gut microbiota was altered in PD patients in comparison to healthy controls (HC) (Scheperjans et al., 2015; Yan et al., 2021), and gut microbiota restoration via fecal microbiota transplantation (FMT) as well as probiotic supplementation could improve the motor and non-motor symptoms of PD patients (Kuai et al., 2021; Mirzaei et al., 2022). In a well-established mouse model of PD by Sampson et al. (2016), antibiotic treatment alleviated the pathophysiology of PD, while microbial re-colonization had a promoting effect on the disease progression. The interplay among the gut, gut microbiota, and the brain has been illustrated and termed the microbiota-gut-brain axis (Cryan and Dinan, 2012). Given the above-mentioned evidence, gut microbiota may influence bidirectional gut-brain signaling pathways through the microbiota-gut-brain axis (Zeng et al., 2022).

Honeybees, with their high accessibility, short lifespan, easy maintenance, and ease of obtaining a large sample of microbiota-free subjects, serve as a potentially ideal model for studying the relationship between gut microbiota and diseases (Wang et al., 2018; Zheng et al., 2018). In our previous research, we successfully constructed honeybee models of autism (Li et al., 2023) and enteritis (Chang et al., 2022). Furthermore, the reduction in dopamine concentration is a characteristic feature of PD, and dopamine also modulates honeybee behaviors and motor abilities (Maze et al., 2006; Raza and Su, 2020). Moreover, genes associated with honeybee motor behaviors share homology with human PD-related genes (Alaux et al., 2009). Honeybees exhibit phototaxis by climbing toward light sources (Yamaguchi and Heisenberg, 2011). Damage to the honeybee's brain may result in impaired balance and slower movement, consequently leading to an extension of climbing time. Thus, the climbing assay can be employed to assess their locomotion (Luo et al., 2021). Hence, we hypothesized the feasibility of constructing a PD honeybee model. Additionally, rotenone, a pesticide that inhibits mitochondrial complex I, can induce symptoms resembling PD in mice and *Drosophila melanogaster* (Coulom and Birman, 2004; Chia et al., 2020), which are characterized by significant locomotor impairments and degeneration of dopaminergic neurons. According to our preliminary experiments, we have identified a sublethal dose of rotenone suitable for constructing a PD honeybee model.

Studies have reported that FMT from patients with schizophrenia (Zhu et al., 2020) or autism spectrum disorder (Sharon et al., 2019) in germ-free mice could induce specific behavioral symptoms of the corresponding disease. Therefore, to investigate whether dysbiosis of the gut microbiota can directly induce PD symptoms and pathology, and to investigate the underlying role of pathogenic microorganisms, we transplanted the gut microbiota from PD patients into microbiota-free honeybees. We observed and compared the motor abilities of four groups: honeybees with conventional (CV) gut microbiota, honeybees transplanted with gut microbiota from healthy individuals or PD patients, and honeybees treated with rotenone.

Methods

Fecal microbiota preparation

Fecal samples were collected from PD patients diagnosed at the Chinese PLA General Hospital and from healthy donors matched as closely as possible to PD patients. The exclusion criteria for PD subjects were as follows: (1) atypical or secondary parkinsonism; (2) use of probiotics or antibiotics within 3 months before sample collection; (3) history of chronic gastrointestinal diseases; (4) unstable medical, neurological, or psychiatric conditions. The inclusion criteria for healthy subjects were as follows: (1) normal blood tests; (2) no neurological diseases; (3) no gastrointestinal diseases, including irritable bowel syndrome, inflammatory bowel disease, colitis, cancer, etc.; (4) no use of probiotics or antibiotics within 3 months before sample collection. This study was approved by the Ethics Committee of Chinese PLA General Hospital (S2019-061-02).

The fresh stool samples were suspended with sterile phosphate-buffered saline (PBS, 7.5 mL/g of feces) and centrifuged at 800 g for 5 min at 4°C. The supernatants were mixed with an equal volume of 40% glycerol-PBS, resulting in a final glycerol concentration of 20%, and vortexed for 5 min. The procedures were conducted in a biosafety cabinet. The fecal suspensions containing microbiota were stored at −80°C before being used for transplantation.

Honeybee rearing and experimental grouping

Honeybees (worker bees) were purchased from Shunyi County of Beijing in July 2022. Microbiota-free bees were obtained as described by Zhang et al. (2022a). Briefly, late-stage bee pupae were manually

removed from mature brood frames and placed in a sterilized feeding box. The bees then naturally emerged at a temperature of 35°C and 50% humidity. After emergence, bees (0 days old) were fed in separate sterilized cup cages with sterile sucrose syrup (50%, wt/vol). After 24 h, guts from random 1-day-old bees are collected for sterility verification using blood agar plates.

One-day-old bees were then divided into four groups: (1) CV bees; (2) PD bees; (3) HC bees; and (4) rotenone (ROT) bees. A total of 75 bees were allocated to each group, and every 25 bees were kept in one cup cage. For the CV group, each cup cage of bees was fed with a mixture consisting of 10 μL of gut homogenates, which included normal gut microbiota from freshly collected guts of nurse bees from their hives of origin, 900 μL of 1 × PBS, 600 μL of sterile sucrose solution (50%, wt/vol), and 500 μL of sterile pollen. For the PD group, each cup cage of bees was fed with a 2 mL mixture, consisting of 1 mL of fecal suspensions containing microbiota from PD patients and 1 mL of sterile sucrose solution. Likewise, for the HC group, each cup cage of bees was fed with a 2 mL mixture, consisting of 1 mL of fecal suspensions containing microbiota from healthy subjects and 1 mL of sterile sucrose solution. For the ROT group, each cup cage of bees was fed with 2 mL of rotenone suspension (500 μM, dispersed in sucrose solution). After 24 h, CV, PD, and HC bees were then fed with sterile sucrose solution and pollen until day 8, while after 6 days, RT bees were fed with sterile sucrose solution and pollen until day 8. Animal maintenance and experimental procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals.

Climbing assays

The climbing assays were performed on day 8. The behavioral apparatus was a black acrylic sand-textured box (55 cm in length, 35 cm in width, 4 cm in height) containing 10 lanes (50 cm long), with LED lights placed at the endpoint, as described by Zaluski et al. (2015) with some modifications. The tests were conducted in the dark. The apparatus was inclined at a 45° angle, with each bee placed at the starting point of each lane. After the partition was removed, the bees climbed upward due to phototaxis. The time taken to reach the endpoint was recorded. All tests were performed in triplicate.

Tissue collection

The entire gut and brain of bees were collected after behavioral tests. The dissection method for bees followed the procedure described by Zhang et al. (2022a). Briefly, microscopic dissection was performed to collect the entire gut and brain with non-brain structures such as compound eyes, pigments, and glands removed. The dissected tissues were snap-frozen in liquid nitrogen and stored at −80°C.

RNA extraction and RT-qPCR

Brain tissues were homogenized using an electric tissue homogenizer (TIANGEN). Total RNA was extracted utilizing the FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, China) under the manufacturer's instructions. The RNA concentration and purity were then determined using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was synthesized from the extracted RNA using the HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). RT-qPCR was performed to assess tyrosine hydroxylase (TH) gene expression levels using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) on a Real-Time PCR Instrument (Thermo Fisher Scientific, Waltham, MA, United States). RT-qPCR was performed under the following conditions: (1) initial denaturation: 95°C for 30 s. (2) Cycling (40 cycles): denaturation: 95°C for 10 s; annealing: 60°C for 30 s. (3) Melting curve analysis: 95°C for 15 s, 60°C for 60 s, 95°C for 15 s. The following primers were used: TH: Forward: 5′-AACCGCGAGATGTTCGCTAT-3′, Reverse: 5′-AGTC CAGCGTCATTCGCC-3′; Actin: Forward: 5′-TGCCAACACTGTCC TTTCTG-3′, Reverse: 5′-AGAATTGACCCACCAATCCA-3′. The relative gene expression levels were normalized to the *Apis mellifera* actin gene and calculated using the $2^{-\Delta\Delta CT}$ method (Song et al., 2022). Each RT-qPCR experiment was conducted in triplicate to ensure reproducibility.

Dopamine ELISAs

The dopamine concentrations in the brain homogenates were determined using the Insect DA ELISA Kit (Mlbio, China) following the manufacturer's instructions. Standard curves were generated using known concentrations of dopamine provided in the kit.

Hematoxylin-eosin staining

The gut tissues of honeybees were fixed in 4% paraformaldehyde for 24 h. Following fixation, tissues underwent dehydration through a graded series of ethanol solutions. Subsequently, the tissues were cleared using xylene. After dehydration and transparency, the tissues were embedded in paraffin. Paraffin-embedded tissues were sectioned at 4 μm using a microtome. The sections were mounted on glass slides. Deparaffinization in xylene and rehydration through a graded series of ethanol solutions were performed on the sections. Hematoxylin was used for staining nuclei, followed by differentiation using acid alcohol. Eosin was applied for cytoplasmic staining. Dehydration was performed using graded ethanol solutions, and the sections were cleared with xylene. Finally, the sections were coverslipped using a neutral resin. Microscopic observation was conducted using an upright microscope (Leica, Germany) to examine the morphological structure of the gut tissues.

Metagenomic sequencing of human fecal samples and taxonomic identification

Fecal samples from human subjects were analyzed by metagenomics sequencing on the Illumina NovaSeq platform. Microbiota analyses were executed on the Majorbio Cloud Platform.[1] The raw sequencing data underwent quality control using Fastp (v0.20.0). Alignment to the host (*Homo sapiens*) genome was performed using BWA (v0.7.17),

1 https://cloud.majorbio.com/page/tools/

enabling the identification and removal of host-derived sequences. The host genome was referenced using the Ensembl database (Release 104) for precise mapping and subsequent analyses. *De novo* assembly of the sequencing data was conducted using MEGAHIT (v1.1.2). Prodigal (v2.6.3) was employed to predict open reading frames (ORFs) within the assembled contigs obtained from *de novo* assembly. The gene sequences predicted from the samples underwent clustering using CD-HIT software (v4.7). Employing BLASTP (Version 2.2.28+), the non-redundant gene set was aligned against the NR (non-redundant) protein database to obtain species annotation results. Alpha and beta diversity indexes were calculated using QIIME2 software. Linear Discriminant Analysis Effect Size (LEfSe) was applied to identify species features contributing significantly to group differences.

Full-length 16S rRNA sequencing and analysis

Microbial DNA was extracted from the gut contents of honeybees. Full-length 16S rRNA gene sequences were amplified using primers designed to span the entire gene. Subsequently, the amplified 16S rRNA gene products underwent library preparation using PacBio's SMRTbell prep kit 3.0. Sequencing was performed on the PacBio platform. Raw data were processed using PacBio's SMRTLink v11.0 software, specifically utilizing the Circular Consensus Sequencing (CCS) module, resulting in high-fidelity reads in fastq format. Denoising was performed using the DADA2 method to optimize and obtain Amplicon Sequence Variants (ASVs).

Microbiota analyses were executed on the Majorbio Cloud Platform (see text footnote 1). ASVs were taxonomically annotated using the QIIME2 bioinformatics platform (version 2022.2). The Ribosomal Database Project (RDP) Classifier served as the annotation method, with the nt_v20221012/16s_bacteria database used as the reference. Alpha and beta diversity indexes were calculated using QIIME2 software. LEfSe was applied to identify species features contributing significantly to group differences, utilizing a linear discriminant analysis (LDA) threshold of 2.5. Functional genes were annotated to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using PICRUSt2 (version 2.2.0-b). The relative abundance of the KEGG orthologs (KO) was further analyzed by the Wilcoxon rank-sum test.

Statistical analysis

Data obtained from the honeybee climbing assays, as well as the brain dopamine and TH gene relative expression measurements across different groups, were processed and analyzed using R software (Version 4.2.0). The normality of data was assessed using the Shapiro–Wilk test, and the homogeneity of variance was evaluated with Bartlett's test. Data exhibiting normal distribution and homogeneity of variance were expressed as mean ± standard deviation and analyzed using the independent samples *t*-test (for comparisons between two groups) or analysis of variance (for comparisons between multiple groups). In cases of non-normally distributed or heteroscedastic data, median and interquartile ranges were utilized, and comparisons were made using the Mann–Whitney *U* test (for comparisons between two groups) or Kruskal–Wallis test (for comparisons between multiple groups) and the Dunn's test is conducted as a post-hoc test. Statistical significance was defined as a *p*-value less than 0.05.

Results

PD microbiota induces motor dysfunction in bees

The flowchart of the study is illustrated in Figure 1. To investigate the causal relationship between gut microbiota and the onset of PD, we compared the behavioral performance of PD-transplanted bees with those transplanted with HC microbiota, CV bees, and ROT bees. The results revealed that the CV group exhibited the shortest median running time (time taken by the bees to climb a 50-cm-long lane), followed by the HC group, and the PD and ROT groups showed the longest running time, as shown in Table 1. Within the PD group, the running time of bees exhibited a positive correlation with the corresponding PD patient's age and disease severity overall, but the differences were not statistically significant ($p > 0.05$). Upon combining the five PD groups and three HC groups, respectively, for statistical analysis, we observed that the running speed of the CV group was significantly faster than that of the HC group ($p < 0.0001$). Additionally, the running speed of the HC group was significantly faster than that of the PD ($p = 0.0001$) and ROT groups ($p = 0.0493$), with no statistically significant difference found between the running speeds of the PD and ROT groups (see Figure 2).

PD microbiota reduces brain TH expression in bees

RT-qPCR was performed to assess the expression levels of the TH gene in the brains of honeybees. We combined the five PD groups and three HC groups, respectively, for statistical analysis. The expression levels of the TH gene were significantly downregulated in both the PD ($p = 0.00084$) and ROT ($p = 0.00013$) groups compared to the HC group. Interestingly, no significant difference was observed between the HC group and the CV group. The results are presented in Figure 3.

We further performed dopamine ELISAs to investigate the dopamine concentrations in the brain among groups. However, although the dopamine concentrations in the HC and CV groups appeared higher than those in the PD and ROT groups, the differences were not statistically significant (Supplementary Figure S1).

PD microbiota disrupt gut barriers in bees

We performed hematoxylin and eosin (HE) staining on the midgut and ileum of honeybees. The histopathological examination of gut tissues revealed alterations indicative of gut barrier disruption in PD bees and ROT bees, compared to CV bees and HC bees (Figure 4). The observed changes included epithelial cell swelling, dilation of the intestinal lumen, and disruption of villous structures. These pathological findings collectively suggest compromised gut barrier function, highlighting the potential implications for gut health and homeostasis.

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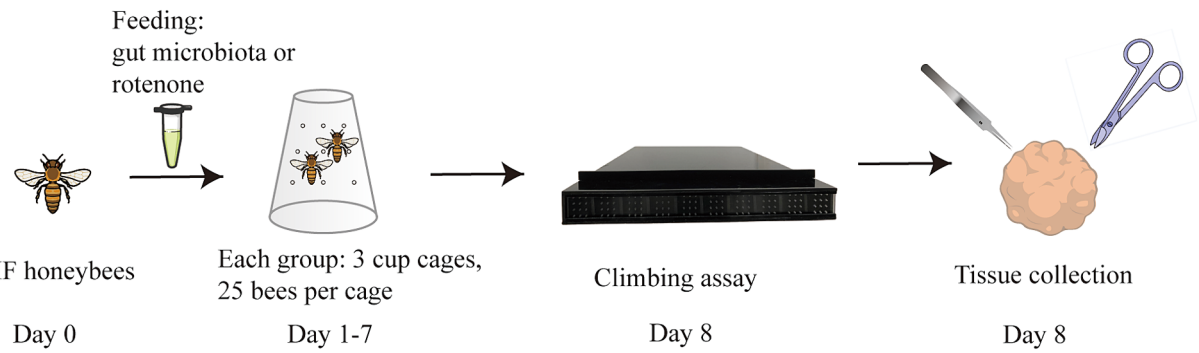

FIGURE 1
Flowchart of the study. One-day-old microbiota-free (MF) honeybees were divided into four groups: (1) PD group: colonized with gut microbiota from PD patients (5 patients, 5 PD subgroups); (2) HC group: colonized with gut microbiota from healthy controls (3 individuals, 3 HC subgroups); (3) CV group: colonized with the gut microbiota of normal honeybees; (4) ROT group: fed with rotenone. Behavioral tests were conducted on day 8, followed by tissue collection.

TABLE 1 Results of the climbing assays of honeybees.

Group	Time (second), median	Age	Disease duration	Hoehn–Yahr stage	UPDRS part III
CV	34	NA	NA	NA	NA
HC1	49	63	NA	NA	NA
HC3	56.5	67	NA	NA	NA
HC2	58	50	NA	NA	NA
PD3	60	53	8	1.0	29
ROT	63.25	NA	NA	NA	NA
PD1	65.33	76	6	4.0	45
PD5	68.5	63	4	3.0	37
PD2	69.5	71	6	4.0	80
PD4	82.5	71	6	4.0	53

Gut microbiota dysbiosis in bees

We collected fecal samples from eight human donors, specifically five patients with PD and three matched healthy controls. Detailed demographic and clinical information were collated in Supplementary Table S1. The beta diversity analysis of the gut microbiota in human donors revealed that the microbial composition of patients with PD was different from that of HC subjects at the genus and species levels (Supplementary Figure S2).

In the gut microbiota analysis of honeybees, alpha diversity analysis showed that microbial richness indices (Chao index) were significantly higher in CV bees compared to HC bees, and diversity indices (Shannon index) were significantly higher in CV bees compared to PD bees (Supplementary Figure S3). The beta diversity analysis showed that the microbial composition among CV, HC, and PD bees was significantly different at the genus and species levels (Supplementary Figure S4). We used a logarithmic LDA score threshold of 2.5 to identify significant taxonomic differences between the PD and HC bees. The results of LEfSe analysis revealed a significant difference in fecal microbiota composition between the PD and HC bees, as illustrated in Figure 5A. In these differentially abundant microorganisms, eight species also exhibited differences in the microbial population of human donors, including *Morganella morganii* and *Erysipelatoclostridium ramosum*, which showed significantly increased relative abundance in the HC group, as well as *Dorea longicatena*, *Collinsella aerofaciens*, *Lactococcus garvieae*, *Holdemanella biformis*, *Gemmiger formicilis*, and *Blautia obeum*, which exhibited significantly increased relative abundance in the PD group.

By the functional prediction analysis of the 16S rRNA gene sequence data, we found that in the HC group, there was an observed increase in the predicted functional pathway related to lysine biosynthesis, suggesting an upregulation of processes associated with lysine production. Conversely, in the PD group, an enhancement in the predicted pathways related to methane synthesis, hydrogen sulfide (H_2S) synthesis, and pathogenicity was identified (Figure 5B).

Discussion

The microbiota-gut-brain axis plays a crucial role in the pathophysiology of PD, and our research further provides evidence supporting this perspective. We obtained normally developed CV bees whose guts were colonized with conventional microbiota, humanized honeybees whose guts were re-colonized with the fecal microbiota of PD patients or healthy controls, and ROT bees treated with rotenone. We observed that the conventional microbiota of wild honeybees and a fraction of the donor's microbiota successfully colonized the honeybee's digestive tract, manifesting distinct microbial enrichment patterns among the three groups of honeybees. Phototaxis is a behavior observed in many insects (Yamaguchi and Heisenberg, 2011), such as honeybees and *Drosophila*. Honeybees climb towards a light source due to phototaxis, and damage to the honeybee's brain would result in impaired balance and slowness of movement, consequently leading to an extension of climbing time. Thus, the climbing assay can be employed to assess their locomotion (Luo et al., 2021). In this study, we hypothesized that a significant decrease in the motor abilities of honeybees could in part parallel motor symptoms of PD. As expected, CV bees displayed the highest motor capabilities, followed by HC bees with a reduced motor capacity. Nevertheless, HC

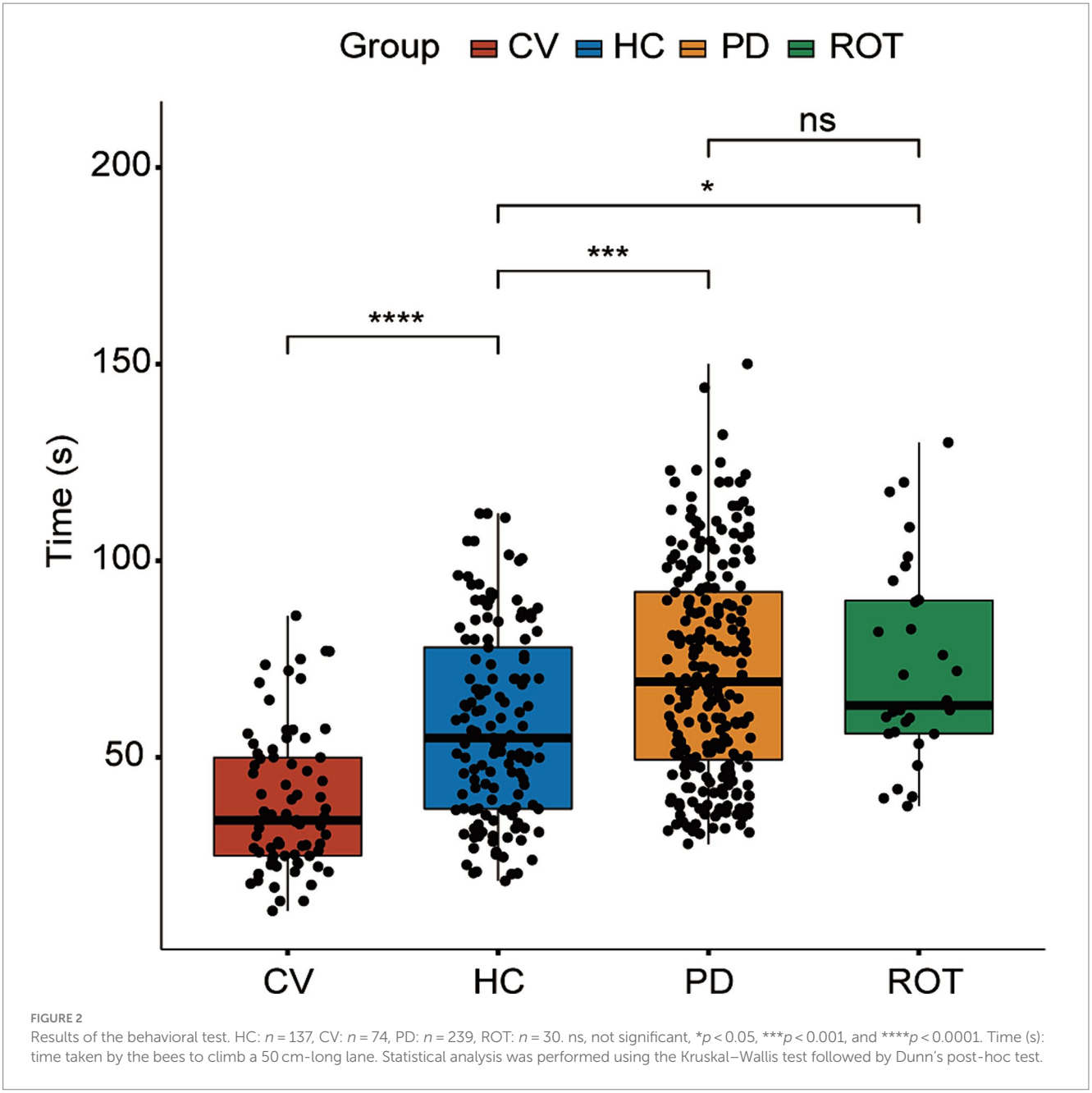

FIGURE 2
Results of the behavioral test. HC: $n = 137$, CV: $n = 74$, PD: $n = 239$, ROT: $n = 30$. ns, not significant, $*p < 0.05$, $***p < 0.001$, and $****p < 0.0001$. Time (s): time taken by the bees to climb a 50 cm-long lane. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post-hoc test.

bees exhibited superior motor abilities compared to PD and ROT bees. *Drosophila* has been used as a PD model for over two decades (Feany and Bender, 2000; Rahul and Siddique, 2022). Exposure to rotenone can induce motor impairments and a dramatic and selective loss of dopaminergic neurons in all of the brain clusters of *Drosophila* (Coulom and Birman, 2004). The results of our study indicated that a sublethal dose of rotenone could also impair the motor ability of honeybees, suggesting that rotenone may also cause cell apoptosis, oxidative damage, and trigger endoplasmic reticulum stress in honeybees (Coulom and Birman, 2004). The results of the climbing assay also indicated the feasibility of utilizing the compromised climbing ability in honeybees as a reflection of motor symptoms of PD.

At the molecular level, TH gene expression levels were significantly downregulated in both the PD and ROT groups compared to the HC group. These expression patterns aligned with the outcomes of the behavior phenotype. In dopaminergic neurons, TH is the rate-limiting enzyme in dopamine synthesis (Kostrzewa et al., 2005) and is used as a marker of the dopaminergic neurons (Hou et al., 2021). In invertebrates, extensive research indicates that dopamine signaling significantly influences locomotion (Chase et al., 2004; Lima and Miesenböck, 2005; Draper et al., 2007). Moreover, dopamine impacts locomotor behavior at various levels, encompassing modulation of the neuromuscular junction, regulation of central pattern generators, and modulation of general arousal levels (Dasari and Cooper, 2004; Andretic et al., 2005; Puhl and Mesce, 2008). A study by Mustard et al. (2010) revealed that dopamine modulates various motor behaviors in honeybees, encompassing walking, halting, upside-down movement, grooming, flying, and fanning. In our study, TH gene expression levels were reduced in both the PD and ROT groups, indicating the potential use of TH gene expression as an indicator of impaired motor function.

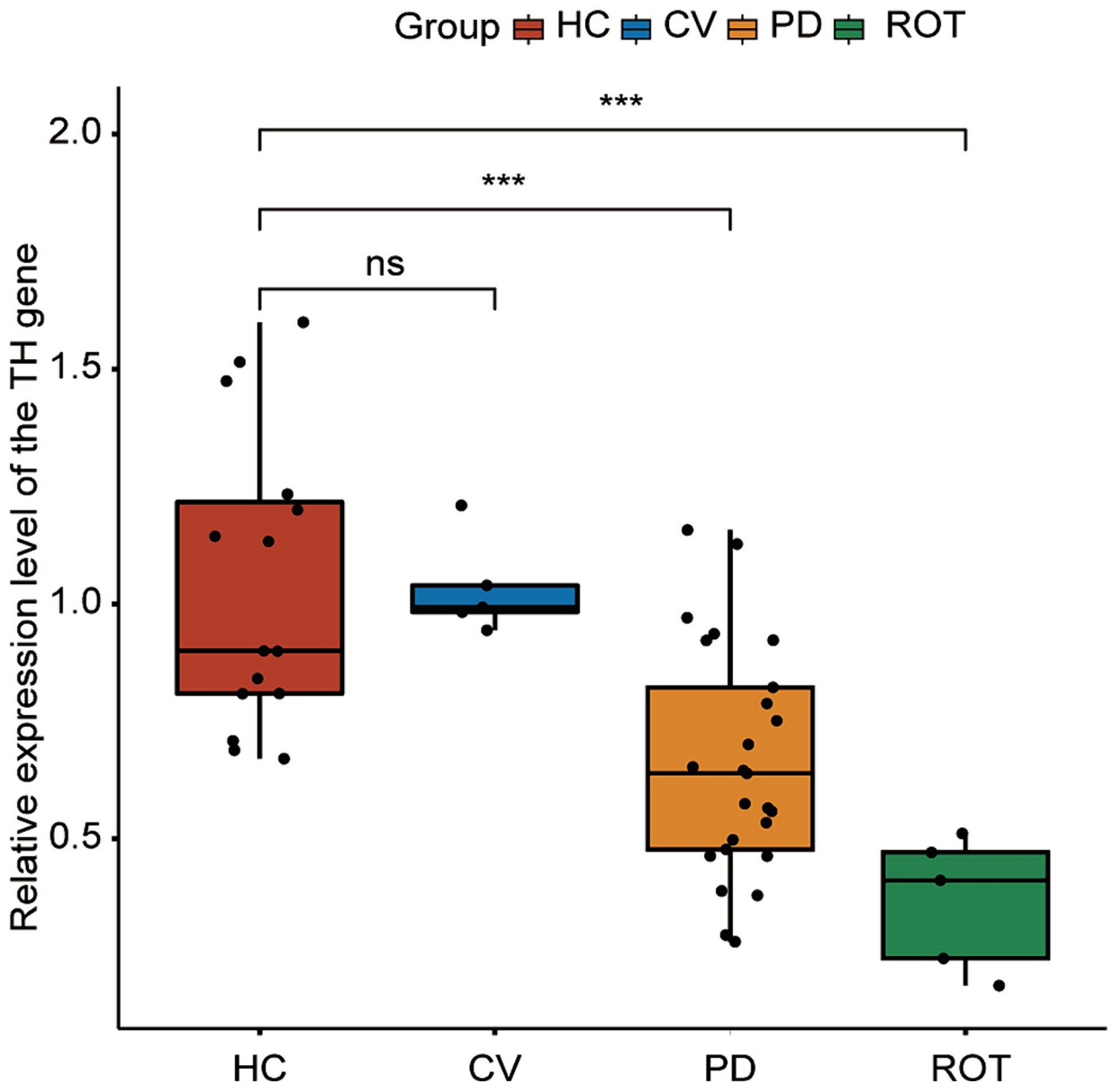

FIGURE 3
Expression of the tyrosine hydroxylase (TH) gene in the brain of honeybees, as measured by RT-qPCR. HC: $n = 15$, CV: $n = 5$, PD: $n = 25$, ROT: $n = 5$. ns, not significant, ***$p < 0.001$. Statistical analysis was conducted using the Mann–Whitney U test.

Nevertheless, the dopamine levels measured by ELISAs did not exhibit a significant difference among the four groups. Several factors may contribute to such results. Firstly, a previous study found that levels of dopamine are lower in microbiota-free honeybees (Zhang et al., 2022b). However, our study found that the microbiota-free ROT group did not show a significant reduction in the levels of dopamine, suggesting potentially insufficient sample size. Secondly, the concentration of dopamine is influenced by its metabolism and reuptake. Even with a reduction in TH gene expression, mechanisms such as increased dopamine metabolism or slowed reuptake by dopamine transporter (DAT) may exist, leading to the relatively stable maintenance of dopamine levels (Klein et al., 2019). Thirdly, there might be a time lag between changes in TH gene expression and dopamine levels. TH, as the rate-limiting enzyme in dopamine synthesis, may not immediately reflect alterations in dopamine levels. It is possible that after a decrease in TH gene expression, a certain amount of time is needed for noticeable changes in dopamine levels to occur. Fourthly, PD involves neurotransmitters beyond dopamine. Abnormalities in non-dopaminergic neurotransmitters, such as acetylcholine, glutamate, norepinephrine, and serotonin, also contribute to the manifestation of PD symptoms (Kalia and Lang, 2015). Further studies should be undertaken to investigate the aforementioned possibilities.

The histology of guts revealed that gut barriers were disrupted in PD bees and ROT bees, causing enteritis-like pathology. A similar pathology was identified in our previous honeybee model of enteritis (Chang et al., 2022). In patients with PD, elevated levels of pro-inflammatory cytokines including TNF-α, IF-γ, IL-6, and IL-1β

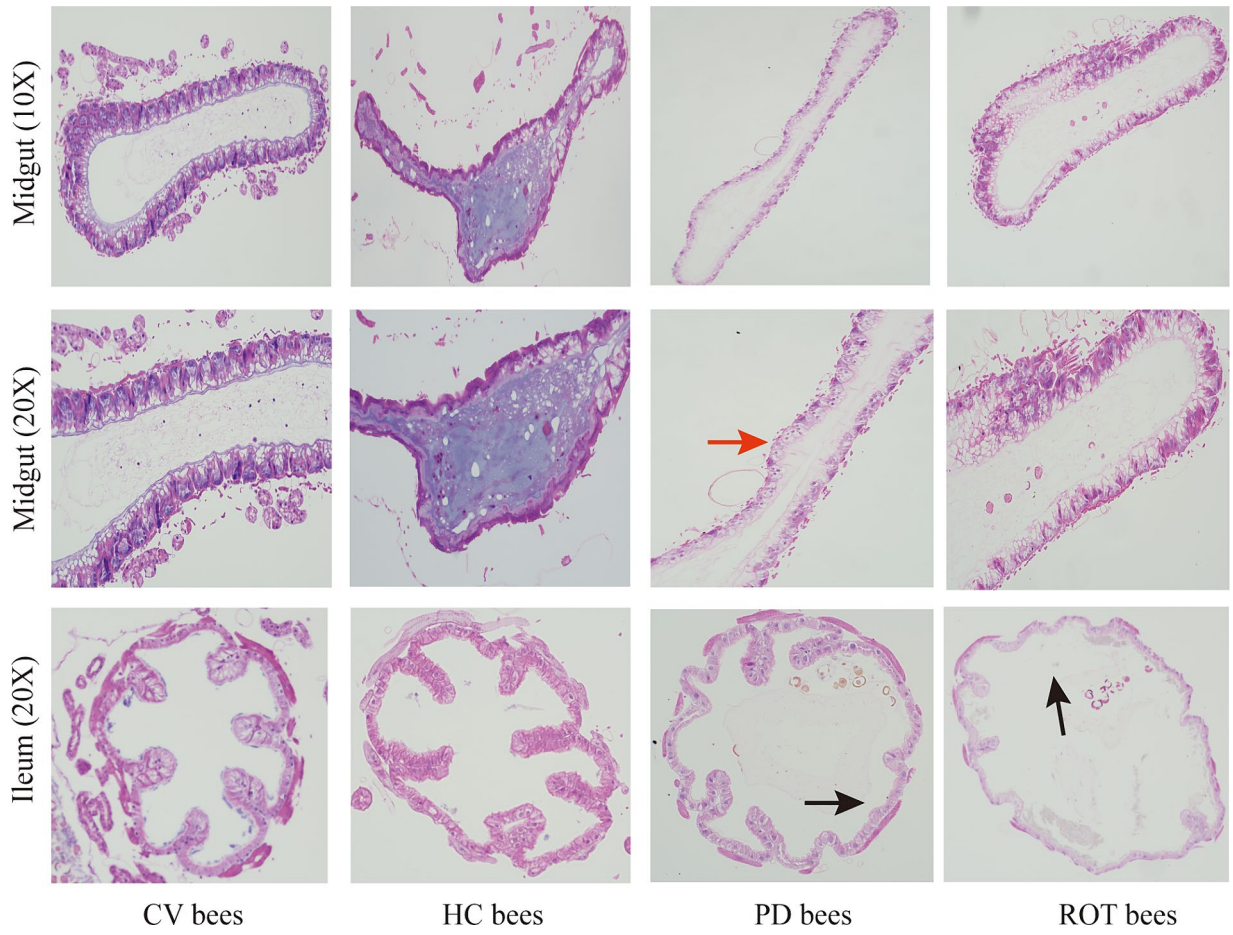

FIGURE 4
Hematoxylin and eosin staining on the midgut and ileum of honeybees. The red arrow indicates epithelial cell swelling, and the black arrows indicate dilation of the intestinal lumen and disruption of villous structures.

have been detected in both the colons and peripheral blood (Devos et al., 2013; Qin et al., 2016). Gastrointestinal inflammation is implicated in the increased intestinal permeability. Traversed gut microbiota and their metabolites may induce the production of proinflammatory cytokines in the enteric nervous system and aggregation of pathological α-synuclein (Zeng et al., 2022). Instead of synthesizing pro-inflammatory cytokines in response to microbiota, honeybees produce antimicrobial peptides (AMPs) as a major part of their non-specific defense system against infections. Honeybee-produced AMPs include abaecin, apidaecin, defensin, and hymenoptaecin (Keitel et al., 2013; Horak et al., 2020). To advance the development of the honeybee PD model, future studies are necessary to explore the role played by AMPs in the microbiota-gut-brain axis.

The elevated alpha diversity in the CV group suggests a richer microbial community within the gut compared to the HC and PD groups, reflecting a healthier status in the gut microbial ecosystem of the CV group. However, it is also possible that this increased alpha diversity reflects that the microbiota from bees may colonize bees more effectively than that from humans. The honeybee microbiota plays a pivotal role in preserving the individual's health, and disturbance to the microbiota renders the insect susceptible to a range of problems (Nowak et al., 2021). Various studies have substantiated the presence of microbiota-gut-brain axis, signifying that gut microorganisms instigate modifications in neurophysiology and induce alterations in the behavior of insect hosts by biogenic amines such as serotonin, octopamine, and dopamine (Westfall et al., 2018; Leger and McFrederick, 2020).

Previous studies using gnotobiotic bees have revealed that gut microbiota alters both brain and behavioral phenotypes. Microbiota-free bees have been reported to show deficits in appetitive olfactory learning and memory, sucrose responsiveness, and social interactions when compared to CV bees (Cabirol et al., 2023). Our results showed that the differentially abundant microbiota between groups may impair the hosts' health in several ways. *M. morganii* and *E. ramosum*, the relative abundance of which increased in the HC group, are recognized as gut commensal microbiota in humans and other mammals (Zrelovs et al., 2022) and may not cause impairment to the honeybees. As for gut microbiota enriched in the PD group, *D. longicatena* can produce formate, a toxic metabolite inhibiting mitochondrial cytochrome C oxidase (Nicholls, 1976). Moreover, formate was found to increase in the serum of PD patients (Nagesh Babu et al., 2018). *C. aerofaciens* induces the expression of IL-17 and the chemokines CXCL1 and CXCL5, causing a loss of gut epithelial integrity under pro-inflammatory conditions (Kalinkovich and Livshits, 2019). *B. obeum*, and *D. longicatena* were also enriched in dystonia subjects (Ma et al., 2021), a disease with neuronal damage and neurotransmitter abnormalities. In summary, these findings indicate dysbiosis of the

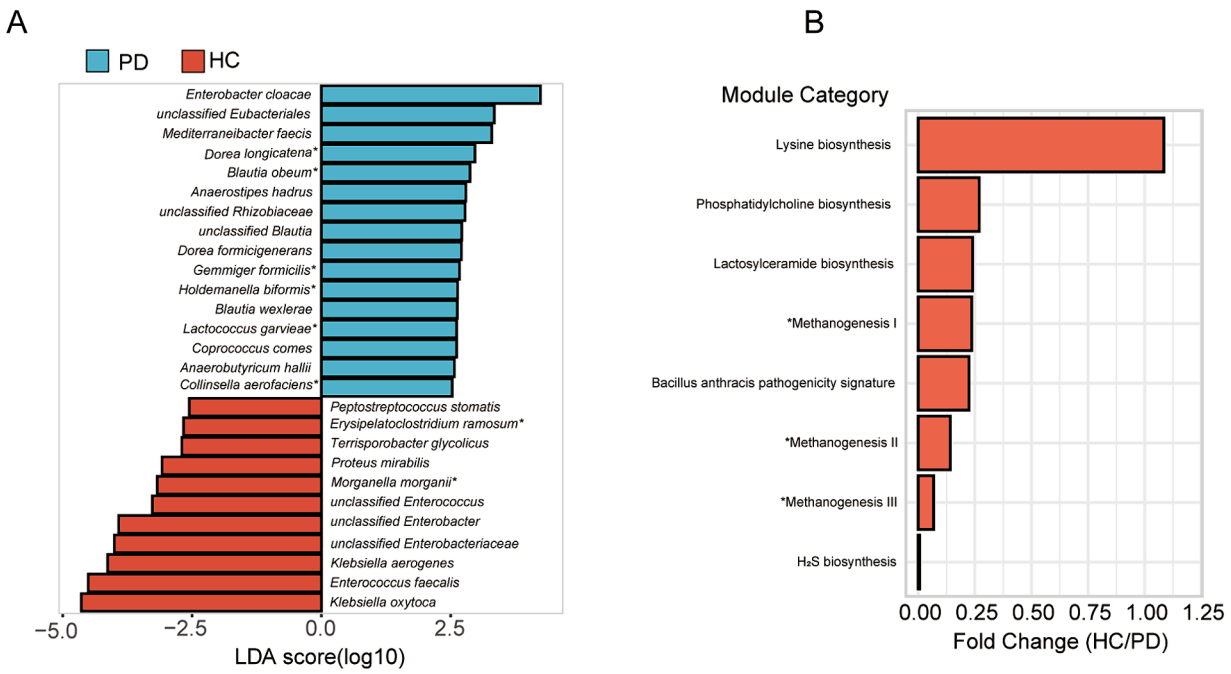

FIGURE 5
Differential taxonomic and functional analysis of gut microbiota in honeybees. **(A)** LEfSe analysis of microbiota composition between PD and HC bees. *Eight species also exhibited differences in the microbial population of human donors. **(B)** Functional prediction analysis unveiling altered metabolic pathways. *Methanogenesis I: CO_2 ⇒ methane; methanogenesis II: methylamine/dimethylamine/trimethylamine ⇒ methane; methanogenesis III: methanol ⇒ methane.

gut microbiota in the PD group, and it may negatively impact host health through various pathways, including modulation of the host immune system, generation of toxic metabolites, and damage to gut mucosal integrity.

For the predicted functional pathways, lysine biosynthesis was potentially downregulated in the PD group. Lysine, an α-amino acid that is a precursor to many proteins, is essential in humans and cannot be synthesized by humans. The pathways involving methane metabolism and H_2S production were elevated in the PD group. Emerging evidence suggests that methane delays intestinal transit, possibly acting as a neuromuscular transmitter. Additionally, methane has been epidemiologically and clinically associated with constipation-related diseases, such as constipation-predominant irritable bowel syndrome and chronic constipation (Pimentel et al., 2006; Triantafyllou et al., 2014). Previous studies have shown that methane slows down bowel movements, which indicates that the abnormal methane metabolism observed in our results may be related to the common symptom of constipation in patients with PD (Jang et al., 2020). H_2S is produced by microbes of the colon (Paul and Snyder, 2015). H_2S has cytoprotective properties by maintaining gut mucus integrity but is toxic to the host at high concentrations (Buret et al., 2022). High H_2S concentrations are toxic to cells, causing the inhibition of the cytochrome oxidase, a hemeprotein that is the last enzyme of the electron transport chain in the mitochondria. In addition, H_2S induces the release of cytochrome C protein from the mitochondrial membrane, an event thought to be associated with the etiopathogenesis of PD (Murros, 2022).

The study has the following limitations: first, further metabolomics and proteomics analyses are needed to identify neuroactive metabolites and proteins in the host gut, hemolymph, and brain, and to reveal the link between such substances and the gut microbiota in a strain-specific manner. Second, patients recruited in the study are on medications, which may be a confounder influencing the results. Third, there are inherent differences in the anatomical and physiological structures between honeybees and humans, which necessitate caution when interpreting findings from the bee model and applying them to human clinical contexts. Fourth, some bacterial taxa from human donors may have a higher propensity for colonizing the bee gut, potentially leading to a shift in microbial composition. Fifth, this is the first attempt to use honeybees as PD model animals, and more repeated experiments are needed to verify the practicability and credibility of the model.

In conclusion, despite the above limitations, we found that it was feasible to use rotenone and gut microbiota to construct a PD honeybee model and that the pathogenesis of the microbiota-gut-brain axis might also exist in the PD honeybee model.

Data availability statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive https://ngdc.cncb.ac.cn/gsa (Genomics, Proteomics & Bioinformatics 2021). The data can be accessed in the National Genomics Data Center (Nucleic Acids Res. 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA014586.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Chinese PLA General Hospital (No. S2019-061-02). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

JZ: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. YL: Methodology, Writing – review & editing. JY: Methodology, Writing – review & editing. RC: Methodology, Writing – review & editing. MX: Methodology, Writing – review & editing. GZ: Methodology, Writing – review & editing. JM: Methodology, Writing – review & editing. DL: Methodology, Writing – review & editing. ZM: Conceptualization, Supervision, Writing – review & editing. YY: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1418857/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Dopamine concentrations in the brains of honeybees. HC: $n = 9$, CV: $n = 3$, PD: $n = 15$, ROT: $n = 3$. ns, not significant. Statistical analysis was conducted using the Mann–Whitney *U* test.

SUPPLEMENTARY FIGURE S2
Principal coordinate analysis (PCoA) plots based on the Bray–Curtis distance. **(A)** PCoA on species level. **(B)** PCoA on genus level.

SUPPLEMENTARY FIGURE S3
The alpha diversity analysis of gut microbiota of honeybees. **(A)** Kruskal–Wallis *H* test for Chao index. **(B)** Kruskal–Wallis *H* test for Shannon index.

SUPPLEMENTARY FIGURE S4
The beta diversity analysis of gut microbiota of honeybees. **(A)** Principal component analysis (PCA) on species level. **(B)** PCA on genus level.

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Duodenal microbiota dysbiosis in functional dyspepsia and its potential role of the duodenal microbiota in gut–brain axis interaction: a systematic review

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Background and aims: Functional dyspepsia (FD) is a common gastrointestinal disorder associated with brain–gut interaction disturbances. In recent years, accumulating evidence points to the duodenum as a key integrator in dyspepsia symptom generation. Investigations into the pathological changes in the duodenum of FD patients have begun to focus on the role of duodenal microbiota dysbiosis. This review summarizes duodenal microbiota changes in FD patients and explores their relationship with gut-brain interaction dysregulation.

Methods: Ten databases, including PubMed, MEDLINE, and the Cochrane Library, were searched from inception to 10th October 2023 for clinical interventional and observational studies comparing the duodenal microbiota of FD patients with controls. We extracted and qualitatively summarized the alpha diversity, beta diversity, microbiota composition, and dysbiosis-related factors.

Results: A total of nine studies, consisting of 391 FD patients and 132 non-FD controls, were included. The findings reveal that the alpha diversity of the duodenal microbiota in FD patients does not exhibit a significant difference compared to non-FD controls, although an upward trend is observed. Furthermore, alterations in the duodenal microbiota of FD patients are associated with the symptom burden, which, in turn, impacts their quality of life. In FD patients, a considerable number of duodenal microbiota demonstrate a marked ascending trend in relative abundance, including taxa such as the phylum *Fusobacteria*, the genera *Alloprevotella*, *Corynebacterium*, *Peptostreptococcus*, *Staphylococcus*, *Clostridium,* and *Streptococcus*. A more pronounced declining trend is observed in the populations of the genera *Actinomyces*, *Gemella*, *Haemophilus*, *Megasphaera*, *Mogibacterium*, and *Selenomonas* within FD patients. A negative correlation in the relative abundance changes between *Streptococcus* and *Prevotella* is identified, which correlates with the severity of symptom burden in FD patients. Moreover, the alterations in specific microbial communities in FD patients and their potential interactions with the gut–brain axis merit significant attention.

Conclusion: Microbial dysbiosis in FD patients is linked to the onset and exacerbation of symptoms and is related to the disorder of gut–brain interaction. Larger-scale, higher-quality studies, along with comprehensive meta-omics research, are essential to further elucidate the characteristics of the duodenal microbiota in FD patients and its role in FD pathogenesis.

Systematic review registration: CRD42023470279, URL: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42023470279.

KEYWORDS

microbiota, functional dyspepsia, duodenum, systematic review, gut-brain interaction

1 Introduction

Functional dyspepsia (FD) is one of the most prevalent functional gastrointestinal (GI) disorders of gastroduodenal origin (Stanghellini et al., 2016). The global prevalence of FD is approximately 16% and may vary substantially according to different countries and the definition criteria of the disease (Ford et al., 2020). FD is defined as the presence of one or more of four symptoms: postprandial fullness, early satiation, epigastric pain, and epigastric burning, and cannot be explained by structural or biochemical abnormalities identified in routine clinical settings (Drossman, 2016; Ford et al., 2020). Based on the predominant symptom pattern, Rome IV provides for two functional dyspepsia subtypes of FD: epigastric pain syndrome (EPS) with epigastric pain and/or epigastric burning and postprandial distress syndrome (PDS) with postprandial fullness and/or early satiety (Drossman, 2016). Although FD does not affect survival, the symptoms of FD can be quite troublesome and difficult to treat, and patients usually have a natural history of recurrence and remission. FD is believed to affect the diet (quantity and quality of meals) and quality of life of patients, reduce the productivity of patients, lead to emotional disorders and somatization, and may result in high medical costs, which seriously burden individuals and society (Lacy et al., 2013; Gracie et al., 2015; Aziz et al., 2018; Esterita et al., 2021).

Due to the multifactorial and heterogeneous nature of functional dyspepsia symptoms, many factors influence the pathogenesis of FD. At present, the mainstream theory believes that the key to the pathogenesis of FD is related to the disorder of gut–brain interaction (DGBI) (Zhou et al., 2022). In addition, emerging research in recent years has begun to point toward the duodenum as a key player in the pathogenesis of FD.

The gastric sensorimotor dysfunction observed in FD patients may be attributed to the activation of duodeno-gastric reflexes, which transmit noxious stimuli from the duodenal mucosa via afferent nerves, leading to abnormal gastric motility and hypersensitivity (Vanheel and Ricard, 2013; Miwa et al., 2019; Wauters et al., 2020a). As a pathogenic epicenter, the duodenum induces upper gastrointestinal symptoms in FD patients primarily due to the stimulation of duodenal contents, low-grade inflammation, and increased mucosal permeability in the duodenum. Alterations in the duodenal microbiota, or "dysbiosis," are posited to be a significant factor contributing to the emergence of these duodenal pathologies (Miwa et al., 2019; Wauters et al., 2020a). Recent research has focused on the presence of small intestinal bacterial overgrowth (SIBO) in FD patients (Gurusamy et al., 2021a). Relevant studies have uncovered a dysbiotic state within the duodenal mucosa of FD patients, characterized by increased bacterial load and diversity. This microbial imbalance has been found to be associated with the manifestation of gastrointestinal symptoms in FD patients (Zhong et al., 2017). The gut microbiota may communicate with the central nervous system via neural, endocrine, and immune pathways, thereby influencing brain function. In FD patients, dysbiosis of the gut microbiota is known to affect the gut–brain interactive functions, with the microbiota-gut–brain axis being recognized as playing a significant role in FD (Rupp and Andreas, 2022).

However, in previous studies, due to the difficulty in obtaining duodenal microbiota samples, most studies on the gastrointestinal microbiota of FD patients focused on the microbiota of the stomach, large intestine, and feces; the understanding of duodenal microbiota was relatively limited (Tziatzios et al., 2020; Zhou et al., 2022). The dysbiosis of the duodenal microbiota and its implications for the pathogenesis of FD warrant further exploration.

Drawing from the aforementioned perspectives, we postulate that alterations in the duodenal microbiota represent a crucial yet underappreciated nexus influencing the pathogenesis of functional dyspepsia (FD) while also engaging in the gut–brain axis of FD patients. Recent observational studies have provided pertinent insights into the involvement of the duodenal microbiota in FD onset. Building upon these foundations, this study synthesizes the characteristic changes in duodenal microbiota among FD patients, endeavors to elucidate the mechanisms by which duodenal microbiota participate in gut–brain interactions, explores the role of duodenal microbiota alterations in FD pathogenesis, and endeavors to identify bacterial targets pertinent to FD diagnosis or treatment.

2 Methods

2.1 Protocol and registration

This systematic review followed the recommended approach described in the Preferred Reporting Items for Systematic Review and Meta-Analyses Protocols (PRISMA-P) 2015 Statement Guidelines (PRISMA-P Group et al., 2015; Page et al., 2020).

The protocol has been registered with the International Prospective Register of Systematic Reviews, and the PROSPERO registration number is CRD42023470279.

2.2 Search strategy

We performed a systematic search of 10 electronic databases, including PubMed, MEDLINE (Medical Literature Analysis and Retrieval System Online), Cochrane Library, ClinicalTrials.gov, Excerpta Medica Database (EMBASE), Web of Science (Wos), Wanfang Data, China National Knowledge Infrastructure (CNKI), VIP Information Resource Integration Service Platform (CQVIP), and Chinese Biomedical Literature Database (SinoMed), from inception to 10th October 2023, with the keywords "duodenum," "microbiota," and "functional dyspepsia." We also manually searched for other relevant literature based on the references to the identified articles.

2.3 Study selection and patient population

This review selected observational and interventional studies (i.e., case–control, cohort, and randomized and non-randomized clinical trial studies) that compared the composition of the duodenal microbiota, microbial diversity, or microbial richness between FD patients and non-FD controls. FD patients must have a presumed diagnosis of FD based on the clinical assessment, questionnaire data, or specific symptom-based criteria, including the Rome criteria, and patients must not show any evidence of gastric/duodenal mucosal abnormalities, lesions, or structural changes (based on endoscopic and clinical histology findings). The control group (CG) comprises individuals without functional dyspepsia, including healthy controls, and is required to possess demographic data comparable to that of the FD group. There are no restrictions on the age and gender of the population. We excluded studies in which the experimental group included patients with functional gastrointestinal disorders or FD combined with other diseases. Case reports, expert opinions, and reviews were also excluded.

2.4 Data extraction

Data were extracted by two independent authors (XPZ and LC) and confirmed by a third (XLS). The following data were extracted: (1) study design, including the name of the first author, year of publication, journal, country, study design, inclusion and exclusion criteria, and criterion for FD diagnosis; (2) population characteristics, including age, sex, sample size, body mass index (BMI), follow-up duration, usage of proton pump inhibitor (PPI), and subtype(s) of FD if specified; (3) outcome measures: specimen processing (collection, storage, and DNA extraction), sequencing platforms, method of gut microbiota estimation, alpha diversity, beta diversity index, duodenal microbiota profile, and relative abundance. The primary outcome was the difference in individual duodenal bacterial species taxonomic classification reported in FD patients compared to non-FD controls.

2.5 Quality of studies

Two independent researchers (QYW and ZTZ) evaluated the risk of research bias separately. Study quality (risk of bias) has been assessed by the Newcastle-Ottawa scale (NOS) tool, which evaluates research through population selection, comparability, exposure, or outcomes (Stang, 2010). Any differences between the two researchers will be resolved through discussion or negotiation with the third reviewer (TZ).

3 Results

3.1 Study selection

A total of 588 studies were identified in the initial literature search. Then, 165 duplicates were identified and excluded. Afterward, 404 studies were rejected based on relevance to the title abstract. After full-text screening, 10 articles were excluded, and six full-text articles and three abstract studies were finally included (Figure 1).

3.2 Characteristics of reviewed studies

Table 1 provides an overview of the study's characteristics. The nine studies consist of 391 FD patients and 132 non-FD controls. Out of all the studies included, only one had a sample size greater than 100, with 257 cases (Tziatzios et al., 2021). The remaining studies had sample sizes below 100. Five studies were conducted in Australia (Zhong et al., 2016, 2017; Schooth et al., 2021, 2022; Shanahan et al., 2023), while others were conducted in Belgium (Wauters et al., 2021), China (Zheng et al., 2022), Greece (Tziatzios et al., 2021), and Japan (Fukui et al., 2020). This review contains one prospective cohort study (Wauters et al., 2021), one prospective cross-sectional study (Tziatzios et al., 2021), and seven case–control studies (Zhong et al., 2016, 2017; Fukui et al., 2020; Schooth et al., 2021, 2022; Zheng et al., 2022; Shanahan et al., 2023). The search did not include interventional studies carried out on FD patients. Out of all the studies included, three were explicitly presented as FD patients compared to healthy controls (Fukui et al., 2020; Wauters et al., 2021; Zheng et al., 2022), while the rest compared FD patients to non-FD patients (Zhong et al., 2016, 2017; Schooth et al., 2021, 2022; Tziatzios et al., 2021; Shanahan et al., 2023). Among the studies in which FD patients were compared with healthy controls, one study (Wauters et al., 2021) compared FD patients who had not been treated with PPI therapy (4-week healing dose) or other acid suppression < 3 months before inclusion ("FD-starters") with FD patients who had persistent symptoms after >1 month of at least one daily dose of PPI ("FD-stoppers") and healthy controls (Table 1).

3.3 Microbiome assessment methods

Of the nine studies included in this systematic review, four studies (Zhong et al., 2016; Schooth et al., 2021, 2022; Shanahan et al., 2023) collected samples through duodenal biopsy only, and one (Fukui et al., 2020) performed upper gut brushing, while two others (Wauters et al., 2021; Zheng et al., 2022) used both duodenal biopsy and brushing. One study (Wauters et al., 2021) collected gastric and duodenal fluids, and another (Tziatzios et al., 2021) used duodenal fluid aspiration along with gastric biopsy. In terms of microbial assessment, 16S rRNA gene sequencing was used by most studies ($n = 8$) (Zhong et al., 2016, 2017; Fukui et al., 2020; Schooth et al., 2021, 2022; Wauters et al., 2021; Zheng et al., 2022; Shanahan et al., 2023). There were differences in the variable region sequenced among the eight studies that used bacterial 16S rRNA gene sequencing. One study (Wauters et al., 2021) used the V4 hypervariable region, one study (Fukui et al., 2020) used the V3-V4 hypervariable region, and two studies sequenced V6–V8 (Schooth et al., 2022; Shanahan et al., 2023), which was the commonly studied variable region (Table 2).

3.4 Alterations in the duodenal microbiota composition of FD patients

Three studies (Fukui et al., 2020; Zheng et al., 2022; Shanahan et al., 2023) have delineated variations in the duodenal microbiota at the phylum level. Two studies (Zheng et al., 2022; Shanahan et al., 2023) found an increase in the amounts of *Fusobacteria* in FD patients, whereas a third study (Fukui et al., 2020) showed no difference

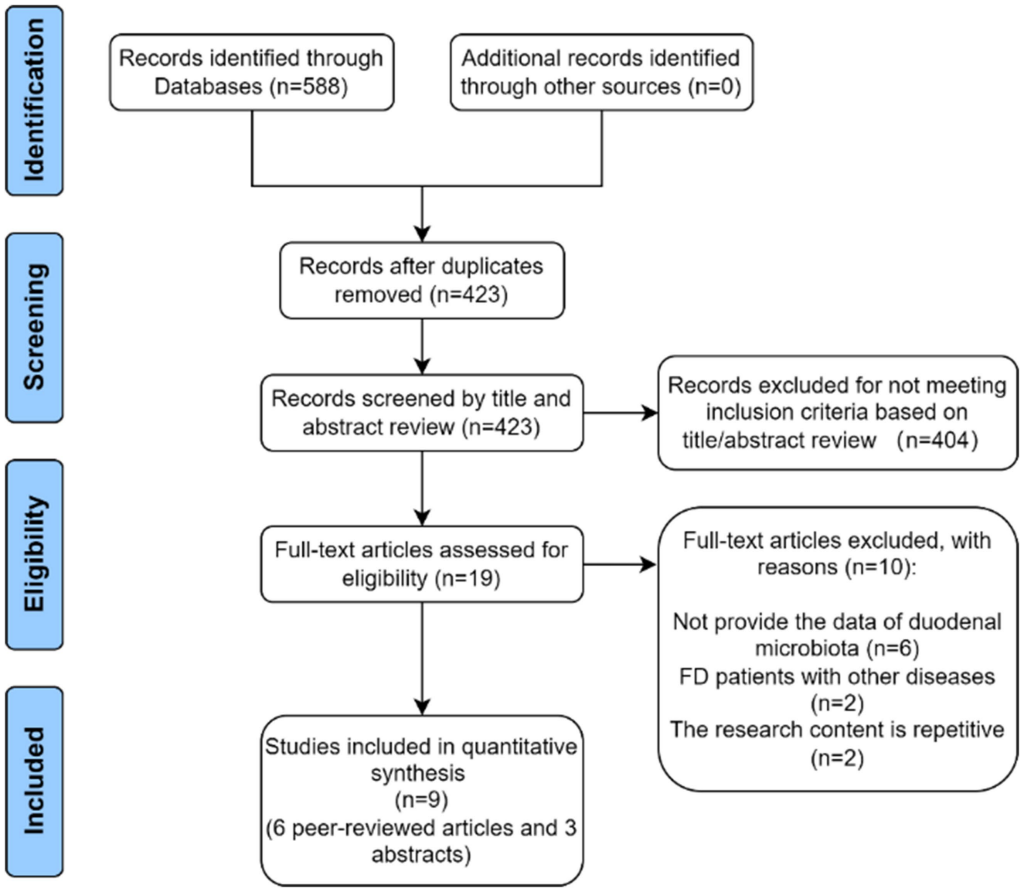

FIGURE 1
Flow diagram of study selection.

compared to healthy controls. Findings from one study (Zheng et al., 2022) suggest that the phylum *Firmicutes* constitutes a substantial proportion of the total sequences in both FD patients and healthy individuals and represents the dominant bacteria of the duodenal flora. One study (Fukui et al., 2020) demonstrated a significant increase in the phylum *Firmicutes* in FD patients. While another study (Shanahan et al., 2023) did not observe a marked difference in the relative abundance of *Firmicutes* between FD patients and healthy controls, it did discern a correlation between the abundance of this phylum and the severity of FD symptoms, with an escalation in *Firmicutes* accompanying an increased symptom burden in FD patients. In one study (Zheng et al., 2022), the phylum *Bacteroidetes* and *Proteobacteria* were identified as the dominant species of duodenal flora. However, three investigations (Fukui et al., 2020; Zheng et al., 2022; Shanahan et al., 2023) collectively found no significant differences in the abundance of *Bacteroidetes* and *Proteobacteria* between FD patients and the control group. Additionally, one study (Zheng et al., 2022) remarked that there was no significant difference in the composition of duodenal flora at the phylum level between FD patients and healthy controls.

At the genus level, there were three Australian studies (Zhong et al., 2016, 2017; Shanahan et al., 2023) demonstrating a significantly decreased number of bacteria in the genus *Actinomyces*. The genus *Selenomonas* was evaluated in three studies (Wauters et al., 2021; Zheng et al., 2022; Shanahan et al., 2023). Of these, one study (Zheng et al., 2022) showed a significant decrease in the amount of the genus *Selenomonas* in FD patients, while the other two studies (Wauters et al., 2021; Shanahan et al., 2023) showed a non-significant trend toward decreased amounts. The results of a study in China (Zheng et al., 2022) showed significantly increased numbers of the genera *Staphylococcus* and *Peptostreptococcus* in FD patients. In contrast, an Australian study (Shanahan et al., 2023) showed a non-significant trend of increase for these genera. Another study identified the genus *Staphylococcus* as a predominant component of the duodenal microbiota in FD patients (Zhong et al., 2016). Two studies evaluated the genus *Alloprevotella* in 76 FD patients (35 healthy controls) and showed a significant increase in one study (Zheng et al., 2022) and a non-significant increase in another (Shanahan et al., 2023). Genus *Haemophilus* showed a decreasing trend in two studies (Wauters et al., 2021; Shanahan et al., 2023), whereas in one study of 9 FD patients compared to 9 healthy controls (Zhong et al., 2017), the relative abundance of the genus *Haemophilus* was not found to be significantly different. Another study from China (Zheng et al., 2022) found that the genus *Haemophilus* was one of the most important microorganisms in the duodenal flora of both FD patients and healthy controls. Furthermore, two studies (Zhong et al., 2017; Shanahan et al., 2023) compared a total of 65 FD patients with 39 healthy controls and concluded that the microbial counts of genus *Megasphaera* showed a decreasing trend in FD patients.

TABLE 1 Characteristics of each study included.

Author, Year	Country	Study design	FD patients						Control group					
			Inclusion criteria	Exclusion criteria	*N* (*n*=)	Sex (M/F)	AGE (years): Mean ± SD	BMI (kg/m^2): mean ± SD	Inclusion criteria	Exclusion criteria	*N* (*n*=)	Sex (M/F)	AGE (years): Mean ± SD	BMI (kg/m^2): mean ± SD
Shanahan et al. (2023)	Australia	Case–control study (FD and Controls)	FD patients based on Rome IV or FD with additional irritable bowel syndrome-like symptoms were included. Only those who did not show any evidence of gastric/duodenal mucosal abnormalities, lesions, or structural changes (based on endoscopic and clinical histology findings) were included in the study.	Patients who reported the onset of GI symptoms after an acute infection have not been included in the study.	56	27/29	47 (17–77)[a]	25.2 ± 5.6	Control subjects were symptom-free (non-FD), with either documented iron deficiency (ID) with and without anemia or individuals undergoing screening following a positive fecal occult blood test. Only those who did not show any evidence of gastric/duodenal mucosal abnormalities, lesions, or structural changes (based on endoscopic and clinical histology findings) were included in the study.	Patients who reported the onset of GI symptoms after an acute infection have not been included in the study.	30	15/15	59 (22–74)[a]	27.5 ± 6.1
Zheng et al. (2022)	China	Case–control study (FD and Healthy Controls)	All enrolled patients were consistent with the diagnostic criteria of Rome IV.	Patients with *helicobacter pylori* infection were excluded.	20	3/17	36.6 ± 14.00	20.81 ± 12.98	The health control group excluded digestive systems and other systemic diseases by the examination of gastroscopy and abdominal ultrasound.	NR	5	1/4	28.8 ± 9.00	20.94 ± 13.10
Wauters et al. (2021)	Belgium	Prospective cohort study (FD-starters and FD-stoppers and Healthy Controls)	Patients with predominant FD symptoms were diagnosed according to Rome IV criteria.	All subjects had no *Helicobacter pylori*, active psychiatric, atopic, inflammatory, or metabolic conditions. Use of immunosuppressants, anti- or probiotics <3 months were exclusionary.	"FD-starters": 28	"FD-starters": 4/24	"FD-starters": 27 (23.5–34.5)[b]	"FD-starters": 22 (19-24)[b] [a]	Age- and gender-matched healthy volunteers without GI symptoms were recruited as controls by advertisement.	All subjects had no *Helicobacter pylori*, active psychiatric, atopic, inflammatory, or metabolic conditions. Use of immunosuppressants, anti- or probiotics <3 months were exclusionary.	30	9/21	27 (24–33.5)[b]	23 (20–25.3)[b]
			"FD-starters": no standard course of PPI therapy (4 weeks healing dose) and/or acid suppression <3 months before inclusion.		"FD-stoppers": 19	"FD-stoppers": 5/14	"FD-stoppers": 32 (26.8–49.5)[b]	FD-stoppers": 21.5 (20.8–24.3)[b]						
			"FD-stoppers": refractory symptoms after >1 month of at least one daily dose of PPI.											

(Continued)

10.3389/fmicb.2024.1409280

TABLE 1 Continued

Author, Year	Country	Study design	FD patients						Control group					
			Inclusion criteria	Exclusion criteria	*N* (*n*=)	Sex (M/F)	AGE (years): Mean ± SD	BMI (kg/m^2): mean ± SD	Inclusion criteria	Exclusion criteria	*N* (*n*=)	Sex (M/F)	AGE (years): Mean ± SD	BMI (kg/m^2): mean ± SD
Zhong et al. (2017)	Australia	Case–control study (FD and Controls)	Patients presenting with FD based on Rome III.	NR	9	Two groups were matched for age, sex, and body mass index.			NR	Controls were screened for iron-deficiency anemia, and people with mucosal/ coeliac disease were excluded.	9	Two groups were matched for age, sex, and body mass index.		
Fukui et al. (2020)	Japan	Case–control study (FD and Healthy Controls)	The groups were age- and sex-matched. All 18 persons fulfilled the following criteria: no antibiotics, corticosteroids, immunosuppressants, and acid-suppressing agents were taken within 1 month. No mucosal inflammation or carcinoma was detected.	NR	11	6/5	56 (29–75)[a]	NR	The groups were age-and sex-matched. All 18 persons fulfilled the following criteria: no antibiotics, corticosteroids, immunosuppressants, and acid-suppressing agents were taken within 1 month. No mucosal inflammation or carcinoma was detected.	NR	7	4/3	52 (33–68)[a]	NR
Tziatzios et al. (2020)	Greece	Prospective cross-sectional study (FD and Controls)	Enrolled patients met Rome IV criteria for FD and reported no other miscellaneous gastrointestinal symptoms that could be indicative of potential overlap with other functional gastrointestinal disorders, i.e., IBS.	Patients aged <18 years, use of antibiotics or probiotics within the last 3 months, inability to provide informed consent, comorbidities, systemic diseases, diabetes mellitus, chronic constipation. Previous gastrointestinal surgeries with postsurgical structural changes, cirrhosis of any cause, and chronic pancreatitis or malignancies were excluded. Patients reporting other miscellaneous gastrointestinal complaints, i.e., diarrhea, malnutrition, weight loss indicative of SIBO, and current or recent use of PPI or H2 blockers, laxatives, and drugs that affect intestinal motility, were also excluded.	227	89/138	51.7 ± 13.8	NR	Individuals with a negative medical history of comorbidities undergoing upper GI endoscopy were considered eligible for inclusion. Those having neither pathological findings on endoscopy nor histological evidence of *Helicobacter pylori* (*H. pylori*) infection – identified as non-erosive reflux disease patients – were enrolled.	Individuals with any drug intake, current or recent (within the last 3 months), use of PPI or H2 blockers, and evidence of overlapping FD-type symptoms were excluded.	30	12/18	50.9 ± 14.6	NR

(Continued)

10.3389/fmicb.2024.1409280

TABLE 1 Continued

Author, Year	Country	Study design	FD patients						Control group					
			Inclusion criteria	Exclusion criteria	*N* (*n*=)	Sex (M/F)	AGE (years): Mean ± SD	BMI (kg/m^2): mean ± SD	Inclusion criteria	Exclusion criteria	*N* (*n*=)	Sex (M/F)	AGE (years): Mean ± SD	BMI (kg/m^2): mean ± SD
Zhong et al. (2016)	Australia	Case–control study (FD patients and Controls with no evidence of mucosal disease)	FD patients undergoing upper GI endoscopy were recruited after informed consent.	NR	9	NR	NR	NR	Individuals with no evidence of mucosal disease undergoing upper GI endoscopy	NR	9	NR	NR	NR
Schooth et al. (2022)	Australia	Case–control study (FD and non-FD controls)	NR	NR	6	NR	NR	NR	NR	NR	6	NR	NR	NR
Schooth et al. (2021)	Australia	Case–control study (FD and non-FD controls)	NR	NR	6	NR	NR	NR	NR	NR	6	NR	NR	NR

N, number; SD, Standard Deviation; M, male; F, female; NR, not reported; [a]Data was presented as average age [minimum age, maximum age]; [b]Data shown in median (interquartile range).

10.3389/fmicb.2024.1409280

TABLE 2 Characteristics of gut microbiota assessments and factors influencing the microbiota.

Study	Type of specimen	Sample collection and storage	Microbiome assessment method	Alpha-diversity index					Beta-diversity index			Factors influencing microbiota
				Shannon	Simpson	Chao1	ACE	Observed species	Bray-Curtis distance	PCoA analysis	Amova analysis	
Shanahan et al. (2023)	Duodenal biopsies	Duodenal biopsies were taken from the second part of the duodenum utilizing the Brisbane Aseptic Biopsy device. Biopsy samples were immediately placed under aseptic conditions into a sterile tube containing RNA later (Qiagen). Samples were allowed to incubate at room temperature for 30 min, then frozen and stored at-80°C.	16S rRNA gene V6-V8 regions	NS	NR	↑	NR	NR	NS	NR	NR	PPI use: There were no differences between the two subgroups in the combined analysis. FD symptom burden: The relative abundances of predominant members of the Firmicutes, Bacteroidota, and Fusobacteriota phyla were linked to symptom burden in FD. Gastric emptying time: The study found inverse relationships between the relative abundances of Streptococcus and Prevotella and the relative abundance of Veillonella spp. with gastric emptying time. Habitual diet: The duodenal MAM is not significantly impacted by habitual diet.
Zheng et al. (2022)	Duodenal brushings and biopsies	The inclusive FD patients and healthy groups underwent the endoscopic examination, using a cell brush to brush the intestinal contents in the duodenum and using the biopsy to randomly clamp the mucosal tissue of the descending duodenum. The samples were stored in a −80°C refrigerator.	16S rDNA gene sequencing	↑*	NR	NR	↑*	↑*	NR	Slight difference	Significant difference	NR
Wauters et al. (2021)	Duodenal brushings and biopsies	During upper GI endoscopy, aseptic biopsies from the second portion of the duodenum were collected using the sheathed and sealed Brisbane aseptic biopsy device (BABD), with additional precautions to avoid contamination. Next, a sterile brush was advanced while leaving the sheathed BABD in place for luminal brushing on the opposite side from where the biopsy sample was taken. Routine duodenal biopsies (for histology) and fluids (pH and bile salts) were collected in all subjects at baseline and follow-up. Samples were immediately snap-frozen and stored at −80°C.	16S rRNA gene V4 hypervariable region	Brush: ↓ Biopsy: ↓	Brush: ↓ Biopsy: ↓	Brush: ↓ Biopsy: ↑	NR	Brush: ↓ Biopsy: ↑	NR	NR	NR	PPI use: Baseline differences and effects of short-term PPI therapy were only found for specific luminal genera and diversity, while long-term PPI therapy may have a certain effect on duodenal Dysbiosis, with stable duodenal luminal and mucosal bacterial communities in the absence of PPI therapy.

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10.3389/fmicb.2024.1409280

TABLE 2 Continued

Study	Type of specimen	Sample collection and storage	Microbiome assessment method	Alpha-diversity index					Beta-diversity index			Factors influencing microbiota
				Shannon	Simpson	Chao1	ACE	Observed species	Bray-Curtis distance	PCoA analysis	Amova analysis	
Zhong et al. (2017)	NR	NR	16S rRNA gene sequencing and qPCR	NR	NR	NR	NR	NR	NR	NR	NR	Quality of life: There is a negative correlation between duodenal mucosal bacterial load and reported quality of life. Symptom responses: More severe symptom responses to the standardized meal positively correlated with mucosal bacterial load.
Fukui et al. (2020)	Upper gut brushings: the middle of the esophagus, gastric body, gastric antrum, and descending portion of the duodenum	The upper gut samples were collected from the middle of the esophagus, gastric body, gastric antrum, and descending portion of the duodenum by upper endoscopy. Under endoscopic examination, the samples were taken by gently brushing each 10 times with a Cytology brush. Each sample was placed into 500 μL of sterile phosphate buffer saline. The samples were frozen and stored at −80°C in a freezer.	16S rRNA gene V3–V4 hypervariable region	NS	NR	NS	NR	NS	NR	Significant difference	NR	Gastrointestinal symptoms: In the species level in Streptococcus, the relative abundance of OTU 90 (the most abundant sequence of *Streptococcus infantis*) was positively correlated with PDS and EPS scores. OTU 90 is a bacterium strongly correlated with upper gastrointestinal symptoms.
Tziatzios et al. (2020)	Duodenal juice aspirated and gastric biopsy	From the greater and lesser curvature of the antrum, incisura, and corpus, researchers extracted five gastric biopsy specimens, which were placed into separate vials and grouped by site. The endoscopist passed the ERCP catheter through the biopsy channel of the endoscope and advanced it into the third and fourth portions of the duodenum for collection of the small bowel aspirate. Small bowel aspirates were quantitatively cultured under aerobic conditions. Plates were cultured for 24 h at 37°C under aerobic conditions.	NR	NR	NR	NR	NR	NR	NR	NR	NR	FD subtypes: Among dyspeptics, SIBO is more frequent in PDS and overlaps PDS-EPS subgroups, but not EPS alone.

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10.3389/fmicb.2024.1409280

TABLE 2 Continued

Study	Type of specimen	Sample collection and storage	Microbiome assessment method	Alpha-diversity index					Beta-diversity index			Factors influencing microbiota
				Shannon	Simpson	Chao1	ACE	Observed species	Bray-Curtis distance	PCoA analysis	Amova analysis	
Zhong et al. (2016)	Duodenum biopsies	NR	16S rRNA gene sequencing and qPCR	NR	NR	NR	NR	NR	NR	NR	NR	Symptom responses: When the duodenal mucosal bacterial load was assessed, an increase in bacterial levels was found to be associated with augmented meal-related symptom responses during the nutrient challenge. Disease-specific quality of life scores: Increased bacterial load was also negatively correlated with disease-specific quality of life scores.
Schooth et al. (2022)	Duodenum biopsies	Duodenal biopsy samples collected with the Brisbane Aseptic Biopsy Device were placed in an anaerobically prepared cryopreservative solution and stored at −80°C. The tissue was later aseptically transferred into a broth medium simulating the duodenal habitat (DHSM-1), cultured for 24 h, and then subsampled for storage as described above. These outgrowth cultures of the duodenal MAM were then used to inoculate 10-mL volumes of DHSM-1 broth for growth studies.	16S rRNA gene V6-V8 region	NR	NR	NR	NR	NR	NR	NR	NR	NR
Schooth et al. (2021)	Duodenum biopsies	Biopsies were collected from FD and non-FD control subjects using the Brisbane aseptic biopsy device and stored anaerobically. Biopsies were transferred to a habitat-simulating medium, incubated at 37°C, and microbial growth monitored. These cultures were sampled and stored anaerobically. Stocks were then used on two occasions to inoculate fresh media and cultured as described above.	16S rRNA gene sequencing	NR	NR	NR	NR	NR	NR	NR	NR	NR

↑, higher relative to comparison group; ↓, lower relative to comparison group; *, significant difference; NS, nonsignificant difference; NR, not reported.

10.3389/fmicb.2024.1409280

At the genus level, there were conflicting results of cumulative evidence for several significant microbiota. Three studies (Zhong et al., 2017; Zheng et al., 2022; Shanahan et al., 2023) have identified the genus *Streptococcus* as the dominant organism in the duodenal flora of both FD patients and healthy controls. A Japanese study (Fukui et al., 2020) showed a significant increase in the duodenal genus *Streptococcus* counts in FD patients compared to controls, and the results of another study (Zhong et al., 2017) also suggested a non-significant trend toward an increase, while the result of an Australian study (Shanahan et al., 2023) showed a non-significant decreasing trend of this genus. Genus *Prevotella* was also identified as a dominant species within the duodenal microbiota in three studies (Zhong et al., 2016; Zheng et al., 2022; Shanahan et al., 2023). However, a significant decrease in the genus *Prevotella* was found in one study (Zhong et al., 2017), and a non-significant increase was found in another study (Shanahan et al., 2023). A study conducted in China (Zheng et al., 2022) found that the genus *Fusobacterium*, genus *Neisseria*, and genus *Porphyromonas* were significant microbiota in the duodenum of both healthy individuals and FD patients at the genus level. Another study (Zhong et al., 2016) also identified the genus *Porphyromonas* as an important component of the duodenal flora in FD patients. Both the genera *Neisseria* and *Porphyromonas* had the same trend of alteration in three articles (Zhong et al., 2017; Wauters et al., 2021; Shanahan et al., 2023). In two of the articles (Zhong et al., 2017; Wauters et al., 2021), the relative abundance of these two genera in FD patients showed a decreasing trend, whereas in another article (Shanahan et al., 2023), their relative abundance exhibited an increasing trend. Four studies (Zhong et al., 2017; Schooth et al., 2021; Wauters et al., 2021; Shanahan et al., 2023) assessed the genus *Fusobacterium*. One study found a significantly increased amount of this genus in FD patients (Shanahan et al., 2023), while two studies (Schooth et al., 2021; Wauters et al., 2021)showed a trend for a decreased amount, and the other (Zhong et al., 2017) revealed a non-significant difference.

Summarizing the abundance changes of genera reported in two or more studies and categorizing them at the phylum level, it can be observed that genera under the phylum *Firmicutes* exhibit the highest number of changes between FD and non-FD control groups, followed by those under the phylum *Actinobacteria* (Figure 2; Table 3).

3.5 Duodenal microbiota diversity in FD patients

Four articles (Fukui et al., 2020; Wauters et al., 2021; Zheng et al., 2022; Shanahan et al., 2023) compared the alpha diversity of FD patients and controls by using different indices and methods. Four studies (Fukui et al., 2020; Wauters et al., 2021; Zheng et al., 2022; Shanahan et al., 2023) reported the Shannon index, three reported the Chao1 index (Fukui et al., 2020; Wauters et al., 2021; Shanahan et al., 2023), three reported observed species (Fukui et al., 2020; Wauters et al., 2021; Zheng et al., 2022), one reported the ACE index (Zheng et al., 2022), and one reported the Simpson index (Wauters et al., 2021). Only one study (Zheng et al., 2022) showed a significant increase in alpha diversity in FD patients, while three other studies (Fukui et al., 2020; Wauters et al., 2021; Shanahan et al., 2023) found no significant difference in alpha diversity between FD patients and the controls.

Three studies analyzed the β diversity of duodenal microbiota in FD patients and their control groups (Fukui et al., 2020; Zheng et al., 2022; Shanahan et al., 2023). Two studies showed the result through the β diversity principal coordinates analysis (PCoA) (Fukui et al., 2020; Zheng et al., 2022). One of the studies (Fukui et al., 2020) showed that there were microbial structural differences between FD and healthy subjects when PCoA was used. The other study (Zheng et al., 2022) based on the results of PCoA showed that the duodenal flora of FD patients and healthy people did not show a clear separate trend, but further Amova analysis of the research revealed that there was a significant difference between the two groups, indicating that the structure of duodenal flora changed in FD patients. One study (Shanahan et al., 2023) independently demonstrated an insignificant difference in β diversity (Bray-Curtis distance; Table 2).

3.6 Factors associated with duodenal microbiota alterations

Two studies have described the relationship between changes in the duodenal microbiota and the use of PPI in FD patients (Wauters et al., 2021; Shanahan et al., 2023). One of the studies showed that (Shanahan et al., 2023) the impacts of PPI use on the duodenal mucosa-associated microbiota (MAM) can be variable but overall limited. The other study (Wauters et al., 2021) showed that baseline differences and effects of short-term PPI therapy were only found for specific luminal genera and diversity, while long-term PPI therapy may have a certain effect on duodenal dysbiosis. Four studies (Zhong et al., 2016, 2017; Fukui et al., 2020; Shanahan et al., 2023) investigated the correlation between duodenal flora changes and FD symptoms burden. A study (Shanahan et al., 2023) suggested that the relative abundances of predominant members of the phylum *Firmicutes* and *Bacteroidota* were linked to symptom burden in FD, in which the relative abundance of taxa affiliated with *Firmicutes* increased with FD symptom burden, whereas taxa affiliated with *Bacteroidota* decreased. The other two studies (Zhong et al., 2016, 2017) suggested that more severe symptom responses to the standardized meal positively correlated with mucosal bacterial load. These two studies (Zhong et al., 2016, 2017)also explored the relationship between duodenal microbiota dysbiosis and the quality of life in FD patients and found a negative correlation exists between duodenal mucosal bacterial load and reported quality of life (Table 2).

3.7 Quality of the evidence

The quality of evidence was assessed by the Newcastle-Ottawa Scale for the six full-text articles, with two of the six articles scoring 7, one scoring 6, and three scoring 5, giving an overall moderately high level of article quality. Among the six full-text articles reviewed, all studies had clear definitions of the study and control populations. Regarding the study population, four studies (Tziatzios et al., 2021; Wauters et al., 2021; Zheng et al., 2022; Shanahan et al., 2023) explicitly diagnosed and included FD patients based on the Rome IV criteria; one study used the Rome III criteria; and only one study did not

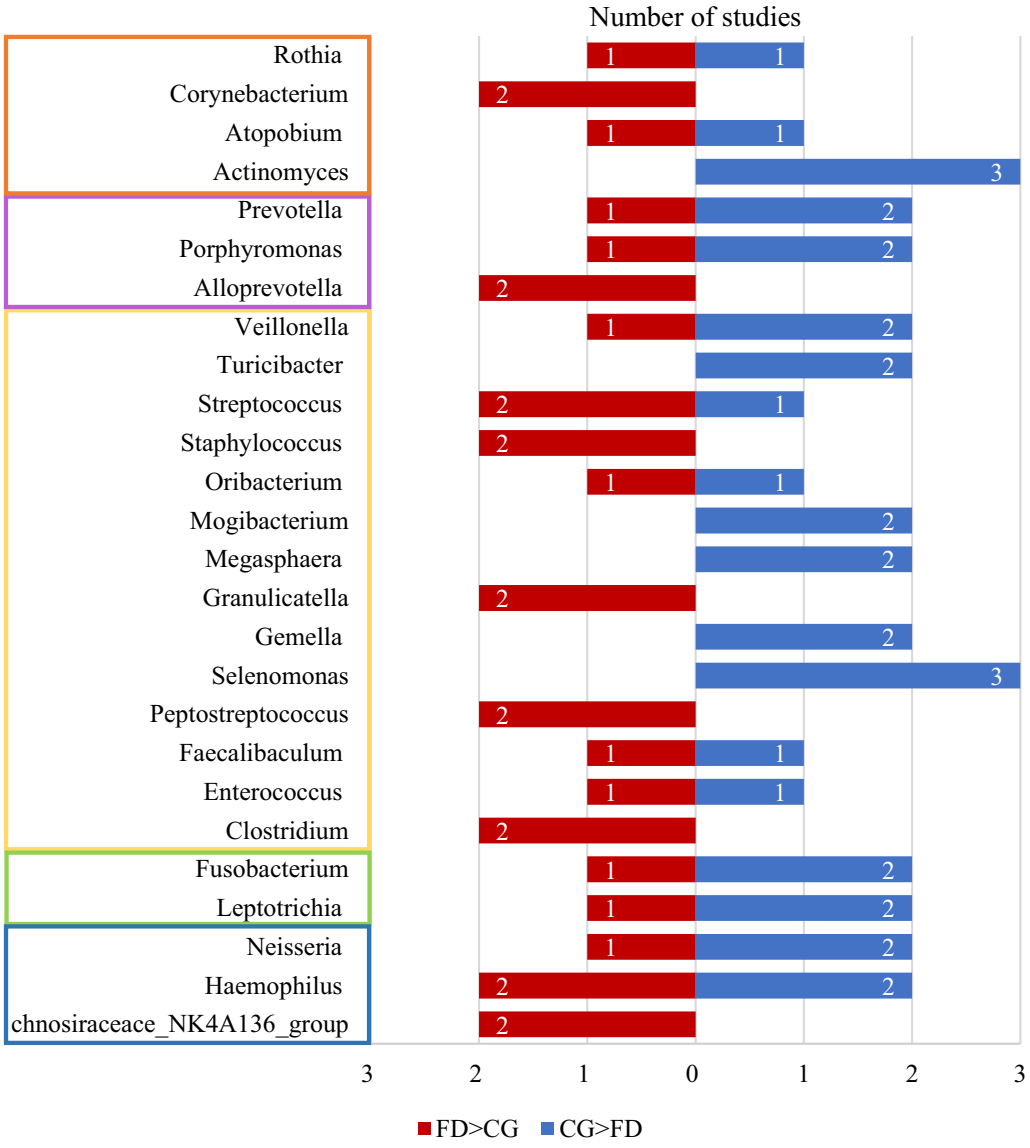

FIGURE 2
The number of studies reporting differences in bacterial genera between the FD group and the CG group across two or more studies. The blue bars represent the number of studies where the abundance of bacterial genera was higher in the CG group compared to the FD group, while the red bars indicate the number of studies where the abundance was higher in the FD group than in the CG group. Genera within the orange frame belong to the *Actinobacteria*, those in the purple frame to the *Bacteroidetes*, those in the yellow frame to the *Firmicutes*, those in the green frame to the *Fusobacteria*, and those in the blue frame to the *Proteobacteria*. CG, control group; FD, functional dyspepsia.

specify the exact diagnostic criteria for FD patients but established standards for endoscopic findings in FD patients. Additionally, five studies (Fukui et al., 2020; Tziatzios et al., 2021; Wauters et al., 2021; Zheng et al., 2022; Shanahan et al., 2023) mentioned that both FD and control groups underwent endoscopic examination to rule out gastric/duodenal mucosal abnormalities, lesions, or structural changes, with only one study (Zhong et al., 2017) not explicitly addressing this point. For the control groups, three studies (Fukui et al., 2020; Wauters et al., 2021; Zheng et al., 2022) used healthy controls, and the other three (Zhong et al., 2017; Tziatzios et al., 2021; Shanahan et al., 2023) used non-FD controls who did not exhibit FD symptoms and were confirmed to have no upper gastrointestinal mucosal lesions via endoscopy. All studies assessed whether baseline data, mainly age and gender, were statistically different between the two groups, with only one study (Shanahan et al., 2023) having a significantly younger group of FD patients than the control group. None of the trials reported whether blinding was used (Table 4).

4 Discussion

The duodenum is currently postulated to play an important role in the pathogenesis of FD. The increased permeability and microinflammation of the duodenal mucosa implicated in the etiology of FD are thought to be associated with alterations in the microbial community (Miwa et al., 2019). FD has been recognized as a disorder of brain-gut interaction, implicating the duodenal microbiota as a potentially significant yet understudied player in the brain-gut dialog. We present the first comprehensive review of existing studies on the duodenal microbiota in FD, attempting to elucidate the composition

TABLE 3 The taxonomic changes to duodenal microbiota.

Study	Trend	Changes at the taxonomic level					
		Phylum level	Class level	Order level	Family level	Genus level	Species level
Shanahan et al. (2023)	↑	TM7 *Proteobacteria* *Fusobacteria** *Bacteroidetes*	NR	NR	NR	*Fusobacterium**, *Alloprevotella*, *Acidovorax*, *Prevotella*, *Ralstonia*, *TM7x*, *Porphyromonas*, *Cloacibacterium*, *Diaphorobacter*, *Granulicatella*, *Neisseria*, *Staphylococcus*, *Parvimonas*, *Comamonas*, *Leptotrichia*, *Atopobium*, *Acinetobacter*, *Peptostreptococcus*, *Oribacterium*, *Methylophilus*, *Actinobacillus*	NR
	↓	*Firmicutes* *Actinobacteria*	NR	NR	NR	*Mogibacterium*, *Faecalibaculum*, *Actinomyces*, *Veillonella*, *Enterococcus*, *Solobacterium*, *Coriobacteriaceae*_UCG-002, *Haemophilus*, *Dubosiella*, *Stomatobaculum*, *Gemella*, *Megasphaera*, *Delftia*, *Allobaculum*, *Lactobacillus*, *Stenotrophomonas*, *Turicibacter*, *Rothia*, *Lachnospiraceae*_NK4A136_group, *Selenomonas*, *Streptococcus*, *Bulleidia*, *Helicobacter*	NR
	Dominant bacteria	NR	NR	NR	NR	NR	NR
Zheng et al. (2022)	↑	*Fusobacteria*	NR	NR	NR	*Alloprevotella**, *chnosiraceace*_NK4A136_group*, *Corynebacterium**, [*Eubacterium*]_nodatum_group*, *Faecalibaculum**, *Lachnoclostridium**, *Lautropia**, *Peptostreptococcus**, *Staphylococcus**, *Sutterella** LEfSe analysis: o_*Corynebacteriales**, c_*Clostridia**, o_*Clostridia*_vadinBB60_group*, o_*Sphingomonadales**, c_*Alphaproteobacteria**, o_*Oscillospirales**, f_*Sphingomonadaceae**, s_*Prevotella*_intermedia*, s_*Prevotella*_jejuni*, f_*Ruminococcaceae**, f_*Peptostreptococcaceae**, g_*Peptostreptococcus**, s_*Haemophilus*_influenzae*	NR
	↓	NR	NR	NR	NR	*Catonella** LEfSe analysis: g_*Johnsonella**, g_*Catonella**, g_*Selenomonas**	NR
	Dominant bacteria	*Firmicutes* *Bacteroidetes* *Proteobacteria*	NR	NR	NR	*Streptococcus*, *Actinobacillus*, *Neisseria*, *Fusobacterium*, *Prevotella*, *Haemophilus*, *Porphyromonas*	NR
Wauters et al. (2021)	↑	NR	NR	NR	NR	NR	NR
	↓	NR	NR	NR	NR	*Fusobacterium*, *Haemophilus*, *Neisseria**, *Porphyromonas**, *Selenomonas*	NR
	Dominant bacteria	NR	NR	NR	NR	NR	NR
Zhong et al. (2017)	↑	NR	NR	NR	Paraprevotellaceae	*Streptococcus*, *Carnobacterium*, *Corynebacterium*, *Gemella*	NR
	↓	NR	NR	NR	NR	*Actinomyces**, *Atopobium**, *Leptotrichia**, *Prevotella**, *Veillonella**, *Megasphaera*, *Neisseria*, *Porphyromonas*	NR
	Dominant bacteria	NR	NR	NR	NR	*Streptococcus*	NR
Fukui et al. (2020)	↑	*Firmicutes** *Actinobacteria*	NR	NR	NR	*Streptococcus**, *Veillonella*	NR
	↓	*Bacteroidetes* *Fusobacteria*	NR	NR	NR	*Oribacterium*, *Lactobacillus*, *Granulicatella*	NR
	Dominant bacteria	*Proteobacteri* *Bacteroidetes* *Firmicutes* *Fusobacteria* *Actinobacteria*	NR	NR	NR	NR	NR
Tziatzios et al. (2020)	↑	NR	NR	NR	NR	NR	*Escherichia coli, Klebsiella pneumonia, Acinetobacter baumannii, Serratia marscecens, Klebsiella spp, Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter cloacae*
	↓	NR	NR	NR	NR	NR	NR
	Dominant bacteria	NR	NR	NR	NR	NR	NR

(Continued)

TABLE 3 Continued

Study	Trend	Changes at the taxonomic level					
		Phylum level	Class level	Order level	Family level	Genus level	Species level
Zhong et al. (2016)	↑	NR	NR	NR	NR	NR	NR
	↓	NR	NR	NR	NR	*Actinomyces**.	NR
	Dominant bacteria	NR	NR	NR	NR	FD Group: *Prevotella*, *Streptococcus*, *Porphyromonas*	NR
Schooth et al. (2022)	↑	NR	NR	NR	NR	NR	*V. atypica*, *V. rogosae*
	↓	NR	NR	NR	NR	NR	*Veillonella ratti/cricetid*, *Veillonella parvula*
	Dominant bacteria	NR	NR	NR	NR	NR	NR
Schooth et al. (2021)	↑	NR	NR	NR	NR	*Enterococcus*, *Granulicatella*	NR
	↓	NR	NR	NR	NR	*Dialister*, *Fusobacterium*, *Gemella*, *Leptotrichia*, *Leuconostoc*, *Mogibacterium*	NR
	Dominant bacteria	NR	NR	NR	NR		NR

Legend:↑, higher relative to comparison group; ↓, = lower relative to comparison group; *, significant difference; NR, not reported.

and characteristics of the duodenal microbiota in FD patients and establish a correspondence between symptom production and disease pathogenesis. The results of this study showed that the diversity of the duodenal microbiota in FD patients was not significantly different from that of non-FD controls but tended to increase and that changes in the duodenal microbiota of FD patients correlated with patient symptom burden and affected patient quality of life. In FD patients, a number of duodenal microbiota have a relatively significant upward trend, and we consider this type of microorganism to be potentially harmful, including the phylum *Fusobacteria*, the genera *Alloprevotella*, *Corynebacterium*, *Peptostreptococcus*, *Staphylococcus*, *Clostridium*, *Streptococcus,* and others. Flora with a more pronounced downward trend in FD patients include the genera *Actinomyces*, *Gemella*, *Haemophilus*, *Megasphaera*, *Mogibacterium*, *Selenomonas*, and others, and we believe that there may be potentially beneficial bacteria for FD patients in these microbiotas. Furthermore, alterations in specific microbial taxonomic groups and their implications for the brain-gut axis interactions in patients with functional dyspepsia warrant attention.

4.1 Correlation of *Streptococcus* and *Prevotella* abundance with FD symptoms

The results of a Japanese study (Fukui et al., 2020) observed a significant increase in the abundance of *Firmicutes* in FD compared to healthy controls in all sites of the upper gut, and the evaluation of taxonomic changes of each genus in the *Firmicutes* revealed that only genus *Streptococcus* was significantly increased in all sites in the upper gut in FD compared to healthy controls, and this study also found a positive correlation between the relative abundance of genus *Streptococcus* and upper gastrointestinal symptoms in FD patients. This finding is consistent with another included Australian study (Shanahan et al., 2023), which also found that the relative abundance of the *Firmicutes* was significantly higher in FD patients and the relative abundance of taxa affiliated with the *Firmicutes* increased with FD symptom burden, and this positive correlation trend continued when the dominant genus of this phylum (i.e., *Streptococcus*) was examined. This suggests that *Streptococcus* spp. may be the main suspected genus closely associated with symptoms in FD patients. In contrast, the results of the study also showed that the relative abundance of *Prevotella* spp. was negatively correlated with the severity of FD symptom burden. In addition, both experiments (Zhong et al., 2017; Shanahan et al., 2023) indicated that the relative abundance of genus *Streptococcus* and genus *Prevotella* in FD patients was inversely related.

The genera *Streptococcus* and *Prevotella* are the main bacteria of the upper gastrointestinal tract. Interestingly, one study found that the changes in the relative abundance of the genera *Streptococcus* and *Prevotella* are thought to correlate significantly with changes in duodenal pH, and the two genera show an opposite trend in correlation with duodenal pH, with *Streptococcus* predominantly exhibiting a positive correlation and *Prevotella* demonstrating a negative association with the changes in duodenal pH (Seekatz et al., 2019). This seems to confirm that the genera *Streptococcus* and *Prevotella* are inversely characterized in the duodenum of FD patients and that changes in the relative abundance of the two genera correlate with the changes in duodenal pH in FD patients. There is a strong correlation and an interactive relationship between the duodenal microbiota and the pH of the duodenal contents. Large fluctuations in environmental pH may select genera such as *Streptococcus*, which can also regulate intracellular pH (Abuhelwa et al., 2017; Seekatz et al., 2019).

The growth of microorganisms, including the genera *Streptococcus* and *Prevotella*, can lead to pH changes by mediating the metabolism of short-chain fatty acids (SCFA; Zoetendal et al., 2012). *Streptococcus* has been demonstrated to elevate concentrations of lactate and butyrate within the duodenum, consequently lowering the pH of the duodenal environment (Zoetendal et al., 2012). *In vitro* studies (Chen et al., 2017) have corroborated that *Prevotella* exhibits a heightened capacity for fiber utilization and serves as an efficient producer of propionate from arabinoxylan and oligofructose. The enhanced capacity for fiber degradation makes the genus *Prevotella* potentially beneficial for glucose homeostasis and host metabolism (Tett et al., 2021). Furthermore, *Prevotella* has been shown to augment bile acid metabolism (Péan et al., 2020), thereby exerting influence on the duodenal pH. Alterations in duodenal pH are posited to contribute to the pathogenesis of FD, with FD patients demonstrating evidence of

TABLE 4 Quality of the included studies by the NOS.

Study	Selection				Comparability		Exposure			Score
	Is the case definition adequate?	Representativeness of the cases	Selection of controls	Definition of controls	Comparability of baseline characteristic 1 (age)	Comparability of baseline characteristic 2 (sex)	Ascertainment of exposure	The same method of ascertainment for cases and controls	Non-response rate	
Shanahan et al. (2023)	*	*	*	*	*	*	NR	NR	*	7*
Zheng et al. (2022)	*	*	NR	*	*	*	NR	NR	*	6*
Wauters et al. (2021)	*	*	*	*	*	*	NR	NR	*	7*
Zhong et al. (2017)	*	NR	NR	*	*	*	NR	NR	*	5*
Fukui et al. (2020)	*	*	*	*	*	*	NR	NR	*	7*
Tziatzios et al. (2020)	*	*	*	*	*	*	NR	NR	*	5*

*, indicates the quality of the studies; NR, not reported.

increased duodenal acid exposure (Bratten and Jones, 2009). Additionally, the sensitivity of the duodenal mucosa to acid is thought to be associated with FD, and acid exposure at the duodenal site in FD patients is believed to worsen FD symptoms (Bratten and Jones, 2009; Ishii et al., 2010).

Based on the included studies, we observed that the duodenal microbiota of FD patients exhibited negatively correlated changes in the genera *Streptococcus* and *Prevotella*. These variations in the two genera may be closely associated with the onset of FD symptoms. The change in duodenal pH may play a significant role in this process.

4.2 Staphylococcal enterotoxin may induce duodenal immune inflammation leading to functional dyspepsia

According to the results from the included studies, the genus *Staphylococcus* exhibits a notably increased trend in the duodenal microbiota of FD patients. Furthermore, one study (Tziatzios et al., 2021) indicated that the proportion of *Staphylococcus aureus* in small intestinal aspirates is higher in FD patients with small intestinal bacterial overgrowth (SIBO) compared to non-FD patients. Duodenal inflammation is considered a novel target in the pathogenesis of FD, and microinflammation in the form of local immune cell infiltration plays an important role in the pathogenesis of FD (du et al., 2018; Wauters et al., 2020b). The increase in the genus *Staphylococcus* presence observed in this study may provide a new avenue of investigation into the mechanisms underlying FD. Previous studies have demonstrated a correlation between the overgrowth of *Staphylococcus* species and inflammatory bowel disease (IBD), which is attributed to the inflammation induced by staphylococcal superantigens (Jun et al., 2003; Collado et al., 2008). *Staphylococcus aureus* is a member of the genus *Staphylococcus*, and the staphylococcal enterotoxin (SE) produced by *S. aureus* is considered to be the causative agent of human food poisoning as well as a potent immune superantigen that readily crosses the intact intestinal epithelium, inducing the proliferation of activated T cells and the release of large amounts of pro-inflammatory cytokines (White et al., 1989; Moretó and Pérez-Bosque, 2009; Principato and Qian, 2014). Staphylococcal enterotoxin A (SEA) produced by *Staphylococcus aureus* has been shown to elicit a rapid immunological response in the stomach and duodenum of rats, and the duodenum displayed a greater leukocytic response than the stomach to SEA, while studies have shown that SEA does not induce gastrointestinal mucous membrane damage encompassing edema, cytolysis, tissue sloughing, luminal necrotic tissue, or alternations in epithelial mitotic (Beery et al., 1984). Liu et al. (2022) reported that SEA significantly upregulated the expression of NLRP3 inflammasome-associated proteins and downregulated the expression of tight junction (TJ) proteins, which triggered the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) signaling pathways in jejunal tissue, inducing intestinal barrier dysfunction and small bowel injury in mice. Staphylococcal enterotoxin B (SEB) was found to evoke significant increases in myeloperoxidase (MPO), macrophage infiltration, T-cell activation, and perturbed epithelialion transport and cause low-grade inflammation of the colonic lumen (Jun et al., 2003). Studies (Moretó and Pérez-Bosque, 2009; Pérez-Bosque and Miquel, 2010) have also shown that SEB exposure decreases the expression of mucosal tight-junction and adherent-junction proteins, leading to increased mucosal permeability and intestinal secretion.

In conclusion, although the definitive studies elucidating the mechanistic role of duodenal *Staphylococcus aureus* in FD are lacking, SE can damage the intestinal mucosal barrier, activate duodenal immunity, and induce an inflammatory response, and its potential inducing effect on the pathogenesis of FD may be worthy of attention.

4.3 *Selenomonas* as a potential "beneficial bacterium" in the duodenum of functional dyspepsia patients

Several studies have found (Ricke et al., 1996; Jordan and Kurt, 2010) that *Selenomonas* have multiple carbon flow routes for carbohydrate catabolism and ATP generation and that catalytically efficientβ-D-xylosidase from *Selenomonas* can hydrolyze cellulose, and *Selenomonas* is thought to promote nutrient digestibility. In addition, recent research has found that (Kim et al., 2022) treatment during the asthma sensitization period with *Selenomonas sputigena* resulted in a significant reduction in airway hyperresponsiveness and a decrease in inflammatory cells present in bronchoalveolar lavage fluid, suggesting that the *Selenomonas* may be able to mitigate the severity of asthmatic phenotypes. In terms of gastrointestinal diseases, the researchers found that the symptoms of the patients with functional constipation (FC) and comorbid depression and anxiety were improved after fecal microbiota transplantation. An increase in the abundance of *Selenomonas* within FC patients' gut microbiota has been noted, leading to the hypothesis that changes in the prevalence of this bacterial genus may be involved in the pathogenesis of constipation presenting with psychiatric symptoms (Yang et al., 2023).

The above results suggest that *Selenomonas* may have a potential "protective" effect on the human body. Current research on *Selenomonas* predominantly focuses on ruminant animals, with a relative paucity of studies elucidating its mechanisms of action in humans. However, in the present study, the genus *Selenomonas* exhibited a relatively significant downward trend in the duodenal microbiota of FD patients. Although it is unclear whether this is the cause or the result of FD, considering the characteristics of this genus, *Selenomonas* may represent a potentially beneficial bacterial genus for FD patients.

4.4 The role of duodenal microbial dysbiosis in gut–brain interactions in functional dyspepsia

Functional GI disorders are classified by the Rome IV criteria as disorders of gut–brain interaction with contributions of both altered brain processing and luminal changes, including dysbiosis (Drossman and William, 2016). Emerging data increasingly points to the duodenum as a key integrator in the generation of dyspepsia symptoms. In patients with dyspepsia, irritation of the duodenum is thought to cause disturbances in duodenal-gastric feedback, leading to gastric motility dysfunction, which in turn causes dyspeptic symptoms (Walker and Nicholas, 2017; Miwa et al., 2019). A study from Leuven (Cirillo et al., 2015) demonstrated a correlation between duodenal eosinophils or mast cells and the Ca^{+} transient amplitude under high-K^{+} depolarization or electrical pulses, suggesting a role for duodenal inflammation in FD neuronal signal transmission.

Current evidence (Fukui et al., 2020) suggests that dysbiosis of the duodenal MAM is considered to play a pivotal role in the development of symptoms in FD patients. The results of a systematic review and meta-analysis (Gurusamy et al., 2021b) further corroborate a link between FD and small intestinal bacterial overgrowth. Additionally, relevant preclinical studies (de Palma et al., 2015) have indicated that stress can induce dysregulation of the gut microbiota, thereby impacting the function and behavior of the central nervous system. Thus, consideration must be given to the potential stimulation of the duodenal mucosa and its neuronal signal transmission by duodenal microbiota and their metabolic products, which may consequently contribute to the pathogenesis of FD through gastrointestinal dysmotility.

The composition and abundance fluctuations of the gut microbiota have been associated with compromised mucosal surface integrity (Zhou et al., 2022). In FD patients, the duodenal mucosa exhibits compromised mucosal integrity and increased permeability, which is believed to permit luminal triggers to initiate local and systemic immune cascades, leading to alterations in neuronal signal transduction and the consequent manifestation of dyspeptic symptoms (Nojkov et al., 2020; Wauters et al., 2020a). Enterotoxins released by *Staphylococcus* are recognized to disrupt small intestinal mucosal barrier function, invade the intestinal mucosa, and elicit immune and inflammatory responses. Genus *Alloprevotella* has also been identified as a key bacterium capable of inducing colonic mucosal damage (Wang et al., 2021). Studies by Zhao et al. (2021) have demonstrated that sodium caprylate (SC) treatment increased the populations of *Prevotella_9* in the ileum and *Lachnoclostridium* and *Roseburia* in the colon but decreased the abundances of *Streptococcus* and *Enterococcus* in the ileum and *Lactobacillus* and *Clostridium_sensu_stricto_1* in the colon, thereby ameliorating intestinal barrier function and maintaining gut health.

The gut microbiota exerts a pivotal influence on the nervous, neuroendocrine, and metabolic systems, producing microbial metabolites, signaling molecules, and hormones with potential implications for alterations in brain signaling (Cowan et al., 2020; Vanuytsel et al., 2023). Short-chain fatty acids (SCFAs) represent metabolites of gastrointestinal microbial communities and are often considered key mediators of communication between the central nervous system and the gut. SCFAs can induce the secretion of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), γ-aminobutyric acid (GABA), and other hormones capable of transmitting stimuli to the central nervous system via the circulatory system or the vagus nerve pathway (Silva et al., 2020). SCFAs are primarily produced by bacteria belonging to genera such as *Prevotella*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*, utilizing dietary fibers, resistant starch, oligosaccharides, and other intestinal compounds as substrates (Markowiak-Kopeć and Katarzyna, 2020). Among the taxa exhibiting a declining trend in this study, genus *Prevotella* demonstrates robust SCFA production capability and high fiber utilization efficiency, serving as proficient propionate producers from arabinoxylan and fructooligosaccharides (Chen et al., 2017). Furthermore, studies (Zhao et al., 2018) have revealed positive correlations between the increased abundance of genera *Prevotella*, *Lactobacillus*, and *Alistipes* and concentrations of saturated long-chain fatty acids (SLCFAs) in the gut, where elevated SLCFA levels promote intestinal smooth muscle contraction, leading to increased gut motility; however, the precise mechanisms through which SLCFAs enhance gastrointestinal motility remain unclear. The gastrointestinal microbiota

also modulates various facets of the gut–brain axis (GBA) through serotonin metabolism. Serotonin, derived solely from tryptophan, serves as a critical monoaminergic neurotransmitter involved in central nervous transmission and intestinal physiological functions (Gao et al., 2020). Numerous bacterial strains, including those from *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Escherichia coli*, and *Klebsiella* genera, have been documented to possess the capability of serotonin synthesis through the expression of tryptophan synthetase (O'Mahony et al., 2015).

Psychosomatic comorbidities play a significant role in the generation of symptoms in functional dyspepsia (Ford et al., 2020). Longitudinal studies examining bidirectional effects between the gut and the brain (Koloski et al., 2012, 2016) have revealed that individuals with functional dyspepsia at baseline are more prone to experiencing anxiety or depression during follow-up compared to those without functional dyspepsia, while individuals with anxiety or depression at baseline are more likely to develop functional dyspepsia than those without anxiety or depression. The gut microbiota can engage in bottom-up signaling through the production of metabolites, interacting with the central nervous system via neural pathways or by crossing the intestinal barrier, and modulating neurotransmitter levels, thereby influencing mental wellbeing (Evrensel and Mehmet-Emin, 2015; Barandouzi et al., 2020). In this study, bacterial genera such as *Corynebacterium*, *Clostridium*, and *Streptococcus*, which showed a significant upward trend in two or more studies, exhibiting significant increases in abundance in the duodenum of FD patients, were also found to significantly increase in the gut microbiota of individuals with depression. Conversely, bacterial genera such as *Lactobacillus* and *Prevotella*, which were found to have decreased relative abundance in two or more included studies, were also found to significantly decrease the gut microbiota of individuals with depression (Barandouzi et al., 2020; Park et al., 2023). Furthermore, ingestion of lactobacilli is believed to regulate central GABA receptor expression via the vagus nerve and reduce stress-induced corticosterone as well as anxiety and depression-related behaviors (Bravo et al., 2011).

In summary, the duodenal microbiota plays a role in the gut-brain axis in FD patients. The duodenal microbiota may influence the crucial duodenal functional status implicated in FD pathogenesis through endocrine, neural signaling, and immune-inflammatory responses. Additionally, it participates in the bidirectional brain-gut interactions in FD patients, thus constituting a dysregulated pathogenesis of FD based on the microbiota-gut–brain axis, ultimately leading to the onset of FD (Figure 3).

5 Conclusion and outlook

In summary, while the overall diversity of the duodenal microbiota does not significantly differ between FD patients and healthy individuals, certain taxa—including *Selenomonas*, *Streptococcus*, *Prevotella*, and *Staphylococcus*—exhibit notable variations that warrant further investigation. Our study suggests that alterations in the duodenal microbiota may be crucial for the onset and manifestation of FD symptoms. The microbial species significantly altered in this study are known to influence the bidirectional interactions between the brain and gut in FD patients by modulating endocrine, neural signaling, and immune-inflammatory responses, thereby contributing to FD pathogenesis. This highlights the significant role of the duodenal microbiota in the brain-gut interactions of FD patients.

The duodenum is increasingly recognized as pivotal in FD pathogenesis, and the gut microbiota's role has garnered considerable attention. However, due to technological limitations in previous studies, research on the duodenal microbiota of FD

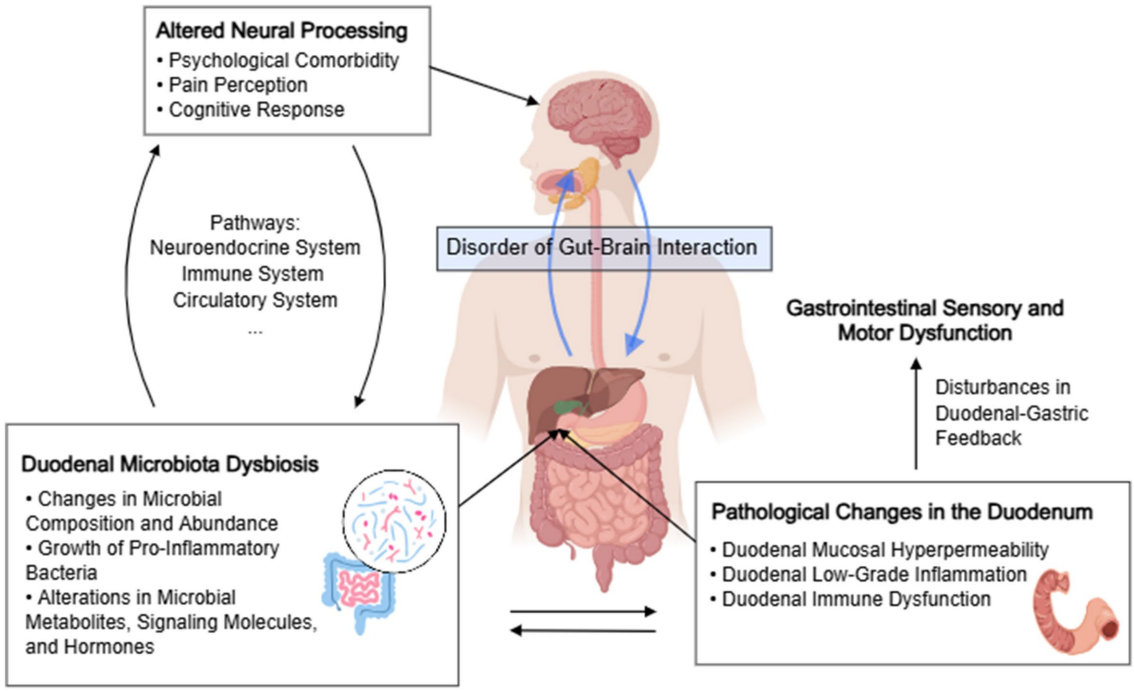

FIGURE 3
The role of duodenal microbial dysbiosis in disorders of gut–brain interactions in functional dyspepsia. The chart was created by MedPeer.

patients remains insufficient. The limited number of studies, small sample sizes, and inconsistent quality of the articles result in contradictory findings, insufficient data for meta-analyses, and challenges in standardized data processing. Consequently, only a general trend of dysbiosis in the duodenal microbiota of FD patients can be discerned, with insufficient evidence to pinpoint key microbial communities.

Future research urgently needs to prioritize higher-quality, larger sample-size studies across diverse populations. Efforts should focus on eliminating bias and incorporating findings from multi-omics studies to further elucidate the characteristics and pathogenic mechanisms of the duodenal microbiota in FD patients. Additionally, attention should be given to confounding factors affecting duodenal microbiota changes in FD patients, such as dietary habits and proton pump inhibitor use. These in-depth studies could provide potential strategic references for the prevention and treatment of FD.

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.

Author contributions

XZ: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. LC: Data curation, Visualization, Writing – review & editing. TZ: Conceptualization, Methodology, Writing – review & editing. RG: Formal analysis, Software, Validation, Writing – review & editing. QW: Data curation, Project administration, Writing – review & editing. ZZ: Methodology, Supervision, Writing – review & editing. MY: Data curation, Writing – review & editing. WW: Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. XS: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Role of inflammatory cytokines and the gut microbiome in vascular dementia: insights from Mendelian randomization analysis

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Background: Both inflammatory cytokines and the gut microbiome are susceptibility factors for vascular dementia (VaD). The trends in the overall changes in the dynamics of inflammatory cytokines and in the composition of the gut microbiome are influenced by a variety of factors, making it difficult to fully explain the different effects of both on the different subtypes of VaD. Therefore, this Mendelian randomization (MR) study identified the inflammatory cytokines and gut microbiome members that influence the risk of developing VaD and their causal effects, and investigated whether inflammatory cytokines are gut microbiome mediators affecting VaD.

Methods: We obtained pooled genome-wide association study (GWAS) data for 196 gut microbiota and 41 inflammatory cytokines and used GWAS data for six VaD subtypes, namely, VaD (mixed), VaD (multiple infarctions), VaD (other), VaD (subcortical), VaD (sudden onset), and VaD (undefined). We used the inverse-variance weighted (IVW) method as the primary MR analysis method. We conducted sensitivity analyses and reverse MR analyses to examine reverse causal associations, enhancing the reliability and stability of the conclusions. Finally, we used multivariable MR (MVMR) analysis to assess the direct causal effects of inflammatory cytokines and the gut microbiome on the risk of VaD, and performed mediation MR analysis to explore whether inflammatory factors were potential mediators.

Results: Our two-sample MR study revealed relationships between the risk of six VaD subtypes and inflammatory cytokines and the gut microbiota: 7 inflammatory cytokines and 14 gut microbiota constituents were positively correlated with increased VaD subtype risk, while 2 inflammatory cytokines and 11 gut microbiota constituents were negatively correlated with decreased VaD subtype risk. After Bonferroni correction, interleukin-18 was correlated with an increased risk of VaD (multiple infarctions); macrophage migration inhibitory factor was correlated with an increased risk of VaD (sudden onset); interleukin-4 was correlated with a decreased risk of VaD (other); *Ruminiclostridium 6* and *Bacillales* were positively and negatively correlated with the risk of VaD (undefined), respectively; *Negativicutes* and *Selenomonadales* were correlated with a decreased risk of VaD (mixed); and *Melainabacteria* was correlated with an increased risk of VaD (multiple infarctions). Sensitivity analyses revealed

no multilevel effects or heterogeneity and no inverse causality between VaD and inflammatory cytokines or the gut microbiota. The MVMR results further confirmed that the causal effects of *Negativicutes*, *Selenomonadales*, and *Melainabacteria* on VaD remain significant. Mediation MR analysis showed that inflammatory cytokines were not potential mediators.

Conclusion: This study helps us to better understand the pathological mechanisms of VaD and suggests the potential value of targeting increases or decreases in inflammatory cytokines and gut microbiome members for VaD prevention and intervention.

KEYWORDS

Mendelian randomization study, vascular dementia, inflammatory cytokines, gut microbiome, genome-wide association study

1 Introduction

Vasculsar dementia (VaD) is defined as cognitive dysfunction and neurological dysfunction caused by cerebrovascular disease and/or reduced cerebral blood flow (O'Brien and Thomas, 2015; Song et al., 2023). VaD is the second most common form of dementia and accounts for approximately 20% of all dementia cases. An individual's risk of VaD doubles approximately every 5.3 years (Wolters and Ikram, 2019; Zhang et al., 2024). VaD affects the ability of patients to live independently and creates a significant socioeconomic burden. With increasing life expectancy and a globally aging population, there is an urgent need for an in-depth study of the pathogenesis of VaD to provide a theoretical basis for new therapeutic approaches.

Neuroinflammation plays a crucial role in the pathophysiological process of VaD onset and progression (Tian et al., 2022). When the expression of inflammatory cytokines is elevated *in vivo*, multiple neuropathological pathways for the onset and development of VaD are initiated (Gao et al., 2023; Liu et al., 2023a), and the release of inflammatory mediators in combination with factors such as oxidative stress increases the permeability of the blood–brain barrier (BBB), allowing immune cells to reach the brain (Zhu et al., 2018; Tashiro et al., 2023; Wang et al., 2023a). Microglia are activated in the brain, triggering processes such as oxidative stress, synaptic disruption, and inhibition of neurogenesis, exacerbating the disruption of the BBB and ultimately leading to neuronal destruction and impaired brain function (Tashiro et al., 2023; Liu et al., 2023a; Wang et al., 2023a). Compared to those of controls, autopsy studies of brain tissue from VaD patients who had undergone *in vivo* serum testing revealed increased hippocampal tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), transforming growth factor beta (TGF-β), inducible nitric oxide synthase, interleukin-23, and interleukin-17 *in vivo* (Belkhelfa et al., 2018; Dubenko et al., 2021). Gao et al. reduced TNF-α, chemokine ligand 9, interleukin-6 (IL-6), and antiangiogenic factors in the cerebrospinal fluid and brain tissues of rats, which attenuated neuronal damage, preserved the integrity of cerebral white matter in VaD rats, and ultimately restored VaD rat cognitive function (Gao et al., 2023). Although the role of inflammatory cytokines in VaD has been partially elucidated, the inflammatory process changes over time, so we need to understand the overall trend of the dynamics of inflammatory cytokines in VaD and its subtypes while analyzing novel inflammatory cytokines critical for VaD and determining their causal relationships.

There is a network of bidirectional communication between the central nervous system and the gut, with the gut microbiome acting as a key node in the communication between the networks (Fung, 2020). Alterations and imbalances in its composition and metabolites (a decrease in dominant genera and an increase in potentially pathogenic bacteria) induce increased intestinal barrier permeability and immune activation, triggering systemic inflammation, which in turn may compromise the BBB, promote apoptosis and neurological damage, and ultimately lead to the development of cognitively dysfunctional diseases (Fung et al., 2017; Angoorani et al., 2022; Pei et al., 2023). The possibility that the gut microbiome is a major risk factor for VaD susceptibility has been confirmed by several studies (Alkasir et al., 2017). Liu et al. reduced neuronal apoptosis in VaD mice by accelerating the rate of butyric acid production in the feces and brain and the content of butyrate in the brain by administering VaD mice a 6-week gavage of *Clostridium butyricum* (Liu et al., 2015). Treatment with probiotics in mice with common carotid artery obstruction not only improved the gut microbiome imbalance but also significantly reduced the number of damaged neuronal cells and apoptotic cells in the hippocampus, ultimately improving spatial learning and memory abilities (Rahmati et al., 2019). However, because of the shortage of randomized controlled trials (RCTs), the existence of mostly basic experimental research, and the presence of limitations such as the difficulty of controlling for confounding factors and the unclear temporal sequence of causal events, evidence for the role of the gut microbiome in VaD and its subtypes is needed.

Mendelian randomization is a method that uses single-nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to infer the causal relationship between exposure and outcome after removing the effects of confounders (Li et al., 2023b). As Mendelian randomization studies can reduce confounding factors, limiting our ability to make causal inferences and interpret the results of observational studies (confounding variables, reverse causal associations, regression dilution bias) and RCTs (representativeness, feasibility, and ethical issues), Mendelian randomization studies have become more common for exploring the underlying biological mechanisms of disease onset, finding new therapeutic targets, and detecting causality between exposures and disease risk, among other goals (Amin et al., 2023; Gong et al., 2023). To date, no animal model or clinical trial has

revealed the relationship between the gut microbiome or inflammatory cytokines and the pathogenesis of the various subtypes of VaD. Mendelian randomization studies provide us with an accurate and stable method to do so. Therefore, the present study aimed to investigate the association between inflammatory cytokines and the gut microbiome on the pathogenesis of different subtypes of VaD by using Mendelian randomization based on the existing research and to provide another perspective with new evidence on the etiology and pathological mechanisms of different subtypes of VaD. In addition, this study used mediation MR to determine whether inflammatory cytokines are a mediating factor for the gut microbiome to affect VaD.

2 Materials and methods

2.1 Study design

The overall design of this study is shown in Figure 1. In this study, the TwoSampleMR (version 0.5.6) and MR-PRESSO (version 1.0) packages in R (version 4.2.1) were used for analyses.

2.2 Data sources

Inflammatory cytokine dataset: Inflammatory cytokine summary data were obtained from a meta-analysis of summary statistics of inflammatory cytokine GWASs published by the University of Bristol.[1] The data contained information on 41 inflammatory cytokines (Ahola-Olli et al., 2017). This is the most recent, largest, and most commonly used dataset.

Gut microbiota dataset: The gut microbiota summary data were obtained from the MiBioGen consortium,[2] which contains 211 taxa (35 families, 20 orders, 16 phyla, 9 classes, and 131 genera) (Kurilshikov et al., 2021). This dataset is the most widely used dataset of a single gut microbiota that we are aware of.

Vascular dementia dataset: Data for six VaD subtypes were obtained from FinnGen Research.[3] The Finnish database has a total sample of 377,277 (210,870 females and 166,407 males) with 20,175,454 variants and 2,272 available phenotypes (Kurki et al., 2023). This dataset is the most detailed and largest sample size dataset available for VaD classification. The six subtypes of VaD selected for this study were VaD (undefined), VaD (subcortical), VaD (other), VaD (mixed), VaD (multiple infarctions), and VaD (sudden onset). Except for the diagnostic criteria for VaD (multiple infarctions), which were based on the ICD-9 and ICD-10, the diagnostic criteria for other vascular dementias were based on the ICD-10.

To prevent population stratification bias from confounding the study results, all SNPs and their accompanying summary data in this study were limited to people of European ancestry. In addition, sample-specific information on the inflammatory cytokines, gut microbiota, and VaD datasets used in this study is presented in Table 1.

2.3 Selection of instrumental variables

In two-sample MR analysis, the choice of IVs largely affects the reliability of causal relationships. First, we eliminated 3 unknown families and 12 unknown genera in the gut microbiota. Moreover, to identify SNPs that were highly predictive of inflammatory cytokine and gut microbiome levels, we initially used a genome-wide significance threshold of $p < 5 \times 10^{-8}$. However, the number of SNPs screened was limited; for inflammatory cytokines, we used a locus-wide significance threshold ($p < 5 \times 10^{-6}$) to rescreen for SNPs associated with exposure; for the gut microbiota, we used a locus-wide significance threshold ($p < 1 \times 10^{-5}$) to rescreen for exposure-related SNPs. The linkage disequilibrium correlation coefficient was set to $r^2 < 0.001$, and the clustering distance was set to 10,000 kb to ensure that there was no linkage disequilibrium among the included IVs. To prevent potential multiple effects, we used a PhenoScanner V2[4] to further exclude IVs associated with confounding factors or risk factors for VaD. Finally, we calculated the F statistic for each SNP and each group of IVs using the formula used to calculate the F statistic in the study of Liu et al. (2023b). Since SNPs with F statistics less than 10 did not have sufficient validity, we deleted them.

2.4 Statistical analysis

Since the statistical power of the inverse-variance weighted (IVW) method is significantly greater than that of other MR methods, IVW was used as the main method in this study (Lin et al., 2021). In addition, MR–Egger, the weighted median, and the weighted mode served as supplements to the IVW method to provide more stable and accurate results. When more than 50% of the SNPs are invalid instruments, the weighted median and weighted mode methods are more robust than the IVW and MR–Egger methods are (Li et al., 2023a). In this study, the MR method was used to follow a consistent beta value direction. The results of the IVW method were significant, but the results of the other methods were not significant. Moreover, on the premise that no pleiotropy or heterogeneity is found, as long as the beta of other methods is consistent, this can also be regarded as a positive result (Wang et al., 2023b). Finally, the Bonferroni-corrected p-value ($p < 0.05/N$, N = number of test methods) was considered to indicate statistical significance (Wu et al., 2020), and the Bonferroni-corrected p-value in this study was 0.0125. The results with p-values between 0.0125 and 0.05 are suggestive results.

2.5 Sensitivity analysis

First, Cochrane's Q test was used to assess heterogeneity among the IVs. If the test results showed heterogeneity ($p < 0.05$), the random effects IVW model was used; if not, the fixed-effects IVW model was used (Barili et al., 2018). Next, we used the MR–Egger intercept and Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) test to measure the level of pleiotropy. A p-value <0.05 in the MR–Egger intercept and MR-PRESSO methods

1 https://data.bris.ac.uk/data/dataset/3g3i5smgghp0s2uvm1doflkx9x
2 https://mibiogen.gcc.rug.nl/menu/main/home/
3 https://r9.finngen.fi
4 www.phenoscanner.medschl.cam.ac.uk

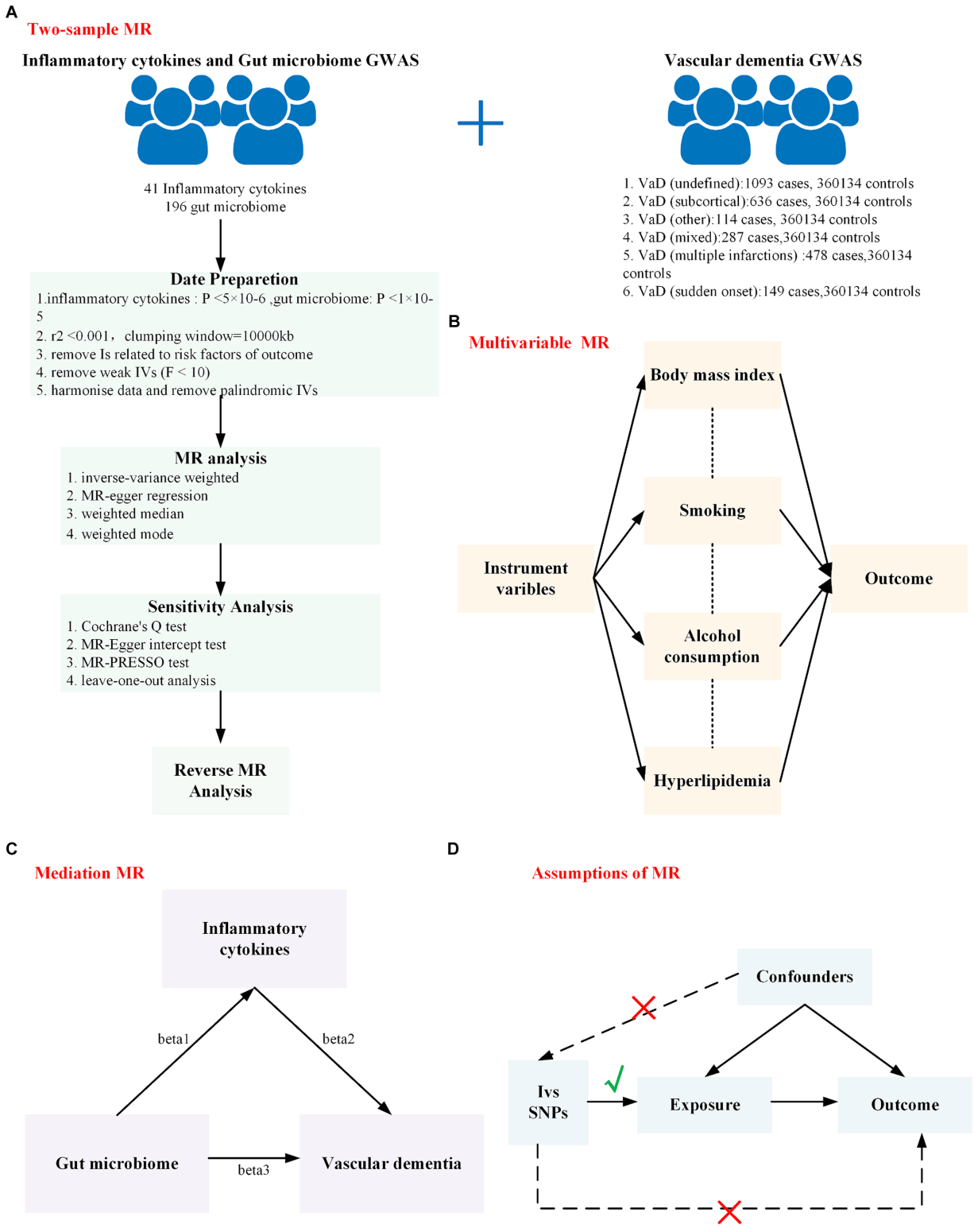

FIGURE 1
The overall design of this study is shown in this figure. **(A)** Workflow of the two-sample Mendelian randomization study; **(B)** Workflow of the multivariable Mendelian randomization study; **(C)** Workflow of the mediation Mendelian randomization study; **(D)** The assumption of Mendelian randomization: (1) There is a strong correlation between genetic instrumental variables and exposure factors. (2) Instrumental variables are independent of any confounders that may affect the exposure and outcomes. (3) Genetic variation can influence the outcome only through exposure factors and not through other factors. MR, Mendelian randomization; GWAS, genome-wide association study.

indicated the occurrence of horizontal pleiotropy, which needed to be removed before MR analysis. The number of MR-PRESSO cycles was 3,000. Moreover, we conducted leave-one-out sensitivity analyses to analyze the significance of the results and to determine whether there were abnormal IVs that significantly affected the causal effect estimate.

TABLE 1 Details of genome-wide association study data.

Phenotypes	Date type	Population	Consortium	Sample size (case/control)
Inflammatory cytokine	Exposure	Europeans	University of Bristol	8,293
Gut microbiota	Exposure	Europeans	MiBioGen	18,340
VaD (undefined)	Outcome	Europeans	FinnGen Biobank	1,093/360,143
VaD (subcortical)	Outcome	Europeans	FinnGen Biobank	636/360,143
VaD (other)	Outcome	Europeans	FinnGen Biobank	114/360,143
VaD (mixed)	Outcome	Europeans	FinnGen Biobank	287/360,143
VaD (multiple infarctions)	Outcome	Europeans	FinnGen Biobank	478/360,143
VaD (sudden onset)	Outcome	Europeans	FinnGen Biobank	149/360,143

VaD, vascular dementia.

TABLE 2 Confounders genome-wide association study details.

Phenotypes	Date type	Population	Consortium	Sample size (case/control)	GWAS ID
Body mass index	Confounder	Europeans	UK Biobank	120,286	ieu-a-1089
Alcohol consumption	Confounder	Europeans	UK Biobank	112,117	ieu-a-1283
Smoking/smokers in household	Confounder	Europeans	MRC-IEU	425,516	ukb-b-960
Hyperlipidemia	Confounder	Europeans	MRC-IEU	3,439/459,571	ukb-b-17462

GWAS, genome-wide association study.

2.6 Reverse MR analysis

Finally, a reverse Mendelian randomization analysis was performed to explore the presence of reverse causality. The dataset, correlation methods, and parameters for the reverse MR analysis were the same as those for the forward MR analysis, but with VaD as the exposure and inflammatory cytokines and gut microbiota as the outcome.

2.7 Multivariable MR analysis

As the incidence of VaD may be influenced by body mass index (BMI), smoking/smokers in the household, alcohol consumption, and hyperlipidemia (Wolters and Ikram, 2019), two-sample MR may not reflect the direct effects of inflammatory cytokines and the gut microbiota on the incidence of VaD. Therefore, we used multivariable MR (MVMR) analysis to clarify whether the significant effects of the significant inflammatory cytokines and gut microbiota on VaD in the results were direct or indirect effects and whether they were driven by potential confounding factors (Burgess and Thompson, 2015). In addition, pleiotropy was assessed using the Egger regression line, with $p < 0.05$ indicating the presence of pleiotropy. The Cochrane Q test was used to assess heterogeneity. The test results showed heterogeneity ($p < 0.05$) (Sanderson et al., 2019). The GWAS data for BMI, smoking/smokers in the household, alcohol consumption, and hyperlipidemia are shown in Table 2.

2.8 Mediation analysis

We performed a mediated MR analysis using a two-step MR approach. First, we calculated the causal effect of the gut microbiota on inflammatory cytokines (beta1); then, we calculated the causal effect of inflammatory cytokines on VaD (beta2). We previously calculated the causal effect of the gut microbiota on VaD (beta3). We used beta3 as the total effect of the gut microbiota on VaD (Figure 1C), beta1 × beta2 as the mediating effect of the gut microbiota on VaD, beta3 − (beta1 × beta2) as the direct effect of the gut microbiota on VaD, and beta1 × beta2/beta3 as the proportion of the mediating effect in the causal relationship (Ahola-Olli et al., 2017).

2.9 Ethical approval

This study was conducted by using previously published, publicly available large-scale GWAS summary datasets. The ethics committee approved the data collection, and all participants provided written informed consent for the corresponding original GWAS.

3 Results

3.1 The two-sample MR analysis

3.1.1 Selection of instrumental variables

IVs were screened according to the conditions described in the Methods section. All SNPs that were included in the analysis of inflammatory cytokines and VaD subtypes after screening are shown in Supplementary Table S1, totaling 331 distinct SNPs with F statistics ranging from 11.16 to 789.146; and all SNPs that were included in the analysis of gut microbiota and VaD subtypes after screening are shown in Supplementary Table S2, comprising 1,658 distinct SNPs with F statistics ranging from 16.913 to 88.429; all SNPs that were included in the reverse Mendelian randomization analysis after screening are

shown in Supplementary Tables S3, S4. When inflammatory cytokines were the outcome, there were 40 distinct SNPs in the VaD subtype, with F statistics ranging from 4,772.892 to 77,865.614. When gut microbiota was the outcome, there were 40 distinct SNPs in the VaD subtype, with F statistics ranging from 6,178.734 to 77,865.614. All F statistics were greater than 10, indicating that no weak IVs were found in the IV strength test. After determining that all IVs were valid under the present conditions, we performed a Mendelian randomization analysis.

3.1.2 Inflammatory cytokines genetically predict the risk of vascular dementia subtypes

We used the IVW method as the main method for identifying nine inflammatory cytokines related to an increase or decrease in the risk of various VaD subtypes. After the Bonferroni correction test, we detected three significant inflammatory cytokines (Figure 2) and six suggestive inflammatory cytokines (Supplementary Table S5). Interleukin-18 levels (OR = 1.375, 95% CI = 1.099–1.721, $p = 0.005$) were positively correlated with an increased risk of VaD (multiple infarctions), macrophage migration inhibitory factor levels (OR = 2.712, 95% CI = 1.277–5.804, $p = 0.010$) were positively associated with an increased risk of VaD (sudden onset), and interleukin-4 levels (OR = 0.210, 95% CI = 0.068–0.647, $p = 0.007$) were positively associated with a reduced risk of VaD (other).

3.1.3 Effect of the genetically predicted gut microbiome on the risk of vascular dementia subtypes

We used the IVW method as the primary method to determine and identify 26 gut microbiotas related to increased or decreased risk for different subtypes of VaD. After Bonferroni correction, we found 5 gut microbiotas with significant associations (Figure 3) and 21 suggestive gut microbiotas (Supplementary Table S6). *Negativicutes* (OR = 0.289, 95% CI = 0.112–0.742, $p = 0.010$) and *Selenomonadales* (OR = 0.289, 95% CI = 0.112–0.742, $p = 0.010$) were negatively associated with the risk of VaD (mixed). *Melainabacteria* (OR = 2.055, 95% CI = 1.260–3.352, $p = 0.004$) was positively associated with the risk of VaD (multiple infarctions). *Ruminiclostridium 6* (OR = 1.680, 95% CI = 1.134–2.487, $p = 0.010$) and *Bacillales* (OR = 0.705, 95% CI = 0.545–0.914, $p = 0.008$) were positively and negatively associated with the risk of VaD (undefined), respectively.

3.1.4 Sensitivity analyses

According to the heterogeneity test, the *p*-values of Cochran's *Q* statistic were all greater than 0.05, indicating that there was no heterogeneity between SNPs. MR–Egger regression and MR-PRESSO tests showed no horizontal pleiotropy. The results of Cochran's *Q* test, MR–Egger regression, and MR-PRESSO test for statistical significance are shown in Tables 3, 4. The results of Cochran's *Q* test, MR–Egger regression, and MR-PRESSO test for the suggestive knot results are shown in Supplementary Tables S7, S8. The MR-PRESSO test removed the *Melainabacteria* outlier SNP, rs113884518. In addition, all the included IVs showed obvious symmetry in the funnel plot, excluding directional pleiotropy (Supplementary Figures S1, S2). The leave-one-out method showed that the significant results were not driven by a single SNP (Supplementary Figures S3, S4). The forest plot and scatter plot are shown in Supplementary Figures S5–S8.

3.1.5 Reverse causality

In the reverse MR analysis results of inflammatory cytokines, there was no $p < 0.05$ for any inflammatory cytokine, and in the reverse Mendelian results of the gut microbiota, there were only two gut microbiotas with $p < 0.05$, genus *Parasutterella* ($p = 0.030$) and *Senegalimassilia* ($p = 0.41$), which were not related to the results shown in Figure 3 and Supplementary Table S6. Thus, the results of the reverse MR analysis did not show a reverse causal relationship between VaD and 9 inflammatory cytokines and 25 gut microbiotas.

3.2 Genetic predictors for multivariable MR

The genetically predicted effects of *Negativicutes*, *Selenomonadales*, and *Melainabacteria* on VaD persisted after adjustment for BMI, smoking/smokers in the household, alcohol consumption, and hyperlipidemia. For *Negativicutes*, after adjustment for BMI (OR = 0.289, 95% CI = 0.114–0.729, $p = 0.009$), alcohol consumption

Exposure	Outcome	Number.of.SNPs	Methods	OR(95%CI)	Pvalue
Macrophage Migration Inhibitory Factor	VaD (sudden onset)	6	IVW	2.71(1.27 to 5.80)	0.010
		6	MR Egger	2.49(0.68 to 9.10)	0.241
		6	Weighted median	3.01(1.18 to 7.69)	0.021
		6	Weighted mode	3.37(0.91 to 12.43)	0.128
Interleukin−18	VaD (multiple infarctions)	15	IVW	1.38(1.10 to 1.72)	0.005
		15	MR Egger	1.64(1.01 to 2.66)	0.067
		15	Weighted median	1.31(0.94 to 1.82)	0.113
		15	Weighted mode	1.21(0.76 to 1.95)	0.435
Interleukin−4	VaD (other)	8	IVW	0.21(0.07 to 0.65)	0.007
		8	MR Egger	0.12(0.01 to 2.36)	0.214
		8	Weighted median	0.18(0.04 to 0.84)	0.029
		8	Weighted mode	0.14(0.01 to 1.48)	0.146

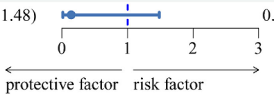

FIGURE 2
Forest plot of the associations between three genetically significant inflammatory cytokines and the risk of vascular dementia. The main results are from inverse-variance weighted analyses. OR, odds ratios; CI, confidence interval; MR, Mendelian randomization; SNPs, single nucleotide polymorphisms; IVW, inverse-variance weighted; VaD, vascular dementia.

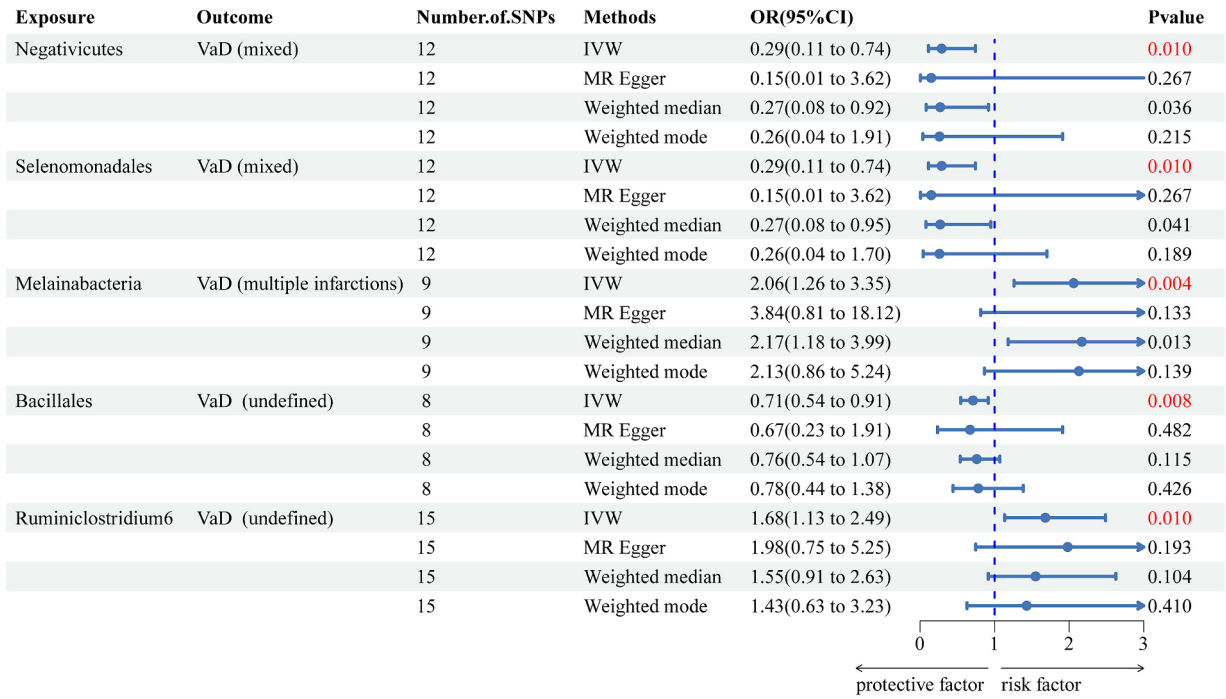

FIGURE 3
Forest plot of the associations between five genetically significant gut microbiomes and the risk of vascular dementia. The main results are from inverse-variance weighted analyses. OR, odds ratios; CI, confidence interval; MR, Mendelian randomization; SNPs, single nucleotide polymorphisms; IVW, inverse-variance weighted; VaD, vascular dementia.

TABLE 3 Sensitivity analysis for the association between three significant inflammatory cytokines and vascular dementia.

Exposure	Outcome	Pleiotropy				Heterogeneity	
		Egger intercept	Intercept's se	Egger *p*-value	MR-PRESSO Global *p*-value	Cochran's Q	Cochran's Q *p*-value
MIF	VaD (sudden onset)	0.028	0.173	0.880	0.954	1.233	0.942
Interleukin-18	VaD (multiple infarctions)	−0.044	0.056	0.440	0.576	12.270	0.585
Interleukin-4	VaD (other)	0.082	0.211	0.711	0.479	6.909	0.438

MIF, macrophage migration inhibitory factor; MR, Mendelian randomization; VaD, vascular dementia.

TABLE 4 Sensitivity analysis for the association between five significant gut microbiomes and vascular dementia.

Exposure	Outcome	Pleiotropy				Heterogeneity	
		Egger intercept	Intercept's se	Egger *p*-value	MR-PRESSO Global *p*-value	Cochran's Q	Cochran's Q *p*-value
Negativicutes	VaD (mixed)	0.045	0.104	0.671	0.945	4.867	0.937
Selenomonadales	VaD (mixed)	0.045	0.104	0.671	0.949	4.867	0.937
Melainabacteria	VaD (multiple infarctions)	−0.071	0.085	0.433	0.315	6.137	0.632
Bacillales	VaD (undefined)	0.008	0.081	0.923	0.595	5.927	0.548
Ruminiclostridium 6	VaD (undefined)	−0.015	0.043	0.724	0.770	10.030	0.760

MR, Mendelian randomization; VaD, vascular dementia.

(OR = 0.281, 95% CI = 0.107–0.743, $p = 0.010$), smoking/smokers in the household (OR = 0.320, 95% CI = 0.129–0.797, $p = 0.014$) and hyperlipidemia (OR = 0.262, 95% CI = 0.086–0.804, $p = 0.019$); and for *Selenomonadales*, after adjustment for BMI (OR = 0.289, 95% CI = 0.114–0.729, $p = 0.009$), alcohol consumption (OR = 0.281, 95% CI = 0.107–0.743, $p = 0.010$), smoking/smokers in the household

(OR = 0.320, 95% CI = 0.129–0.797, $p = 0.014$) and hyperlipidemia (OR = 0.262, 95% CI = 0.086–0.804, $p = 0.019$); and for Melainabacteria, after adjustment for BMI (OR = 2.05, 95% CI = 1.26–3.34, $p = 0.004$), alcohol consumption (OR = 2.28, 95% CI = 1.38–3.77, $p = 0.001$), smoking/smokers in the household (OR = 2.32, 95% CI = 1.43–3.76, $p = 0.001$) and hyperlipidemia (OR = 1.80, 95% CI = 1.05–3.10, $p = 0.033$), the above results strongly support the association of *Negativicutes* and *Selenomonadales* with a reduced risk of developing VaD and *Melainabacteria* with an increased risk of developing VaD. However, the remaining association of inflammatory cytokines and the gut microbiome with VaD was partially attenuated (Figure 4). Finally, sensitivity analysis showed no heterogeneity and no pleiotropy (Supplementary Table S9).

3.3 Mediating role of inflammatory cytokines between gut microbiome and VaD

We first used two-sample MR to analyze whether the gut microbiome in the outcomes had a causal effect on the inflammatory cytokines in the outcomes. Then, inflammatory cytokines and the gut microbiome with causal effects were included in the mediation analysis (Supplementary Table S10) to explore whether inflammatory cytokines are mediating factors for the incidence of VaD influenced by the gut microbiota. Our results show that inflammatory cytokines associated with each subtype of VaD do not play a mediating role in the causal pathways between the gut microbiota associated with each subtype of VaD and each subtype of VaD (Table 5).

Finally, the STROBE-MR checklist was checked and uploaded as Supplementary Table S11.

4 Discussion

Because the changes in inflammatory cytokines and gut microbiota composition are dynamic processes, high (low) values observed at a single time point may not accurately represent the overall trends of change in both. Therefore, this study serves as the first Mendelian randomization study using 41 inflammatory cytokines and 196 gut microbiota datasets with various subtypes of VaD as exposures to predict the inflammatory cytokines and gut microbiota that influence the risk of developing VaD. In addition, this study also used multivariate MR analysis and mediation analysis to determine

Exposure	Adjustment	Number.of.SNPs	Method	OR(95%CI)	P value
Macrophage migration inhibitory factor	Body mass index	13	IVW	3.77(1.73 to 8.26)	0.001
	Alcohol consumption	38	IVW	2.07(0.91 to 4.73)	0.082
	Smoking/smokers in household	22	IVW	2.75(1.32 to 5.73)	0.007
	Hyperlipidemia	9	IVW	3.92(0.70 to 21.95)	0.120
Interleukin−18	Body mass index	18	IVW	1.20(0.91 to 1.58)	0.203
	Alcohol consumption	45	IVW	1.19(0.93 to 1.53)	0.174
	Smoking/smokers in household	29	IVW	1.37(1.09 to 1.72)	0.008
	Hyperlipidemia	15	IVW	1.41(1.03 to 1.93)	0.033
Interleukin−4	Body mass index	14	IVW	0.14(0.03 to 0.74)	0.020
	Alcohol consumption	42	IVW	0.27(0.09 to 0.80)	0.019
	Smoking/smokers in household	24	IVW	0.24(0.06 to 0.97)	0.045
	Hyperlipidemia	12	IVW	0.41(0.07 to 2.23)	0.301
Negativicutes	Body mass index	25	IVW	0.29(0.11 to 0.73)	0.009
	Alcohol consumption	51	IVW	0.28(0.11 to 0.74)	0.010
	Smoking/smokers in household	39	IVW	0.32(0.13 to 0.80)	0.014
	Hyperlipidemia	24	IVW	0.26(0.09 to 0.80)	0.019
Selenomonadales	Body mass index	25	IVW	0.29(0.11 to 0.73)	0.009
	Alcohol consumption	51	IVW	0.28(0.11 to 0.74)	0.010
	Smoking/smokers in household	39	IVW	0.32(0.13 to 0.80)	0.014
	Hyperlipidemia	24	IVW	0.26(0.09 to 0.80)	0.019
Melainabacteria	Body mass index	22	IVW	2.05(1.26 to 3.34)	0.004
	Alcohol consumption	49	IVW	2.28(1.38 to 3.77)	0.001
	Smoking/smokers in household	34	IVW	2.32(1.43 to 3.76)	0.001
	Hyperlipidemia	22	IVW	1.80(1.05 to 3.10)	0.033
Bacillales	Body mass index	18	IVW	0.70(0.55 to 0.90)	0.006
	Alcohol consumption	42	IVW	0.77(0.58 to 1.01)	0.060
	Smoking/smokers in household	29	IVW	0.65(0.50 to 0.84)	0.001
	Hyperlipidemia	20	IVW	0.70(0.51 to 0.95)	0.023
Ruminiclostridium6	Body mass index	27	IVW	1.60(1.08 to 2.38)	0.019
	Alcohol consumption	51	IVW	1.50(1.02 to 2.20)	0.038
	Smoking/smokers in household	41	IVW	1.76(1.17 to 2.65)	0.007
	Hyperlipidemia	21	IVW	1.30(0.69 to 2.43)	0.415

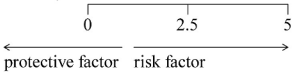

FIGURE 4
Forest plot of multivariate Mendelian randomization analysis of significant effects of inflammatory cytokines and the gut microbiota on vascular dementia after adjustment for confounders. The main results are from inverse-variance weighted analyses. OR, odds ratios; CI, confidence interval; SNPs, single nucleotide polymorphisms; IVW, inverse-variance weighted.

TABLE 5 Mediation analysis of inflammatory cytokines on vascular dementia subtypes.

Outcome	Mediator	Exposure	Total effect	Direct effect	Mediation effect	
			(95% CI)	(95% CI)	(95% CI)	*p*-value
VaD (mixed)	Eotaxin	*Haemophilus*	2.206 (1.116–4.362)	2.385 (1.198–4.749)	0.925 (0.840–1.019)	0.100
VaD (multiple infarctions)	IL-18	*Melainabacteria*	2.055 (1.260–3.352)	1.193 (1.166–3.140)	1.074 (0.993–1.162)	0.064
VaD (other)	MIF	*Actinobacteria*	3.968 (1.202–13.103)	3.259 (0.954–11.126)	1.218 (0.916–1.620)	0.151

VaD, vascular dementia; IL-18, interleukin-18; MIF, macrophage migration inhibitory factor.

whether inflammatory cytokines and the gut microbiota act as independent risk factors in the pathogenesis of VaD and to determine the relationships among them. Seven inflammatory cytokines and 15 gut microbiotas were associated with an increased risk of developing VaD subtypes, and two inflammatory cytokines and 11 gut microbiotas were associated with a decreased risk of developing VaD subtypes (Figure 5). Fibroblast growth factor basic and *Veillonella* increase the risk of developing VaD, and the finding that *Prevotella 9* reduces the risk of developing VaD is consistent with the findings of relevant studies (Ji et al., 2024). After the Bonferroni correction test, we obtained the results of the significance study. The inflammatory cytokines interleukin-18 (IL-18) and macrophage migration inhibitory factor (MIF) were associated with increased VaD risk, and interleukin-4 (IL-4) was associated with decreased VaD risk. The gut microbiota: *Ruminiclostridium 6* and *Melainabacteria* were associated with increased VaD risk, and *Negativicutes*, *Selenomonadales*, and *Bacillales* were associated with decreased VaD risk.

Furthermore, the effects of *Negativicutes*, *Selenomonadales*, and *Melainabacteria* on VaD remained significant after adjusting for the confounders BMI, smoking/smokers in the household, alcohol consumption, and hyperlipidemia. Unfortunately, the data on inflammatory cytokines and the gut microbiota analyzed in this study did not reveal the role of inflammatory cytokines as mediators of the role of the gut microbiota in VaD.

4.1 Inflammatory cytokines

MIF is a cytokine involved in various inflammatory responses and immune processes and is expressed by various cell types, such as immune cells, neurons, and glial cells, in response to stimuli such as hypoxia or ischemia (Thiele et al., 2022). During MIF expression, MIF can first induce the degradation of connexin and simultaneously change the actin cytoskeleton from cortical actin rings to stress fibers, which further leads to the destabilization of cell–cell junctions, resulting in vascular leakage (Chen et al., 2015). Moreover, MIF receptor expression in endothelial cells can induce endothelial cell autophagy to disrupt tight junctions, leading to BBB injury (Liu et al., 2018). Moreover, MIF stimulates the release of proinflammatory cytokines such as TNF-α and IL-1β (Sumaiya et al., 2022), and when inflammatory cytokines enter the CNS through the damaged BBB and bind to their receptors on microglia, astrocytes, or infiltrating inflammatory cells, they activate an inflammatory cascade response to induce neuronal apoptosis, neuroinflammation, and neurodegeneration, which can lead to disruption of brain homeostasis; this is one of the reasons for the onset of VaD (Liang et al., 2018; Liu et al., 2018; Zhao et al., 2023a). Atherosclerosis is a major cause of chronic hypoxia and hypoperfusion (Nam et al., 2020), whereas MIF is upregulated in atherosclerotic plaques and induces the expression of chemokines and adhesion molecules, which promote monocyte adhesion to the endothelium, thereby facilitating the migration and recruitment of atherosclerotic leukocytes, which can indirectly cause damage to neurons (Sinitski et al., 2019). MIF not only enhances intraplaque inflammation through macrophage secretion of proinflammatory cytokines, including MIF, leading to plaque instability but also accelerates foam cell formation by promoting macrophage uptake of oxidized low-density lipoproteins, which further contributes to atherosclerosis (Asare et al., 2013; Sinitski et al., 2019), ultimately triggering the development of cardiovascular disease, which decreases cerebral blood flow and leads to cerebral ischemia and hypoxia. When MIF levels are increased *in vivo*, inflammation within the white matter and hippocampus is also increased, and executive function and spatial learning are disrupted in mice and subjects (Bancroft et al., 2019; Zhao et al., 2023b). Therefore, MIF impairs cognitive function by increasing endothelial dysfunction, reducing intercellular tight junctions, increasing the permeability of the BBB, promoting atherosclerosis, enhancing reactive oxygen species production, and enhancing inflammatory responses. Our study showed that strategies to reduce MIF levels or block its activity might improve cognitive function, but the results were no longer significant after adjusting for alcohol consumption and hyperlipidemia.

IL-18 is a proinflammatory cytokine that is released mainly by microglia (Zhang et al., 2023c). Clinical and basic studies have shown increased plasma IL-18 levels in VaD patients and mice compared with those in non-demented controls (Malaguarnera et al., 2006; Poh et al., 2021). IL-18 not only promotes microglial activation and induces immune cells to produce IL-1β and TNF-α to promote neuroinflammatory responses but also activates interferon-γ to promote the production of IL-8 and chemokines (Yasuda et al., 2019; Li et al., 2022), which in turn promotes the production of IL-18, leading to the formation of a vicious cycle of severe inflammatory responses (Yasuda et al., 2019). A significant increase in BBB permeability is an important pathological change in VaD (Hussain et al., 2021). When IL-18 crosses the BBB, it can accelerate the disruption of BBB integrity by promoting the expression of proinflammatory cytokines and matrix metalloproteinases, which allows the accelerated infiltration of inflammatory cytokines and immune cells into the brain, exacerbating neuroinflammation and neuronal damage and causing neuronal loss (Weekman and Wilcock, 2016; Qin et al., 2020). Additionally, inflammatory vesicle (e.g., NLRP3)-mediated caspase-1 activation can activate cellular pyroptosis and cleave the precursor of IL-18 to its active and secreted form and sustain its release, thereby amplifying and enhancing the inflammatory response and creating a lingering inflammatory milieu that can hinder neuronal function and sustain cognitive dysfunction (Fu et al., 2019;

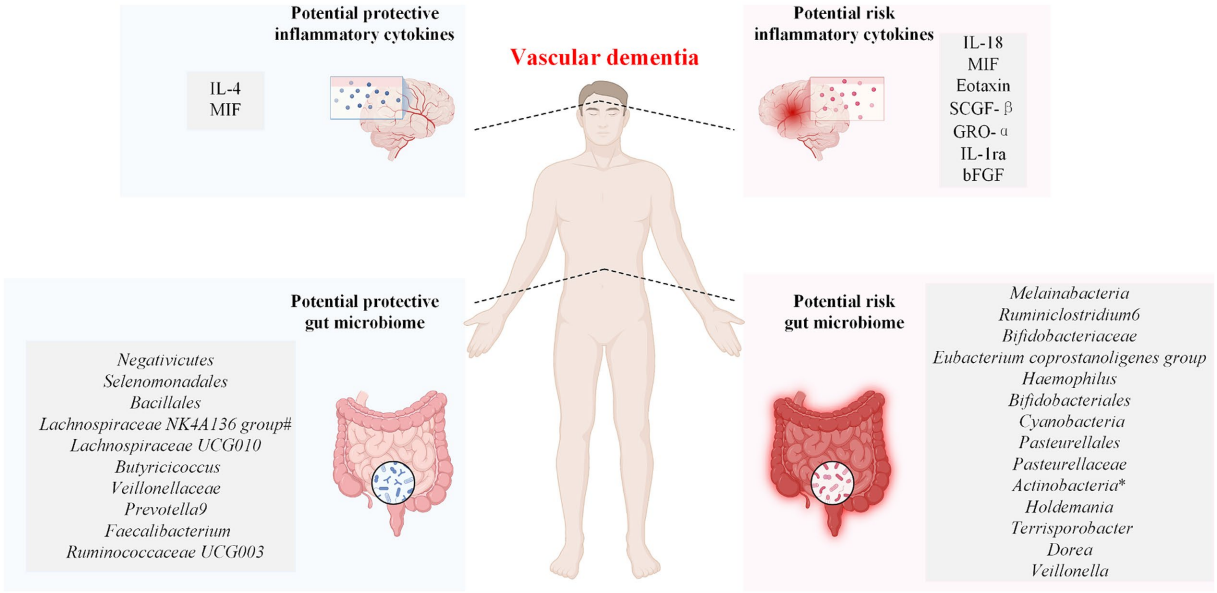

FIGURE 5
Associations between the genetically determined gut microbiome and inflammatory cytokines and the risk of vascular dementia. IL-4, interleukin-4; IL-18, interleukin-18; SCGF-β, stem cell growth factor beta; MIF, macrophage migration inhibitory factor; GRO-α, growth-regulated protein alpha; IL-1ra, interleukin-1-receptor antagonist; bFGF, fibroblast growth factor basic. The symbol * indicates the phylum *Actinobacteria* and the class *Actinobacteria*. The symbol # indicates that the *Lachnospiraceae NK4A136 group* has a protective effect on both vascular dementia (sudden onset) and vascular dementia (mixed).

Ising et al., 2019; Yu et al., 2021). Inhibiting the activation of inflammatory vesicles induced by chronic cerebral insufficiency of cerebral perfusion reduces the release of proinflammatory cytokines, the production of apoptotic substances and cellular pyroptosis and further reduces white matter damage and neuronal cell death to restore cognitive function in VaD mice, which has been proven to be possible by Poh et al. (2021). Finally, high IL-18 levels may be strongly associated with the development of cerebrovascular disease through their association with cardiovascular risk factors (Yasuda et al., 2019). For example, enhanced IL-18 mRNA expression may not only increase the severity of internal carotid artery stenosis (Arapi et al., 2018) but also cause a similar increase in blood pressure (Li et al., 2022). The above findings support our conclusions that elevated peripheral levels of IL-18 may lead to alterations in multiple signaling pathways accompanied by instability of the neurovascular unit, increasing the degree of damage to brain function in VaD patients and indirectly showing that elevated levels of IL-18 increase the likelihood of VaD. In addition, our results also showed that after adjusting for BMI and alcohol consumption, the effect of IL-18 on the increased incidence of VaD was no longer significant.

IL-4 is an anti-inflammatory cytokine (Pu et al., 2021b). It not only inhibits the production of proinflammatory cytokines such as IL-1β and TNF-α but also promotes the production of other anti-inflammatory cytokines such as interleukin-10 (IL-10) and TGF-β (Gärtner et al., 2023). IL-4 induces the differentiation of M2-type macrophages, which have tissue-repairing properties, and M2-type microglia, which have anti-inflammatory effects, to modulate immune cell activity (Dang et al., 2023; Kang et al., 2023). It prevents neuronal loss in the hippocampus, amygdala, and white matter; increases oligodendroglial production or regeneration; improves synaptic connectivity; restores the structural and functional integrity of the brain; and contributes to the restoration of neurological function (Zhang et al., 2019; Pu et al., 2021a,b, 2023). Increasing the expression level of IL-4 in the hippocampus and prefrontal cortex of VaD rats or mice inhibited the release of proinflammatory cytokines and reduced microglia/macrophage activation, stabilizing the anti-inflammatory environment. These findings show that it has the potential to attenuate the deleterious effects of neuroinflammation and improve cognitive function in VaD rats and mice (Zhu et al., 2020; Zhang et al., 2021b; Wang et al., 2023a). IL-4 plays an important role in attenuating the inflammatory response, promoting the release of anti-inflammatory cytokines, regulating the activity of immune cells, promoting neural repair, and reducing apoptosis and oxidative stress; thus, the current evidence indirectly supports our view that elevated IL-4 slows the progression of VaD pathogenesis. Unfortunately, after adjusting for hyperlipidemia, the beneficial effects of IL-4 on VaD were not significant.

4.2 The gut microbiome

Negativicutes are a class of bacteria in the phylum *Bacillota* (Rands et al., 2019). The reduced abundance of *Negativicutes* in fecal samples from Alzheimer's disease (AD) patients in a clinical study of 43 patients with AD may indicate a protective role for *Negativicutes* in dementia (Zhuang et al., 2018). In addition, *Negativicutes* can produce propionic acid through synergistic metabolism (Duncan et al., 2023). *Selenomonadales*, on the other hand, is an order of bacteria within *Negativicutes* that can metabolize not only propionic acid but also lactic and acetic acid (Reichardt et al., 2014). Acetic acid and propionic acid have various potential healthful and neuroprotective effects. Propionic acid acts as a mediator of fatty acid metabolism by

improving lipid biosynthesis, lowering cholesterol, and reducing cardiovascular disease incidence. It also has some inhibitory effects on neuroinflammation (Hu et al., 2018; Cuevas-Sierra et al., 2021); acetic acid not only enhances intestinal mucosal barrier function but can also be transported to the central nervous system through the circulatory system to promote the repair of the BBB, which in turn can indirectly reduce neuroinflammation and enhance synaptic function (Mirzaei et al., 2021; Zhu et al., 2023). Dai et al. improved the intestinal health status of voles after increasing the abundance of *Negativicutes* and *Selenomonadales* in their gut, which ultimately favored their overall health status (Dai et al., 2022). After Naumova et al. administered probiotic treatment to obese patients, *Selenomonadales* and *Negativicutes* increased in the gut along with a decrease in blood glucose in obese patients, which may indicate their role in the beneficial effects of probiotics on host health (Naumova et al., 2020). Therefore, the above studies not only revealed the indirect protective effect of *Negativicutes* and *Selenomonadales* on this organism, but also supported our findings that *Negativicutes* and *Selenomonadales* have beneficial effects on VaD even after adjusting for BMI, smoking/smokers in the household, alcohol consumption, and hyperlipidemia factors.

Research on the role of *Melainabacteria* in neurological diseases has been scarce. Only a few studies have speculated that *Melainabacteria* may synthesize β-N-methylamino-L-alanine (BMAA), which can cause erroneous protein folding and aggregation in the body, triggering endoplasmic reticulum stress responses and cell apoptosis, thereby causing neurodegeneration (Silva et al., 2020). Moreover, BMAA can also activate innate immune responses in neurons, leading to neuroinflammation. Most importantly, BMAA can cause excessive phosphorylation of the Tau protein and the accumulation of Aβ (Silva et al., 2020). The above results indicate that *Melainabacteria* may indirectly participate in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Silva et al., 2020; Hu and Rzymski, 2022). Although studies on the role of *Melainabacteria* in neurological disorders are limited, our findings add to the recent evidence that an increase in the abundance of *Melainabacteria* is positively associated with an increase in the incidence of VaD, even after adjusting for four confounders.

Bacillales are an order of gram-positive bacteria within *Bacillota* (Parte, 2014). Research has shown that *Bacillales* species exhibit excellent potential probiotic characteristics, lowering cholesterol levels to prevent cardiovascular disease; thus, they could reduce the number of causative factors of VaD and lower the risk of VaD (He et al., 2021; Farnsworth von Cederwald et al., 2022). Therefore, our results suggest that an increase in *Bacillales* abundance may indirectly reduce the incidence of VaD. However, the results were not significant after adjustment for alcohol consumption, the reason for which needs to be investigated in our further research.

Ruminiclostridium 6 abundance may be positively associated with the risk of developing AD (Ning et al., 2022), implying that *Ruminiclostridium 6* may be pathogenic in individuals with cognitive dysfunction. The *Ruminiclostridium 6* level is positively correlated with the expression level of aconitate decarboxylase 1 (a protein that is abnormally expressed in ulcerative colitis) and simultaneously promotes the release of proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α, which in turn indirectly increase intestinal permeability, exacerbate microbial imbalances, and contribute to the development of inflammatory diseases of the intestinal tract (Ge et al., 2021; Zhang et al., 2023b). A longitudinal study showed that patients with inflammatory bowel disease (IBD) were more than twice as likely to develop dementia as the general population (Zhang et al., 2021a). Similarly, our findings showed that the abundance of *Ruminiclostridium 6* in the gut of VaD patients was positively correlated with the incidence of VaD even after adjusting for BMI, smoking/smokers in the household, and alcohol consumption.

4.3 Multivariable MR

Hyperlipidemia is one of the known risk factors for VaD (Shang et al., 2024) and is characterized by elevated blood lipid levels and can lead to an imbalance between pro-and anti-inflammatory responses in the body (Collado et al., 2021). IL-4 reduces adiposity, inhibits adipocyte differentiation, and promotes lipolysis (Tsao et al., 2014; Shiau et al., 2019). However, the presence of hyperlipidemia exacerbates the inflammatory process (Don-Doncow et al., 2021), which may reduce the anti-inflammatory effects of IL-4. In addition, hyperlipidemia can cause atherosclerosis, and MIF is upregulated in atherosclerotic plaques (Sinitski et al., 2019) and accelerates the exacerbation of atherosclerosis (Zhao et al., 2023a). In addition, hyperlipidemia can alter the intestinal environment, affecting the composition, metabolism, and function of the gut microbiome (Liang et al., 2021; Di Vincenzo et al., 2024), so that the beneficial independent effect of *Ruminiclostridium 6* in the context of hyperlipidemia is weakened. For the above reasons, it is possible that adjustment for hyperlipidemia, while reducing the influence of confounding factors, may ultimately weaken the independent effects of MIF, IL-4, and *Ruminiclostridium 6* on the incidence of VaD.

BMI is a measure of body fat that can be based on height and weight (Khanna et al., 2022), and a higher BMI is not only a risk factor for developing VaD (Hakim, 2021) but is also commonly associated with increased adipose tissue in the body (Agrawal et al., 2023). Studies have shown that adipose tissue is not only a major source of inflammation but also secretes inflammatory cytokines such as IL-18 (Ahmad et al., 2017). Therefore, a higher BMI may indicate that the body is often in a low-grade chronic inflammatory state and may affect the expression level of IL-18 in the body, masking the independent effect of IL-18 on VaD and rendering the relationship between it and VaD insignificant.

Studies have shown that excessive alcohol consumption can alter the body's immune system and inflammatory response (Crews et al., 2017; Petralia et al., 2020), affecting the levels, functions, and pathways of MIF and IL-18 (Kazmi et al., 2022; Zhao et al., 2023a). Therefore, alcohol consumption directly or indirectly interferes with the expression of inflammatory cytokines, which in our findings, interferes with the independent effects of MIF and IL-18 on the pathogenesis of VaD. In addition, excessive alcohol consumption impairs the intestinal barrier function, leads to increased intestinal permeability, alters the intestinal pH and microenvironment (Gorky and Schwaber, 2016; Bishehsari et al., 2017), affects intestinal immune homeostasis, and causes alterations in the abundance (Engen et al., 2015) and function (Couch et al., 2015) of the gut microbiome, ultimately leading to an imbalance in the gut microbiome. This, in turn, allows alcohol to interfere with the potentially beneficial effects of *Bacillales* on VaD and reduces the beneficial effects of *Bacillales* in

reducing the incidence of VaD. Finally, the inflammation associated with excessive alcohol consumption leads to brain damage and cognitive dysfunction, which is itself a risk factor for the development of VaD (Rehm et al., 2019). These factors explain why the independent effects of IL-18, MIF, and *Bacillales* on the incidence of VaD were attenuated after adjustment for alcohol consumption.

4.4 Gut microbiome-inflammatory cytokines-vascular dementia

The exact relationships among the gut microbiome, inflammatory cytokines, and VaD remain an area of ongoing research. Studies have shown that gut microbiome imbalance is associated with a variety of central nervous system disorders, and there is a link between gut microbiome imbalance and cognitive dysfunction and inflammatory cytokine responses (Song et al., 2024). A metabolically dysregulated gut microbiome or an altered composition of VaD patients may affect the activation of peripheral immune cells, both cellular and humoral, and further lead to elevated levels of proinflammatory factors, such as IL-1β, IL-6, and TNF-α (Parker et al., 2020), as well as further damage to the vasculature and BBB, leading to systemic inflammation and neurodegeneration, which may contribute to the development of VaD (Alkasir et al., 2017; Sun et al., 2021; Kaur et al., 2023). On the other hand, the gut microbiome can produce short-chain fatty acids (SCFAs), including acetic, butyric, and propionic acids (Morrison and Preston, 2016), through innervation of the enteric nervous system. SCFAs can enter the circulation, regulate microglial development and maturation, reduce proinflammatory factors in the host, reduce synaptic dysfunction and neuronal death, regulate gut homeostasis, and ultimately improve cognitive dysfunction (Sarkar et al., 2016; Zhu et al., 2023).

However, our research revealed that the 41 inflammatory cytokines we selected are not the mediators of the 196 common gut microbiomes in increasing or decreasing the incidence of VaD, and our results are in line with the findings of Ji et al. (2024). We believe this is because (1) the amount of GWAS data on inflammatory cytokines and the gut microbiome is limited, which limits our discovery of inflammatory cytokines with mediating effects; (2) Mendelian randomization studies themselves have many gene–environment interactions, in which case the effect of genes on disease may be influenced by environmental factors that change over time or between different groups. This phenomenon deserves further investigation; (3) The gut microbiome does not directly act on inflammatory cytokines to intervene in the pathogenesis and progression of VaD. It is possible that one or more combinations of SCFAs, immune cells, or trimethylamine N-oxide (TMAO) act on inflammatory factors in VaD patients, ultimately enhancing or ameliorating the course of VaD. Research suggests that the gut microbiome can alleviate cognitive dysfunction due to systemic inflammation through acetic and propionic acids (He et al., 2020). According to our results, *Negativicutes* can metabolize propionic acid, and *Selenomonadales* can metabolize propionic acid and acetic acid, among others. Propionate and butyrate inhibit histone deacetylase activity and induce the differentiation of peripheral CD4+ T cells into Treg cells, which produce the anti-inflammatory cytokine IL-10 and suppress the function of Th2 and Th17 cells (Arpaia et al., 2013; Anania et al., 2022). In addition, increasing the abundance of the indole-producing gut microbiome in AD mice can reduce the release of inflammatory factors such as IL-18 and attenuate the inflammatory response, which improves cognitive deficits in AD mice (Zhang et al., 2023a). In addition, trimethylamine N-oxide (TMAO) is another gut microbiome metabolite that not only causes systemic inflammation and neuroinflammation with age but also leads to peripheral and central inflammatory responses and cognitive deficits in mice with elevated circulation levels (Brunt et al., 2021). Studies have also shown that IL-18 and MIF are related to the occurrence of TMAO-mediated inflammatory processes in a variety of diseases (Fang et al., 2021; Zarbock et al., 2022; Constantino-Jonapa et al., 2023), but unfortunately, studies on cognitive dysfunction diseases are still sparse and need to be explored further. Therefore, to obtain more definite evidence on whether inflammatory cytokines act as intermediary factors for the gut microbiome to act on VaD or how the gut microbiome improves or exacerbates VaD through inflammatory cytokines, further research is needed.

To date, our understanding of neurological disorders has evolved from a single-organ, brain-centered view to a more integrated, whole-body view. A good way to control the first site of action in the gut and produce a new generation of safer treatments for central nervous system disorders is to target the gut microbiome (Long-Smith et al., 2020). Research has shown that dietary modulation of the composition and increased diversity of the gut microbiota may be a way to combat VaD (Livingston et al., 2017; Merra et al., 2020) and that probiotic supplementation significantly improves cognitive function in patients with Alzheimer's disease, mild cognitive dysfunction, and VaD mice (Liu et al., 2015; Xiang et al., 2022). However, recent studies have shown that the gut microbiome has coevolved with the host and is interdependent (Cryan et al., 2019), that there is a high degree of interindividual variability and heterogeneity, and that the composition of the gut microbiome evolves continuously with age (Falony et al., 2016). Both the host genome and the associated microbial genome contribute to genetic variation in organisms, as shown by our findings; inflammatory cytokines and the gut microbiome influence the pathogenesis of VaD. In addition, aging alters the gut microbiota (Xu et al., 2019; Wilmanski et al., 2021), and the beneficial gut microbiome that produces SCFAs is reduced in the gut of older or older mice compared to that in younger or younger mice (Kim et al., 2019; Lee et al., 2020), which in turn contributes to the body's inflammatory response, which in turn contributes to accelerated aging and may promote the development of age-related diseases (Xia et al., 2016; Yang et al., 2017). On the other hand, changes in the composition of the gut microbiota in elderly individuals have also been suggested to contribute to inflammation (Thevaranjan et al., 2017). Unfortunately, the impact of age-related dysbiosis on VaD has not been extensively studied; therefore, based on the above points, in the future, we need to understand the latest impact of the microbiome on host health in an evolutionary context through macroeconomics, metabolomics scores, brain imaging techniques, and the use of forward and reverse gut microbiome approaches (Peh et al., 2022). Genetic and functional analyses of gut microbes have been performed, and where possible, the direct effects of gut microbiome metabolites on CNS function have been studied in conjunction with other pathways (e.g., immune or neuronal pathways) to clarify the involvement of a wider range of metabolic pathways with a greater number of relevant inflammatory factors (Bonaz et al., 2017). In addition, the inclusion of aging and dysbiosis as independent but interdependent biological variables in related studies to understand the role of coevolution of the host and its microbiota will contribute to new therapeutic insights into neurodevelopment and decline in patients with cerebrovascular disease and provide new directions to combat

age-associated neurodegeneration and cognitive decline (Cryan et al., 2019; Boehme et al., 2020).

5 Conclusion

We assessed the potential pathogenic role of inflammatory cytokines and the gut microbiome in the etiogenesis of various subtypes of VaD. In addition, *Negativicutes*, *Selenomonadales*, and *Melainabacteria* were identified as playing critical roles in the pathogenesis of vascular dementia (VaD). Thus, the present study may provide new insights into the gut microbiome and inflammatory cytokine-mediated reduction in VaD disease risk.

6 Limitations

Our study has the following limitations: (1) Only participants from the European population were included, which may limit the generalization of our results to other ethnicities. In the future, we will include more ethnicities in our studies. (2) Mendelian randomization studies are different from randomized controlled trials. Therefore, the results from MR analysis may differ to some extent from those expected in previous or future RCTs, which can be interpreted as a life-course effect. (3) Our study included datasets for only 196 common gut microbiomes and 41 common inflammatory cytokines, which resulted in an incomplete set of exposure tools. In the future, we will collect more data to increase the comprehensiveness of our study. (4) There was some overlap between the inflammatory cytokine and VaD sample data. Due to the lack of individual-level GWAS data, we were unable to remove overlapping samples. Therefore, we calculated the maximum overlap rate to be 2.3% (the largest sample size for inflammatory cytokines was 8,337, and the smallest sample size for VaD was 360,248). According to the study by Burgess et al. (2016), the estimated bias from a 30% sample overlap rate is less than 0.1%. Therefore, we assume that the sample overlap between exposure and outcome in our study has little impact on the results.

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.

Author contributions

YY: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. TR: Investigation, Methodology, Writing – review & editing. SW: Conceptualization, Data curation, Formal analysis, Visualization, Writing – review & editing. JCh: Methodology, Supervision, Validation, Visualization, Writing – review & editing. YZ: Conceptualization, Investigation, Software, Writing – review & editing. TL: Investigation, Project administration, Validation, Writing – review & editing. JcC: Formal analysis, Funding acquisition, Resources, Writing – review & editing. XZ: Writing – review & editing. YJ: Writing – review & editing, Writing – original draft. SY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1398618/full#supplementary-material

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Exploring the pathogenesis of insomnia and acupuncture intervention strategies based on the microbiota-gut-brain axis

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Insomnia is a common sleep disorder observed in clinical settings, with a globally rising prevalence rate. It not only impairs sleep quality and daytime functioning but also contributes to a range of physiological and psychological conditions, often co-occurring with somatic and mental disorders. Currently, the pathophysiology of this condition is not fully understood. Treatment primarily involves symptomatic management with benzodiazepine receptor agonists, melatonin and its receptor agonists, sedative antidepressants, atypical antipsychotics, and orexin receptor antagonists. However, due to the adverse side effects of these drugs, including dependency, addiction, and tolerance, there is an urgent need for safer, more effective, and environmentally friendly treatment methods. In recent years, research on the microbiota-gut-brain axis has received significant attention and is expected to be key in uncovering the pathogenesis of insomnia. Acupuncture stimulates acupoints, activating the body's intrinsic regulatory abilities and exerting multi-pathway, multi-target regulatory effects. A substantial body of evidence-based research indicates that acupuncture is effective in treating insomnia. However, the unclear mechanisms of its action have limited its further clinical application in insomnia treatment. Therefore, this study aims to elucidate the pathogenesis of insomnia from the perspective of the microbiota-gut-brain axis by examining metabolic, neuro-endocrine, autonomic nervous, and immune pathways. Additionally, this study discusses the comprehensive application of acupuncture in treating insomnia, aiming to provide new strategies for its treatment.

KEYWORDS

microbiota-gut-brain axis, insomnia, pathogenesis, acupuncture intervention, strategies

1 Introduction

Insomnia is characterized by subjective symptoms such as difficulty falling asleep or frequent awakenings with dreams, resulting in poor sleep quality, short sleep duration, and subsequent daytime fatigue, drowsiness, and lack of concentration. Epidemiological studies indicate that nearly 30% of the global population suffers from insomnia (Morin and Jarrin, 2022), with the

prevalence rate in China reaching up to 45.5% (Chinese Society Of Neurology and Sleep Disorders Group, 2018). Chronic sleep deprivation can lead to reduced immunity, daytime lethargy, and fatigue, significantly affecting patients' lives and work. In severe cases, it can cause a range of physiological and psychological conditions such as anxiety, depression, hypertension, diabetes, dementia, and cancer, imposing a substantial economic burden on society.

The pathophysiological mechanisms underlying insomnia have not been fully elucidated. Modern medicine attributes insomnia to abnormal neuronal activity in the brain, affecting neurotransmitters, hormone secretion, and the immune system. Primary clinical treatments for insomnia currently include benzodiazepines and medications based on melatonin. However, due to their significant side effects, including tolerance, dependency, and addiction (Zengjie et al., 2017), many patients find these treatments unacceptable. Acupuncture, an external therapy guided by principles of traditional Chinese medicine, lacks drug-related side effects. Acupuncture functions by stimulating acupoints to activate the body's intrinsic regulatory abilities, exerting multi-pathway and multi-target regulatory effects. Evidence-based medical research (Kim et al., 2021) has demonstrated significant efficacy of acupuncture in treating insomnia. However, the mechanisms underlying its action continue to be a focus of investigation among scholars worldwide, thus limiting its broader clinical application in treating insomnia.

The microbiota-gut-brain axis (MGBA) integrates the gut microbiota with the nervous, endocrine, and immune systems, establishing bidirectional regulatory pathways crucial for maintaining the dynamic balance of the sleep–wake cycle. This axis is closely linked to the pathogenesis of insomnia. However, existing studies often focus on mechanisms from individual perspectives, lacking a systematic approach. Therefore, this study aims to comprehensively understand insomnia from the perspective of the MGBA, elucidating metabolic, neuro-endocrine, autonomic nervous, and immune pathways. Additionally, this research explores the broader application of acupuncture in insomnia treatment, aiming to propose novel therapeutic strategies (Figure 1).

2 The microbiota-gut-brain axis

The MGBA refers to the bidirectional communication network between the gut microbiota and the central nervous system (CNS). This network is comprised of the nervous, endocrine, and immune systems, and includes several key components such as metabolic pathways, neuroendocrine regulatory pathways, the autonomic nervous system (ANS), and immune signaling. The following outlines the various aspects of how the MGBA influences the CNS.

The MGBA affects the CNS through metabolic pathways. Gut microbiota can impact host metabolism; for instance, neuroactive metabolites such as short-chain fatty acids (SCFAs) produced by the microbiota can influence neural development and function (Rothhammer et al., 2016). SCFAs produced by gut bacteria such as Clostridia and Bacteroides can cross the blood–brain barrier and directly enter the CNS (Onyszkiewicz et al., 2019), regulating the epigenetics of brain function genes and thus affecting CNS development. During early life, gut microbiota actively participates in the normal formation and functional construction of the nervous system. Gut-derived tryptophan metabolites can bind to aryl hydrocarbon receptors in the fetus through the placenta (Wei et al., 2021), significantly influencing fetal CNS development.

The MGBA influences the CNS through neuroendocrine regulatory pathways. Gut microbiota can alter host behavior and cognition by affecting the release of various neurotransmitters, including norepinephrine, serotonin (5-HT), and dopamine (Zhang et al., 2021), Additionally, gut microbiota can secrete various neurotransmitters such as gamma-aminobutyric acid (GABA), catecholamines, and histamine, which transmit signals to the CNS (Chen et al., 2021). These products can regulate neuronal activity in the brain through the MGBA, significantly impacting brain development and behavior. Furthermore, gut microbiota can influence brain function and emotional states by producing neurotransmitters and metabolic products, thereby regulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Animal experiments have shown that early microbial colonization can affect the development and stress responses of the HPA axis in mice (Sudo et al., 2004). Additionally, certain probiotics may help reduce the release of stress hormones such as cortisol, thereby alleviating stress responses (Crumeyrolle-Arias et al., 2014).

The MGBA influences the CNS by regulating autonomic neural pathways. The gastrointestinal (GI) tract is densely innervated and regulated by the CNS, the ANS, and the enteric nervous system (ENS). Sensory information from the GI tract can be directly transmitted to the brain via the ENS or the vagus nerve (Doifode et al., 2020), Additionally, gut microbiota can influence the development and function of gut neurons by activating toll-like receptors (Doifode et al., 2020). Animal experiments have shown that electrical stimulation of vagal afferent fibers can alter the concentration of 5-HT in the brain, indicating that the brain and gut can communicate signals via the vagus nerve (Burgos et al., 2014). By stimulating the vagus nerve, gut microbiota can transmit gut inflammation signals to the CNS. Further studies have revealed that gut microbiota also regulate extrinsic sympathetic nerves in the gut. When the gut microbiota is lost, the expression of the neuronal transcription factor c-Fos in gut sympathetic ganglia is upregulated. Conversely, bacteria colonizing germ-free (GF) mice can inhibit c-Fos expression, thereby affecting the development and function of the CNS (Kuwahara et al., 2020).

The MGBA influences the CNS by regulating the immune system. The GI tract is the body's largest immune system, with 70% of the lymphatic tissue attached to the intestinal mucosa. Immune cells in this lymphatic tissue play a protective role for the body. Cytokines produced by immune responses, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), circulate through the bloodstream to the hypothalamus, transmitting immune signals. These cytokines have potent immune functions, significantly impacting CNS activities.

In summary, the MGBA constructs a complex bidirectional communication network between the gut microbiota and the central nervous system through multiple mechanisms, including metabolic pathways, neuroendocrine regulation, autonomic nervous pathways, and the immune system. Through these pathways, gut microbiota can significantly influence the

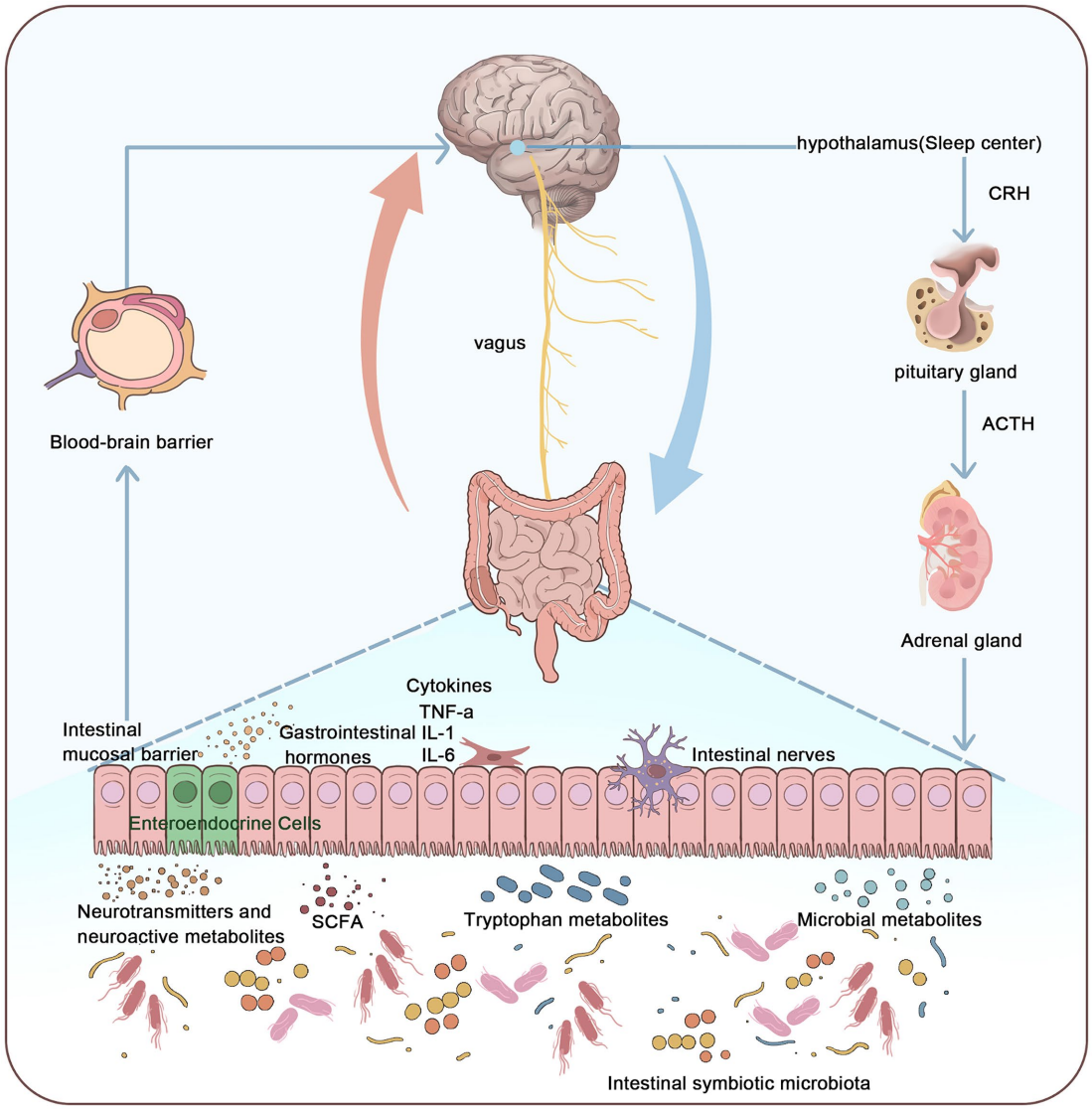

FIGURE 1
Relationship between the microbiota-gut-brain axis and the pathogenesis of insomnia. This figure illustrates that the pathogenesis of insomnia can be understood through the microbiota-gut-brain axis system. The microbiota-gut-brain axis connects the enteric nervous system (ENS) and the central nervous system (CNS), primarily influencing the CNS through the vagus nerve, gut microbiota, metabolites, neurotransmitters, and gastrointestinal hormones. This interaction activates the hypothalamic-pituitary-adrenal (HPA) axis, thereby affecting sleep. By integrating the gut microbiome, nervous system, endocrine system, and immune system, the microbiota-gut-brain axis forms a multidimensional, bidirectional regulatory pathway, thus maintaining the dynamic balance of the sleep–wake cycle.

development, function, behavior, and emotional state of the central nervous system, revealing the critical role of gut microbes in neural health and disease.

3 The relationship between the microbiota-gut-brain axis and insomnia

Insomnia is closely linked to abnormalities in CNS function. The MGBA can regulate the CNS through various mechanisms, including metabolic, neuroendocrine, autonomic neural, and immune pathways, thereby potentially alleviating insomnia.

3.1 Metabolic pathways

The dysregulation of the MGBA can worsen CNS disorders by producing abnormal metabolites, thus affecting sleep. Examples include gut bacteria, SCFAs, and bile acids (BAs).

The genus Bacteroides is the largest Gram-negative bacterial group in the gut microbiota. Its production of lipopolysaccharides (LPS) and other endotoxins can activate the peripheral immune system, promoting infiltration of peripheral immune cells into the brain, thereby triggering CNS inflammation. According to reports (Szentirmai and Krueger, 2014), peripheral administration of LPS can increase the duration and intensity of slow-wave sleep while reducing paradoxical sleep.

Sleep deprivation may alter the composition of the gut microbiota, affecting the overall health and metabolic functions of the host. Specific probiotic strains can improve insomnia symptoms and may modulate physiological mechanisms related to sleep. Animal experiments (Wang et al., 2021), have found that chronic sleep deprivation in mice reduces the relative abundance of beneficial bacteria in the gut, suppressing the number of beneficial bacteria in feces nearly twofold. Other studies have shown that chronic insomnia patients have decreased levels of certain beneficial bacteria in the gut, such as bifidobacteria and lactobacilli, while potentially pathogenic bacteria like Enterobacteriaceae increase in number. The quantity of lactobacilli is negatively correlated with subjective sleep quality scores (Wang et al., 2021), suggesting that these microbial communities may play a role in sleep responses in humans. Another study (Chen et al., 2022a,b), using 16S rRNA gene sequencing of fecal samples from insomnia patients, revealed specific gut microbiota associated with reduced sleep efficiency, such as decreased levels of *Akkermansia muciniphila* correlating with lower sleep quality. In a randomized double-blind controlled trial (Chen et al., 2022a,b), insomnia patients treated with *Lactobacillus plantarum* PS128 probiotics showed significantly improved sleep quality, increased sleep efficiency, and longer sleep maintenance. Another study confirmed (Patterson et al., 2024) that intake of *Bifidobacterium infantis* 35624 probiotics had a positive impact on patients with irritable bowel syndrome and insomnia symptoms, improving their sleep quality and gut function.

Additionally, in the feces of insomnia patients, the concentration of SCFAs significantly decreases. SCFAs, derived from the microbial breakdown of carbohydrates such as acetate, propionate, and butyrate, are metabolic products produced by intestinal microbiota fermenting cellulose. They have been found to influence sleep through the gut-brain axis. Changes in the gut microbiota of insomnia patients may reduce the synthesis of SCFAs (Lehahn, 2022). Experiments have shown (Szentirmai et al., 2019; Silva et al., 2020) that butyrate can significantly increase non-rapid eye movement (NREM) sleep time in mice. SCFAs affect vagus nerve activity via the GPR43 receptor (Silva et al., 2020), thereby influencing reward and emotion regulation areas in the brain, which contributes to improving sleep.

BAs are among the most important metabolites of the intestinal microbiota, possessing multiple signaling functions. In a mouse model of insomnia, levels of BAs are generally elevated in both serum and ileum (Tianyue et al., 2023). Various BA receptors have been found in immune cells throughout the gastrointestinal tract. Evidence also suggests (Hang et al., 2019) that BAs have protective and anti-inflammatory effects on the brain. Therefore, activation of bile acid receptors in immune cells can trigger immune responses and modulate the CNS.

3.2 Neuroendocrine pathways

The neuroendocrine regulatory pathway is a crucial mechanism of the MGBA, influencing the CNS by adjusting levels of neurotransmitters and brain-gut peptides, thereby affecting sleep.

Brain-gut peptides (BGPs) are a significant class of gastrointestinal hormones in the neuroendocrine signaling pathway, exerting dual actions as hormones and neurotransmitters. They are widely distributed in gastrointestinal and brain tissues. Certain BGPs such as serotonin (5-HT), melatonin (MT), substance P (SP), and gamma-aminobutyric acid (GABA) play regulatory roles in sleep.

5-HT, an important neurotransmitter in the gastrointestinal tract, accounts for 95% of the total body serotonin produced in the gut (Jones et al., 2020). It is considered a crucial biological substrate in the pathogenesis of mood disorders. Besides, 5-HT plays a significant role in regulating the structural integrity of the enteric nervous system (ENS), immune responses, and epithelial cell integrity. 5-HT initiates and sustains slow-wave sleep to facilitate sleep regulation (Rancillac, 2016). During wakefulness, 5-HT is released from nerve terminal axons, influencing the synthesis of specific sleep-related substances in the brain. Gut microbiota stimulate 5-HT production through TpH1 activation (Legan et al., 2022), and critical metabolites such as SCFAs and secondary bile acids also impact 5-HT synthesis.

Gastrointestinal hormones are distributed in gastrointestinal tissues and transmit endocrine-neural impulse signals bidirectionally through the blood–brain barrier. Current research indicates that the mechanism of gastrointestinal hormones regulating central nervous signals may be related to the activation of G protein-coupled receptors. SCFAs and BAs can activate the G protein-coupled bile acid receptor TGR5, thereby stimulating the release of 5-HT (Chen et al., 2022a,b). Additionally, MT derived from 5-HT can regulate the body's circadian rhythm and sleep cycle (Pandi-Perumal et al., 2008), and it also inhibits gastric acid secretion, protects the gastric mucosa, and promotes gastrointestinal motility.

MT is a hormone secreted by the pineal gland in the brain, playing a crucial role in regulating the human circadian rhythm and sleep patterns. MT can influence the sleep–wake cycle through its receptors MT1 and MT2 (Rashid et al., 2024), and it is involved in regulating sleep structure and sleep quality. The gut microbiota can indirectly affect the synthesis and secretion of MT through the gut-brain axis. Some metabolic products produced by gut bacteria, such as SCFAs, have been shown to influence MT production. Additionally, dysbiosis of the gut microbiota can affect MT synthesis, thereby impacting sleep. One study demonstrated (Wang et al., 2023) that transplanting the gut microbiota from sleep-deprived mice to normal mice resulted in disrupted MT synthesis and secretion in the recipient mice, leading to decreased sleep quality.

Additionally, substance P (SP) is an excitatory neurotransmitter composed of 11 amino acids. SP can alter sleep or slow-wave sleep activity through its receptors. Studies have shown (Asarnow, 2019) that injecting SP into the lateral ventricle of rats can increase sleep time, categorizing it as a central sleep-promoting substance. It can regulate the expression levels of various substances in brain tissue and promote sleep in the CNS by exciting neurons in the hypothalamic preoptic area (Yanlong, 2004). Furthermore, chronic sleep deprivation can lead to reduced microbial adhesion and penetration in the distal ileum and cecum, elevated serum cytokine levels (Poroyko et al., 2016), and decreased pro-adrenaline levels, with these changes worsening with prolonged sleep deprivation.

GABA is an inhibitory neurotransmitter that plays important physiological roles in humans, including reducing neuronal activity, regulating heart rate, enhancing memory, and modulating hormone secretion. Studies have shown (Yu et al., 2020) that GABA-fermented milk can improve sleep, with its protective mechanism possibly involving the regulation of gut microbiota and increased SCFA levels. Experiments have confirmed (Strandwitz et al., 2019) that GABA serves as a growth factor for certain bacteria, thereby

regulating the gut microbiota. Another study indicated (Mabunga et al., 2015) that GABA extracted from fermented rice germ can counteract caffeine-induced sleep disorders without affecting the general activity of mice.

Additionally, gut microbiota can influence sleep activities by regulating the hypothalamic-pituitary-adrenal (HPA) axis. High levels of HPA axis activity can cause sleep disorders, and correspondingly, sleep disturbances can further dysregulate HPA axis function (Qinghai and Rongyu, 2006). Sleep disorders may be associated with overactivation of the HPA axis (Manosso et al., 2024). Gut microbiota can affect HPA axis levels (Ge et al., 2021), and supplementing with probiotics can regulate HPA axis activity, thereby improving sleep quality (Lan et al., 2023).

3.3 Autonomic nervous pathways

The autonomic nervous system (ANS) includes the sympathetic nervous system, parasympathetic nervous system, and enteric nervous system (ENS), with the primary component of the parasympathetic nervous system—the vagus nerve—playing an important role in regulating sleep and emotions due to its extensive distribution and functions. The vagus nerve pathway is a major route through which the gut microbiota influences the CNS, as activating the vagus nerve has significant anti-inflammatory effects that help mitigate excessive inflammatory responses triggered by the gut microbiota. This neural pathway acts as a bridge between the gut and the CNS. Vagal afferent fibers regulate sleep by translating inflammatory signals between the brain and the periphery. These fibers can transmit inflammatory stimuli (Zielinski and Gibbons, 2022), from peripheral viscera to stimulate the nucleus of the solitary tract (NTS) in the brainstem. The NTS projects to multiple sleep-regulating brain areas, which can induce the expression of pro-inflammatory sleep-inducing molecules and effects. The efferent fibers of the vagus nerve, partly mediated by acetylcholine neurotransmitters in the dorsal motor nucleus (DMN) and the nucleus ambiguus (NA), have anti-inflammatory effects and can reduce the release of peripheral inflammatory factors. The suprachiasmatic nucleus (SCN) can play a role in regulating the circadian rhythms of brain and peripheral molecules. Studies have confirmed (Morais et al., 2020) that *Bifidobacterium longum* may exert anxiolytic effects by signaling to the CNS through the vagus nerve pathway at the ENS level. Hansen et al. (1997) demonstrated through animal experiments that the subdiaphragmatic vagus nerve plays a crucial role in conveying sleep and fever signals to the brain. Yue Jiao and colleagues (Jiao et al., 2020) confirmed through clinical trials that transcutaneous vagus nerve stimulation might be an effective option for treating insomnia. Additionally, vagus nerve stimulation can regulate sleep by altering neurotransmitter levels, and GABA produced by the gut microbiota may influence CNS functions through the vagus nerve (Lucena et al., 2021). Studies have shown (Xian and Aihua, 2015), that stimulating the main trunk or superficial branches (such as the auricular branch) of the vagus nerve increases afferent impulses, which can elevate CNS levels of neurotransmitters like GABA and reduce concentrations of excitatory neurotransmitters like glutamate, thereby improving nighttime sleep quality, reducing daytime sleepiness, and enhancing mood. This process plays a crucial role in sleep regulation, further demonstrating the close link between gut microbiota and sleep quality.

3.4 Immune pathway

The immune regulation pathway is a critical mechanism of the MGBA. The gut microbiota interacts with intestinal epithelial cells, regulates the function of the intestinal barrier, and influences the maturation and activation of immune cells, thereby affecting brain behavior and function.

Gastrointestinal bacteria have been proven to play crucial roles in the initiation, control, regulation, and implementation of the immune system (Belkaid and Hand, 2014). Gastrointestinal bacteria can induce intestinal T cells to migrate to the brain (Zhou et al., 2022). These T cells residing in the brain can regulate the transition of microglia from immature to mature states. In addition to participating in immune functions within the CNS, microglia are also involved in various stages of neuronal activity, including synaptic remodeling to improve neural network connectivity. Researchers have found (Mossad and Erny, 2020) that the lack of a complex host microbiota in the gastrointestinal tract increases the number of microglia in the CNS and leads to defects in microglia maturation, activation, and differentiation, ultimately resulting in impaired immunity to bacterial or viral infections. Additionally, there is growing evidence (Erny et al., 2015) that SCFAs have various impacts on microglial function. Studies have shown that supplementing GF mice with SCFAs can reduce the production of abnormal microglia. Li et al. (2023) demonstrated through animal experiments the critical role of SCFAs in maintaining microglial homeostasis against neuroinflammatory stimuli. Metabolites produced by the gut microbiota, such as SCFAs, can cross the blood–brain barrier, affecting microglia and the synthesis and release of neurotransmitters (Van de Wouw et al., 2018), thereby regulating emotions and behavior. Some researchers believe (Fock and Parnova, 2023) that SCFAs can influence blood–brain barrier permeability, thereby regulating the brain's sensitivity to other sleep-regulating factors.

Approximately 70% of immune cells are located in the gut-associated lymphoid tissue, where they can release various cytokines including interleukins and tumor necrosis factor (TNF). Molecules derived from the gut microbiota may act as antigens in the brain. Several members of the interleukin-1 (IL-1) family have been shown to regulate sleep (Zielinski et al., 2016), including IL-1β, IL-1α, IL-18, and IL-37. The sleep-modulating effects of these IL-1 family members partly depend on the activation of downstream receptors. Researchers suggest (Ingiosi et al., 2015) that increased levels of IL-1 in the brain significantly prolong non-rapid eye movement (NREM) sleep, while reducing IL-1 secretion decreases total sleep time, demonstrating the influence of IL-1 levels on sleep duration. Other studies have found (Krueger et al., 2011) that bacteria can promote sleep by enhancing cytokines such as TNF and IL-1, which act on individual neuronal circuits and sleep-regulating centers in the brain, influencing sleep through substances like nitric oxide, adenosine, and glutamate. Animal experiments have shown (Fernandes, 2006), that TNF-α transiently inhibits the gene expression of melatonin precursors Aa-nat, hiomt, and N-acetyl-5-hydroxytryptamine in the pineal gland of rats, indicating that the nocturnal surge of melatonin is impaired at the onset of inflammation and recovers during acute response shutdown or chronic inflammation. Furthermore, probiotics such as bifidobacteria, lactobacilli, and *Escherichia coli* can affect the levels of inflammatory factors in the blood (Cristofori et al., 2021), and levels of inflammatory

factors like IL-1, interleukin-6 (IL-6), and TNF are significantly elevated in patients with sleep disorders, suggesting a potential link between these microbial communities and immune pathways regulating sleep.

4 Acupuncture intervention strategies

Traditional Chinese Medicine (TCM) places great emphasis on the relationship between the brain and the gastrointestinal system when treating insomnia. The ancient theory that "if the stomach is not in harmony, sleep will be disturbed" aligns with the modern concept of brain-gut interaction, providing an objective basis for the holistic approach of TCM diagnosis and treatment. TCM offers various methods for treating insomnia, among which acupuncture stands out as a green and effective treatment option, demonstrating unique advantages. Acupuncture methods for treating insomnia include body acupuncture, scalp acupuncture, abdominal acupuncture, auricular acupuncture, moxibustion, and other techniques. By stimulating specific acupoints, acupuncture can regulate the body's intrinsic balance and exert a comprehensive regulatory effect.

4.1 The efficacy of acupuncture in treating insomnia and related research

Acupuncture treatment for insomnia has proven effective in clinical practice. Numerous clinical studies and systematic reviews have shown that acupuncture can significantly improve sleep quality in patients with insomnia, shorten sleep onset time, extend sleep duration, reduce the number of nighttime awakenings, enhance sleep efficiency, and decrease daytime sleepiness and anxiety-depressive symptoms. Yichuang (2023) found through a meta-analysis that acupuncture treatment for insomnia is effective and superior to non-acupuncture treatments, with stable results, providing scientific evidence for the efficacy of acupuncture in treating insomnia. A randomized controlled trial (Guo et al., 2013) divided patients into acupuncture, estazolam, and sham acupuncture groups, and found that all groups showed improvement compared to baseline, with the acupuncture group demonstrating significantly better sleep quality and vitality, and reduced daytime dysfunction and sleepiness compared to both the pharmaceutical and sham acupuncture groups. Another study (Qiang et al., 2020) discovered that acupuncture has similar or even better effects compared to medication in improving sleep quality in insomnia patients, and that acupuncture treatment has a longer-lasting effect and lower relapse rate (Xu et al., 2024). Additionally, neuroimaging studies (Zang et al., 2023) have found that acupuncture can regulate brain functional connectivity in insomnia patients and affect brain activity related to sleep, thereby improving insomnia. This indicates that acupuncture not only alleviates insomnia symptoms but also offers stable long-term effects, ultimately enhancing patients' quality of life.

4.2 Acupuncture's regulatory effect on the microbiota-gut-brain axis

Acupuncture, as a holistic regulatory method, stimulates specific acupoints to influence the body's nervous, endocrine, immune, and other systems, thereby regulating the function of the MGBA to treat insomnia.

Research indicates (Hao et al., 2022), that acupuncture can improve intestinal peristalsis and secretion, enhance intestinal mucosal barrier function, and regulate intestinal immune responses, thereby modulating the structure and quantity of gut microbiota. Xu et al. (2023) confirmed through animal experiments that electroacupuncture can significantly increase the production of butyrate and regulate gut microbiota to promote intestinal peristalsis. Qiu et al. (2023) demonstrated that electroacupuncture can exert antidepressant effects by modulating the abundance of lactobacilli and staphylococci. Additionally, acupuncture can regulate metabolic products produced by the gut microbiota, such as SCFAs and bile acids. A clinical trial (Bao et al., 2022) showed that acupuncture treatment three times a week for 12 weeks can increase the ratio of SCFAs and anti-inflammatory bacteria, and effectively reduce the levels of LPS and Th1/Th17-related cytokines in the blood, thereby enhancing intestinal barrier function. These studies suggest that acupuncture may help restore the balance of the gut microbiota and treat insomnia by influencing the metabolic pathways of the MGBA.

In recent years, several studies have found that acupuncture can regulate neurotransmitters and cytokines, thereby improving sleep. Research shows (Provided et al., 2019) that acupuncture can promote the release of neurotransmitters such as 5-HT, GABA, and MT in the brain, thereby regulating neuronal activity and improving insomnia symptoms. Another study (Chen et al., 2023) indicated that depressive-like behaviors in rats significantly improved after 4 weeks of acupuncture treatment, with increased expression of 5-HT. Jinpeng et al. (2022) used retained needle therapy at the Five Heart Points combined with repetitive transcranial magnetic stimulation to treat post-stroke insomnia. The results showed that after 4 weeks of treatment, patients had significantly elevated serum GABA and 5-HT levels compared to before treatment, along with improved sleep quality and reduced anxiety and depression. Hong et al. (2020) confirmed that melatonin levels in the pineal gland of mice receiving acupuncture treatment significantly increased, thereby improving sleep outcomes. Furthermore, acupuncture can regulate the neuroendocrine axis (Yi et al., 2023), reducing levels of stress hormones such as adrenaline and cortisol, thus alleviating sleep disorders. These studies suggest that acupuncture may influence the neuroendocrine pathways of the MGBA by modulating neurotransmitters and cytokines, thereby treating insomnia.

Stimulation of specific acupuncture points can also activate the vagus nerve (Li et al., 2022), thereby balancing the activity of the sympathetic and parasympathetic nervous systems, reducing the body's stress response, promoting physical and mental relaxation, and improving sleep onset and quality.

The research conducted by Liu et al. (2020) suggests that auricular acupuncture can alleviate depressive symptoms, such as anxiety, cognitive impairment, and sleep disturbances, by activating the vagus nerve. Rong et al. (2016) confirmed through clinical trials that transcutaneous auricular vagus nerve stimulation can significantly improve depressive symptoms in patients. These studies indicate that acupuncture may treat insomnia by stimulating the vagus nerve, thereby affecting the autonomic nervous pathways of the MGBA.

By stimulating specific acupoints, acupuncture can regulate the activity of immune cells in the gastrointestinal tract, reduce the levels

of immune factors such as IL-1 and TNF-α, and alleviate sleep disturbances caused by immune responses, thereby helping to restore normal sleep patterns.

Jinfeng et al. (2008) and Yanli and Xianfeng (2012) demonstrated through animal experiments that acupuncture can increase the levels of IL-1β and TNF-α in the hypothalamus of insomnia rat models, confirming that acupuncture can participate in the sleep regulation process by modulating the immune system. Zhao et al. (2008) showed through animal experiments that electroacupuncture can influence the synthesis and release of IL-1β, IL-6, and TNF-α in the hypothalamus, thereby improving the sleep state of rats. Shuangshuang (2013) demonstrated through clinical trials that the mechanism by which acupuncture combined with massage improves sleep quality is related to increasing TNF-α levels and decreasing IL-6 levels. These studies suggest that acupuncture may treat insomnia by regulating immune factors and thereby influencing the immune pathways of the MGBA.

In conclusion, acupuncture, as a safe and effective treatment method, improves sleep quality by influencing MGBA communication through various pathways. In addition to regulating metabolites, neurotransmitters, immune function, and the autonomic nervous system, acupuncture can also promote blood circulation, improve cerebral blood flow, and increase oxygen supply to the brain, thereby enhancing sleep quality. Moreover, acupuncture can alleviate emotional issues such as anxiety and depression, reduce physical and mental stress, and help improve insomnia symptoms such as difficulty falling asleep and frequent waking.

4.3 Acupuncture intervention

In clinical practice, acupuncture has been widely used in the treatment of insomnia. However, there is no standardized protocol for acupuncture prescriptions for insomnia, and it remains in the exploratory stage of operational standardization. Based on the microbiota-gut-brain axis and existing evidence-based medicine (Liang et al., 2022), we believe that the primary acupuncture points for treating insomnia are Baihui (DU20), Shenting (DU24), Shenmen (HT7), Neiguan (PC6), Zhongwan (RN12), Zusanli (ST36), and Sanyinjiao (SP6) (Figure 2).

Baihui (DU20) is located on the head and belongs to the Du meridian. It is the point where all meridians converge, allowing the energy from various meridians to gather and permeate the entire body. Acupuncture at Baihui (DU20) has the effect of regulating the body's yin-yang balance and improving brain function. Modern medicine suggests (Ran et al., 2019) that acupuncture at Baihui (DU20) can maintain the normal sleep–wake cycle rhythm by activating the hypothalamic region and increasing signals in the temporal lobe.

Shenting (DU24) is a meeting point of the Du meridian, Stomach meridian of Foot-Yangming, and Bladder meridian of Foot-Taiyang, where the yang energy of the Du meridian gathers. Acupuncture at Shenting (DU24) can regulate the inflow and outflow of yang energy, balancing it with yin energy. Experiments have shown (Yuanzheng et al., 2021) that electroacupuncture at the Baihui (DU20)–Shenting (DU24) point combination can alleviate autonomic nervous system dysfunction and regulate neurotransmitter levels, thereby improving insomnia.

Shenmen (HT7), the original point of the Heart meridian, serves as the portal for heart energy. Acupuncture at Shenmen (HT7) has the effect of calming the mind and nourishing heart energy. Research (Hui et al., 2011) found that acupuncture at Shenmen (HT7) can improve sleep by regulating the levels of 5-HT in the brain.

Zhongwan (RN12) is located on the abdomen and is the alarm point of the Stomach as well as the meeting point of the bowels, belonging to the Ren meridian. Acupuncture at Zhongwan (RN12) has the effect of harmonizing the stomach and treating all visceral diseases.

Zusanli (ST36) is the He-Sea point of the Stomach meridian of Foot-Yangming and the lower He-Sea point of the Stomach. Acupuncture at Zusanli (ST36) has the function of tonifying the spleen and harmonizing the stomach. Research by Peihong (2018) and others found that acupuncture at Zhongwan (RN12) and Zusanli (ST36) can significantly reduce plasma somatostatin levels and increase hypothalamic GABA levels, improving sleep disorders and thereby enhancing the quality of life for patients.

Neiguan (PC6) is the Luo-Connecting point of the Pericardium meridian. Ancient physicians believed that the pericardium could "act on behalf of the heart to receive pathogens" and is good at opening the chest and regulating qi. Acupuncture at Neiguan (PC6) can expel pathogens and regulate sleep. Fajun et al. (2020) and others confirmed that acupuncture at Zusanli (ST36), Neiguan (PC6), and Zhongwan (RN12) points can significantly improve serum 5-HT levels in insomnia patients, thereby improving sleep quality and maintaining long-term therapeutic effects.

Sanyinjiao (SP6) is the meeting point of the meridians of the Spleen, Kidney, and Liver. It can regulate the body's yin and yang, allowing "yang to enter yin," and improve insomnia symptoms by strengthening the spleen and stomach, regulating the liver, and nourishing the kidney. Research has found (Zhongwen et al., 2022) that acupuncture at Sanyinjiao (SP6) can increase the levels of serum GABA and 5-HT in the body, thereby improving sleep and enhancing sleep quality.

5 Discussion

From the above discussion, it is evident that insomnia is a complex sleep disorder involving dysregulation across multiple systems, including the nervous, endocrine, and immune systems. In recent years, the MGBA has emerged as a novel research field, providing new insights into the pathogenesis of insomnia. Acupuncture, as a traditional Chinese medical therapy, has demonstrated significant efficacy in the treatment of insomnia, and its mechanisms of action have gradually become a focal point of research. Acupuncture may treat insomnia by influencing the MGBA through the regulation of gut microbiota, neurotransmitters, certain hormones, the autonomic nervous system, and inflammatory factors. However, there are still many limitations and challenges at this stage. For instance, the connection between theory and clinical practice is not sufficiently strong. Acupuncture prescriptions for insomnia are varied and often depend on the clinical experience of practitioners, lacking experimental data to validate their scientific basis and feasibility. Additionally, while the feasibility of acupuncture for treating insomnia has been confirmed, the theoretical support for MGBA within traditional Chinese medicine is relatively weak, and

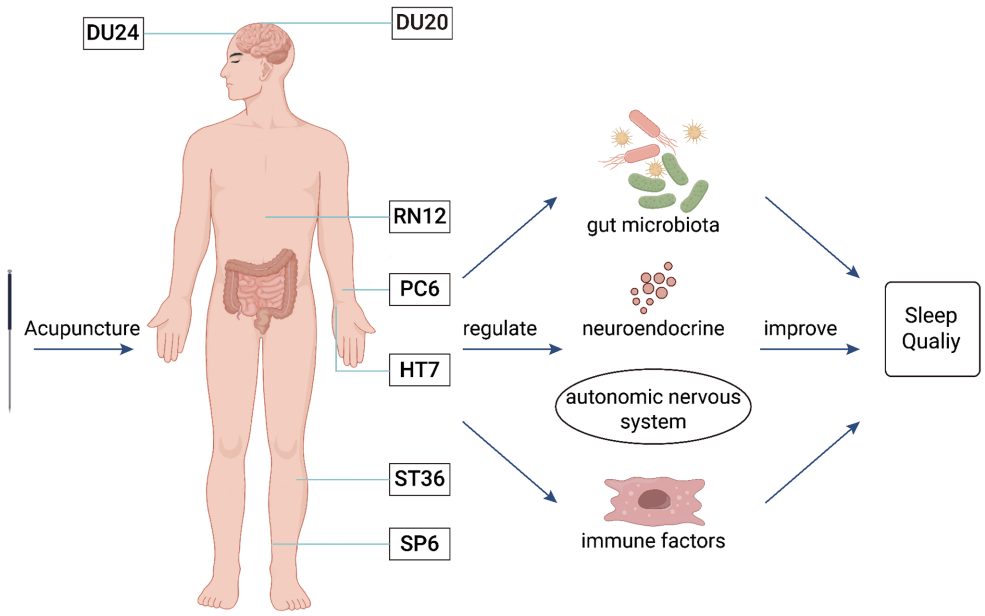

FIGURE 2
The mechanism of acupuncture intervention in the MGBA for the treatment of insomnia. This figure illustrates that acupuncture at Baihui (DU20), Shenting (DU24), Zhongwan (RN12), Neiguan (PC6), Shenmen (HT7), Zusanli (ST36), and Sanyinjiao (SP6) can regulate the gut microbiota, neurotransmitters, autonomic nervous system, and immune factors to improve insomnia.

there is limited research on the mechanisms by which acupuncture treats insomnia through the MGBA. Future research should further integrate basic science with clinical practice to explore the specific regulatory mechanisms of MGBA in insomnia and evaluate the effectiveness and safety of interventions such as acupuncture. This approach will not only deepen the understanding of the pathophysiological mechanisms of insomnia but also potentially lead to the development of more innovative and personalized treatment strategies to address this complex sleep disorder.

In summary, the MGBA offers a new perspective for comprehensively understanding the pathological mechanisms of insomnia, while also providing a scientific foundation for exploring the mechanisms of external therapies, such as acupuncture, in treating insomnia. Therefore, in-depth research on the relationship between MGBA and insomnia, as well as the specific mechanisms of acupuncture in the treatment of insomnia, holds significant practical importance for optimizing clinical treatment strategies. Although current research findings are limited, the field holds vast potential and is worthy of further exploration. With the continuous application of new technologies and theories, such as microbiomics and metagenomics, research on the relationship between the MGBA and insomnia is expected to deepen, leading to more comprehensive results. These advancements could provide innovative treatment methods for clinical practice and potentially open new avenues for the treatment of various diseases.

Author contributions

JiaG: Investigation, Resources, Writing – original draft. JixG: Investigation, Resources, Writing – original draft. XR: Investigation, Supervision, Writing – review & editing. RZ: Investigation, Writing – original draft. QL: Resources, Supervision, Writing – review & editing. KZ: Writing – review & editing, Methodology. SM: Methodology, Writing – review & editing. JZ: Writing – review & editing, Visualization. CJ: Writing – review & editing, Funding acquisition, Supervision.

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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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Fecal microbiota transplantation alleviates cognitive impairment by improving gut microbiome composition and barrier function in male rats of traumatic brain injury following gas explosion

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Background: Dysbiosis of gut microbiota (GM) is intricately linked with cognitive impairment and the incidence of traumatic brain injury (TBI) in both animal models and human subjects. However, there is limited understanding of the impact and mechanisms of fecal microbiota transplantation (FMT) on brain and gut barrier function in the treatment of TBI induced by gas explosion (GE).

Methods: We have employed FMT technology to establish models of gut microbiota dysbiosis in male rats, and subsequently conducted non-targeted metabolomics and microbiota diversity analysis to explore the bacteria with potential functional roles.

Results: Hematoxylin–eosin and transmission electron microscopy revealed that GE induced significant pathological damage and inflammation responses, as well as varying degrees of mitochondrial impairment in neuronal cells in the brains of rats, which was associated with cognitive decline. Furthermore, GE markedly elevated the levels of regulatory T cell (Tregs)-related factors interleukin-10, programmed death 1, and fork head box protein P3 in the brains of rats. Similar changes in these indicators were also observed in the colon; however, these alterations were reversed upon transfer of normal flora into the GE-exposed rats. Combined microbiome and metabolome analysis indicated up-regulation of *Clostridium_T* and *Allobaculum*, along with activation of fatty acid biosynthesis after FMT. Correlation network analysis indirectly suggested a causal relationship between FMT and alleviation of GE-induced TBI. FMT improved intestinal structure and up-regulated expression of tight junction proteins Claudin-1, Occludin, and ZO-1, potentially contributing to its protective effects on both brain and gut.

Conclusion: Transplantation of gut microbiota from healthy rats significantly enhanced cognitive function in male rats with traumatic brain injury caused by a gas explosion, through the modulation of gut microbiome composition and the improvement of both gut and brain barrier integrity via the gut-brain axis. These findings may offer a scientific foundation for potential clinical interventions targeting gas explosion-induced TBI using FMT.

KEYWORDS

gas explosion, cognitive impairment, microbiota-gut-brain axis, fecal microbiota transplantation, gut barrier function, 16S rRNA gene sequencing, metabolomics

1 Introduction

As a critical public health concern in the coal mining industry, gas explosions (GE) pose a significant threat to the safety of workers in male mines and roadways (Shieh et al., 2019). Due to the complex nature of roadways, GE occurrences often result in various injuries to miners. Although there is evidence indicating a decrease in mortality rates among coal miners with advancements in mining technology, the disability rate remains high and the prognosis is poor (Li et al., 2021; Dickstein et al., 2020). The brain tissue serves as a crucial target organ for GE patients, and the shock wave generated during an explosion can lead to damage of brain tissue and subsequent traumatic brain injury (TBI) (Dong et al., 2022). Furthermore, GE-induced TBI presents diverse complexities; our previous studies have established an association between GE and TBI (Dong et al., 2020), while current research primarily focuses on lung injuries (Dong et al., 2021b; Zhang M. et al., 2022). Among these findings, it has been well-documented that GE is linked with neurobehavioral abnormalities and its impact on injuries is more pronounced in developing brains. This is due to markers of GE-induced damage easily crossing through the blood–brain barrier or other means during post-injury recovery periods, exerting detrimental effects on the brain (Dong et al., 2021a). The accumulating evidence from epidemiological and experimental studies has confirmed the impact of TBI on neuronal damage (Thapak et al., 2024), blood–brain barrier disruption (Wang et al., 2021), and other related effects. TBI can be categorized into primary injury and secondary injury (Barrett et al., 2021). Secondary brain injury refers to additional damage, such as pathological inflammation following the initial injury (Khellaf et al., 2019), often leading to neurodegenerative diseases and cognitive impairment (Willis et al., 2020). Furthermore, GE-induced TBI can result in cognitive dysfunction in patients with a poor prognosis, emphasizing the critical need to identify potential therapeutic targets for addressing cognitive impairment.

The neuronal inflammation and neurodegenerative conditions resulting from TBI have implications on gut function via bidirectional communication between the gut and brain pathways (Jameson et al., 2020). Furthermore, various metabolites produced by the gut microbiota impact brain tissue through multiple pathways (Cryan et al., 2019). This underscores the importance of maintaining homeostasis in the gastrointestinal tract as a potential therapeutic target for TBI (Hanscom et al., 2021; Lei et al., 2023). Currently, there is increasing recognition of employing diverse strategies to modulate beneficial populations within the gut microbiome, such as fecal microbiota transplantation (FMT), as a clinical intervention for restoring intestinal flora (Antushevich, 2020; Zhao et al., 2023). FMT serves as an effective method for addressing imbalances in intestinal flora and rebuilding the microecosystem within the intestines (Ooijevaar et al., 2019; Yuan et al., 2024). Additionally, FMT entails transferring donor-derived microbiota to recipients with aims to reshape symbiotic microbial communities in order to confer protective effects against TBI (Koszewicz et al., 2021). Research indicates that alterations in intestinal flora composition disrupts homeostasis within the "gut-brain axis," exacerbating secondary injury following nervous system trauma (Yuan et al., 2021). Our recent findings reveal cognitive deficits and gut microbiota dysbiosis in rats exposed to GE (Dong et al., 2024), underscoring a need for further investigation into how FMT specifically treats GE-induced TBI. Additionally, gut bacteria and their metabolites are capable of alternatively activated macrophages (M2) (Capizzi et al., 2020), reducing neural inflammation (Mossad and Erny, 2020), and increasing macrophage activity to enhance immune response while ameliorating neural degeneration (Soriano et al., 2022). Recent studies on both human populations and laboratory models highlight close associations between FMT efficacy with respect to gut barrier function, microbiota composition, as well as bacterial metabolic product levels (Prochazkova et al., 2019; Ma et al., 2021). Nonetheless, limited knowledge exists regarding long-term effects of FMT treatment during recovery from GE-induced TBI.

The precise mechanisms underlying the connection between gut microbiota and the effects of brain injury have not yet been fully elucidated. Currently, it has been suggested that a stable composition of gut microbiota positively regulates long-term neurodegenerative processes following traumatic brain injury through the microbiota-gut-brain axis (Chiu and Anderton, 2023). Variations in gut microorganisms can be detected by toll-like receptors (TLRs), with TLR4 being particularly adept at recognizing bacterial endotoxins such as lipopolysaccharide (LPS) and triggering the production of proinflammatory cytokines, as demonstrated in my previous study (Dong et al., 2024). Subsequently, LPS and proinflammatory cytokines further disrupt intestinal barrier function, leading to an increase in permeability and translocation into systemic circulation, causing systemic inflammation. Ultimately, these substances may even breach the blood–brain barrier (BBB), affect regulatory T cells (Tregs)-related factors, and result in neuronal death (Paudel et al., 2020). In a stress-induced rat model of depressive-like behavior, the findings demonstrated that FMT treatment ameliorates depressive-like behavior, modulates gut microbiota imbalance, and mitigates intestinal tract inflammation, disruption of intestinal mucosa, and neuroinflammation in rats (Rao et al., 2021). "Further investigations are warranted to assess the potential role of the microbiota-gut-brain axis in mediating the efficacy of FMT treatment for GE-induced TBI, given the observed dysbiosis in gut microbiota following GE.

In this study, we investigated the neuroprotective effects of FMT on neurological deficits, neuropathological changes, and neuroinflammation in GE-induced TBI rats by modulating the microbiota-gut-brain axis. Additionally, we evaluated gut microbiota composition, gut and brain barrier function, as well as the population of Treg cells in the brain. Furthermore, we have confirmed that the potential anti-inflammatory mechanisms identified through integrated microbiome and metabolome profiling are involved in the peripheral immune pathway, providing a promising strategy to alleviate GE-induced TBI.

2 Materials and methods

2.1 Chemicals, reagents, and instruments

Neomycin sulfate (N109017), natamycin (P107822), and sodium butyrate (S102956) were procured from Shanghai Aladdin Biochemical Technology Co., Ltd. Bacitracin (S17005) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. RNA isolate Total RNA Extraction Reagent (R401-01) was purchased from Nanjing Vazyme Biotech Co., Ltd. The Protein Quantification Kit (BCA Assay) (KTD3001) and (PC0020) was acquired from Abbkine (Wuhan) Scientific Co., Ltd. and Beijing Solarbio Science and Technology Co., Ltd., respectively. BeyoECL Star (Ultra hypersensitive ECL chemiluminescence kit) (P0018AS) was sourced from Shanghai Beyotime Biotechnology Co., Ltd. Western blot and IHC revealed the presence of β-Actin (bs-0061R), Interleukin 10 (IL-10) (bs-0698R), programmed death 1 (PD-1) (bs-1867R), Fork head box protein P3 (Foxp3) (bs-10211R), Zonula occludens-1 (ZO-1) (bs-1329R), Occludin (bs-10011R). Claudin-1 (bs-1428 R) and Claudin-5 (bs-1241 R) were obtained from Biosynthesis Biotechnology lnc. (Beijing China).

2.2 Animals and experimental design

The study was conducted in compliance with international standards for the Feeding and use of laboratory animals. Forty healthy male Sprague–Dawley rats (100 ± 5) g were provided by Henan Skobes Biotechnology Co., Ltd, China (License key: SCXK(Yu)2020-0005). The SD rats were housed in standard cages and maintained under typical ambient conditions of temperature (21 ± 1°C) and relative humidity (50 ± 10%) on a regular 12 h light/12 h dark cycle. All rats underwent a one-week acclimatization period prior to formal experiments.

The rats were randomly divided into 5 groups (n = 8/group): the control group (CON) did not undergo any treatment; GE model group (MOD): exposed to GE using an Experimental System for Biological Lethality Testing of Gas Explosions in Shock Tubes designed by Huludao Northern Petrochemical Equipment Factory, China (Utility Model Patent Certificate: CN 214150529 U). Rats were anesthetized with pentobarbital and secured in an iron cage facing the explosion source. GE was simulated using a 10% methane mixture and a 23.6 J shock wave. The rats were positioned 2.4 meters away from the explosion source; GE + fecal microbiota transplantation group (FMT): following exposure to GE, the fecal microbiota suspension derived from normal rat feces was administered via gavage; Antibiotic clearance + GE group (ABX): before exposure to GE, the rats received combined antibiotics to eliminate intestinal flora, followed by normal feeding after exposure to GE; Antibiotic removal + GE + fecal microbiota transplantation group (AF): prior to exposure to GE, the rats received combined antibiotics for intestinal flora elimination, and after exposure to GE, the fecal microbiota suspension derived from normal rat feces was administered via gavage. A shock tube device was simulated a GE in the Institute of Trauma and Orthopedics, Xinxiang Medical University. After anesthesia, the rats were fixed in an explosion cage with their heads and faces facing the explosion source. Neomycin sulfate (5 mg/mL), natamycin (1.25 μg/mL), and bacitracin (5 mg/mL) were dissolved in rat drinking water to prepare combined antibiotic drinking water which was used for rats to achieve the purpose of antibiotic clearance refer to previous study (Bercik et al., 2011). Fresh feces of rats in the control group were collected and dissolved in normal saline to make fecal microbiota suspension. Feces were collected according to the volume to weight ratio of 1:5. This study was approved by the Animal and Medical Ethics Committee of Xinxiang Medical University (No. XYLL-2020007).

2.3 Sample collection and processing

The fecal samples from each rat were collected on the 21st day post-gavage and stored at −80°C for subsequent 16S rRNA gene sequencing. Subsequently, the body weight was recorded, and euthanasia was performed using 1% sodium pentobarbital. Blood was drawn from the abdominal aorta and incubated at 4°C for 2 h before centrifugation at 3000 rpm for 15 min. Following centrifugation, the serum was separated and stored in a refrigerator at −80°C. Brain and colon tissues were then harvested, with simultaneous removal of intestinal contents during colon tissue collection, which were also stored at −80°C for future use. In addition to macroscopic examination of the collected tissues, brain and colon tissues (n = 3) underwent hematoxylin eosin staining (H&E) as well as transmission electron microscopy (TEM). These procedures were conducted to facilitate subsequent observation of histopathological changes and assessment of tissue damage. The remaining tissues were preserved in a − 80°C freezer for molecular biology experiments.

2.4 Fecal microbiota transplantation

FMT was conducted following a well-established protocol (Chang et al., 2015). Briefly, fresh fecal samples were obtained from male rats in the Control group. The samples were collected using a sterile tube and weighed. Subsequently, 0.9% NaCl solution was added to the weighed feces four times, and the mixture was homogenized by stirring with a sterile cotton swab before being centrifuged at 1500 rpm for 5 min. The resulting pellet was then resuspended in 0.9% NaCl to achieve a microbial concentration of 2×10^9 CFU/mL for FMT testing purposes. A fecal bacterial suspension of 1 mL/100 g was orally administered to each gastrointestinal model rat via gavage on a daily basis for 21 consecutive days.

2.5 Blood pressure and heart rate test

Blood pressure and heart rate were measured prior to euthanasia of the rats. All rats in the five groups (n = 8) were tested sequentially. The rats were positioned in the detection device, and tail-end detection was performed with five repetitions for each rat. Data collection was carried out using a Smart Non-Invasive Blood Pressure Monitor (Softron BP-2010 Series, China), as per the operational manual, and statistical analysis was conducted using GraphPad Prism 8.

2.6 Open filed test

The open field test (OFT) apparatus comprises a black square substrate measuring 80 × 80 and a black wall spanning 50 cm. The

substrate is divided into 16 equally spaced squares, with the central area consisting of 4 squares and the remaining 12 squares designated as the periphery area. Each rat is gently placed in the center of the apparatus and allowed to explore freely for 3 min before being captured. Subsequently, all rats are observed in an open field box for 5 min using a video tracking system. Following each session, the open field box is cleaned with a solution of 10% alcohol to mitigate any potential odor influence on subsequent rats. Locomotor activity is evaluated based on distance covered across the entire area. The total moving distance and average moving speed of the rats are utilized to assess neurobehavioral changes. Data collected during these experiments are recorded and analyzed using an animal trajectory tracking system (Etho Vision XT16.0, Noldus).

2.7 Hematoxylin and eosin staining and transmission electron microscope analysis

After sample collection, histopathological examination was conducted to assess the TBI induced by GE. Brain and colon tissues from each group of rats ($n = 3$) were previously fixed in 4% paraformaldehyde, followed by embedding, sectioning, staining, and mounting. Subsequently, H&E-stained samples were examined using an upright optical microscope (Nikon Eclipse E100, Japan), and the resulting images were captured and analyzed with an imaging system (NIKON DS-U3, Japan).

For TEM analysis, the brain and colon tissues of rats in each group ($n = 3$) were fixed with 2.5% glutaraldehyde solution. Each sample was sectioned into 1 mm^3 cubes and immersed in fixative at 4°C for 4 h. Subsequently, the tissues underwent fixation in 1% osmium tetroxide at room temperature for 2 h, followed by dehydration using a gradient alcohol series. The specimens were then embedded in resin and polymerized by baking at 60°C for 48 h. Ultra-thin sections (60 nm) were obtained using an ultramicrotome. Finally, ultrastructural examination of tight junctions in both brain and colon tissues was conducted using a transmission electron microscope (HITACHI, HT7700, Japan).

2.8 IHC staining and western blot assay

For IHC staining, brain and colon tissue sections were incubated with Claudin-1, Occludin, ZO-1, PD-1, Foxp3, and IL-10 antibodies (dilution 1:500) overnight at 4°C. Subsequently, the sections were treated with secondary antibodies (dilution 1:500) for 2 h at room temperature. Following this, the tissue sections underwent incubation in 0.003% hydrogen peroxide in 0.01 M PBS and 0.05% DAB for 10 min in darkness to visualize the immune response; then they were counterstained with hematoxylin for 5 min. The localization and distribution of immunoreactive substances in brain and colon tissues were examined under a microscope. The results were quantified as the percentage of positive area.

For western blot analysis, brain and colon tissues were placed on the operating table, sectioned with a blade, and then immersed in RIPA solution. Following homogenization, EP tubes were utilized for centrifugation to extract the supernatant, and protein concentrations were determined using the BCA kit. Subsequently, proteins underwent separation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred onto PVDF membranes. The membranes were incubated overnight at 4°C with primary antibodies including β-Actin, Claudin-1, Occludin, ZO-1, PD-1, Foxp3 and IL-10 (1:2000). Afterward, corresponding secondary antibodies such as HRP-conjugated anti-rabbit antibody (1:50000) were applied for 1 h followed by washing with TBST and development using an ECL kit. Visualization of blots was achieved utilizing the Amersham ImageQua system while density analysis was conducted using ImageJ software. Results were expressed as the gray value of target protein relative to the internal reference protein.

2.9 RNA extraction and real-time quantitative PCR

The total RNA was extracted using TRIzol reagent (R401-01, Vazyme Biotech Co., Ltd., Nanjing, China), and the concentration of the extraction was determined using a nucleic acid detector. Subsequently, 2 μL of total RNA was mixed with reverse transcriptase to synthesize cDNA using a gradient PCR apparatus. After 10-fold dilution of the cDNA, polymerase chain reaction was performed. The reaction system comprised 0.4 μL forward primer, 0.4 μL reverse primer, 7.2 μL enzyme-free water, 2 μL cDNA and 10 μL SYBR qPCR Master Mix in a total volume of 20 μL. Following the reaction, the number of cycles for each gene was obtained using a multifunctional microplate reader. Each sample underwent triplicate testing to ensure accuracy in determining the relative expression of the target gene in the sample. Results: The relative expression was obtained using the $2^{-\triangle\triangle Ct}$ method combined with β-Actin as reference. Primer names and sequences for mRNA detection are provided in Supplementary Table S1.

2.10 Intestinal contents DNA extraction, 16S rRNA gene sequencing, and Bioinformatic analysis

A total of 5 groups ($n = 5$) comprising 25 qualified samples of intestinal contents were utilized for DNA extraction. The genomic DNA was extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States). Subsequently, the purity and concentration of the DNA were assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. Following this, an appropriate quantity of sample DNAs was taken in a centrifuge tube and diluted to 1 ng/μL with sterile water prior to PCR amplification. The bacterial 16S rRNA genes V3–V4 region was amplified via PCR utilizing the forward primer 338F (5'-ACTCCTACGGGAGGCAGC-3′) and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3′). The resulting 16S rRNA sequencing data were analyzed as previously described (Ma et al., 2020). Post individual quantification steps, amplicons were pooled equitably before undergoing pair-end 2 × 250 bp sequencing on the Illlumina NovaSeq platform with NovaSeq 6,000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

The analysis of microbiome bioinformatics was carried out using QIIME2 2022.11 with minor adjustments in accordance with the

official tutorials.[1] Sequence data were primarily processed utilizing QIIME2 and R packages (v3.2.0). Alpha diversity indices at the amplicon sequence variants (ASV) level, such as Chao1 richness estimator, observed species, Shannon diversity index, and Simpson index, were computed from the ASV table in QIIME2 and presented as box plots. ASV-level ranked abundance curves were generated to compare the richness and evenness of ASVs across samples. Beta diversity analysis was conducted to explore the structural variation of microbial communities among samples using Bray-Curtis's metrics, visualized through principal coordinate analysis (PCoA). Additionally, Principal component analysis (PCA) was performed based on genus-level compositional profiles. The differentiation of microbiota structure among groups was assessed for significance utilizing PERMANOVA (Permutational multivariate analysis of variance) within QIIME2. Furthermore, LEfSe (Linear discriminant analysis effect size) was utilized to identify differentially abundant taxa across groups with default parameters. Microbial functions were predicted by employing PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) based on MetaCyc[2] and KEGG[3] databases.

2.11 Non-targeted metabolomics analysis of the fecal

The fecal samples ($n = 5$/group) from rats in different treatment groups underwent non-targeted metabolomics analysis using ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) at Personalbio Technology Co., Ltd., as previously outlined (Li et al., 2022). The processed data was assessed for overall differences among the three groups utilizing Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Metabolites with a variable importance in projection (VIP) value >1 and a p-value <0.05 (two-tailed Student's t-test) were deemed significant, indicating potentially relevant metabolic markers. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was employed to investigate related metabolic and signal transduction pathways linked to significantly differentially expressed metabolites in the various treatment groups. This study was carried out by Shanghai Personal Biotechnology Co., Ltd., Shanghai, China.

2.12 Statistical analysis

The mean ± standard deviation (SD) was used to express all data, and GraphPad Software (GraphPad Prism version 8, La Jolla, CA, United States) was employed to create statistical charts. Statistical analysis was performed using SPSS software (version 20.0). Group differences were assessed through one-way and two-way analysis of variance (ANOVA) followed by Turkey's multiple comparisons tests. The independent t test was applied for comparing two groups. Spearman correlation analysis conducted by R (V3.5.1) was used to determine the correlations among different groups. In addition, species were clustered by default, that is, UPGMA clustering (default clustering algorithm) was performed according to the Pearson correlation coefficient matrix of their component data, and arranged according to the clustering results. Results with a p value <0.05 were considered statistically significant.

3 Results

3.1 FMT facilitates the amelioration of cognitive dysfunction and brain barrier impairment induced by GE

To investigate the potential impact of intestinal microbiota on ameliorating brain injury in rats with traumatic brain injury (TBI) induced by gastric aspiration, we administered antibiotics to the ABX and AF groups, while fecal bacteria from rats in the CON group were transplanted into the FMT and AF groups (Figure 1A). Following gastric aspiration, abnormal body weight, heart rate, and blood pressure were observed in the rats compared to the CON group; however, these parameters improved following FMT (Figures 1B,C). The results of open field tests (Figures 1D–F) revealed reduced movement distance and speed in MOD and ABX groups compared to the CON group, whereas these impairments were mitigated after FMT in the FMT and AF groups. These findings suggest a potential role for gut microbiota in alleviating cognitive impairment associated with gastric aspiration.

To further investigate the cerebral injury induced by GE and assess potential improvements following FMT, we conducted an assessment of the dentate gyrus of the hippocampus using H&E staining and TEM scanning (Figure 1G). The H&E staining revealed a reduction in cell numbers with loose arrangement in the MOD and ABX groups. Following FMT, there was an increase in cell numbers with darker coloration observed in the FMT and AF groups. TEM scanning demonstrated reduced cell numbers, mitochondrial swelling, cavitation, as well as dilated and degranulated endoplasmic reticulum in the MOD and ABX groups. Post-FMT observations included intact nuclear membrane structure, reduced mitochondrial count with visible cristae, along with persistent dilation of endoplasmic reticulum in both FMT and AF groups.

To further assess striatal damage, we examined alterations in the expression of tight junction proteins within the striatum (Figures 1H–K). In comparison to the CON group, there was a significant reduction in the positive area ratio of Claudin-5 and Occludin protein (Figures 1I,J) in the MOD and ABX groups ($p < 0.001$). Following FMT, these ratios increased in the FMT group ($p < 0.01$), while no significant change was observed in the AF group (Figure 1K). Notably, there were no substantial differences in ZO-1 protein expression levels (Figure 1K). Our findings demonstrate that GE can induce blood–brain barrier damage, which is ameliorated by FMT treatment, thereby exerting a protective effect.

3.2 FMT induced the upregulation of regulatory T cell-related factors proteins expression in the striatum

To investigate whether the protective effect of FMT against GE-induced blood–brain barrier damage is associated with immune

1 https://docs.qiime2.org/2022.11/tutorials/
2 https://metacyc.org/
3 https://www.kegg.jp/

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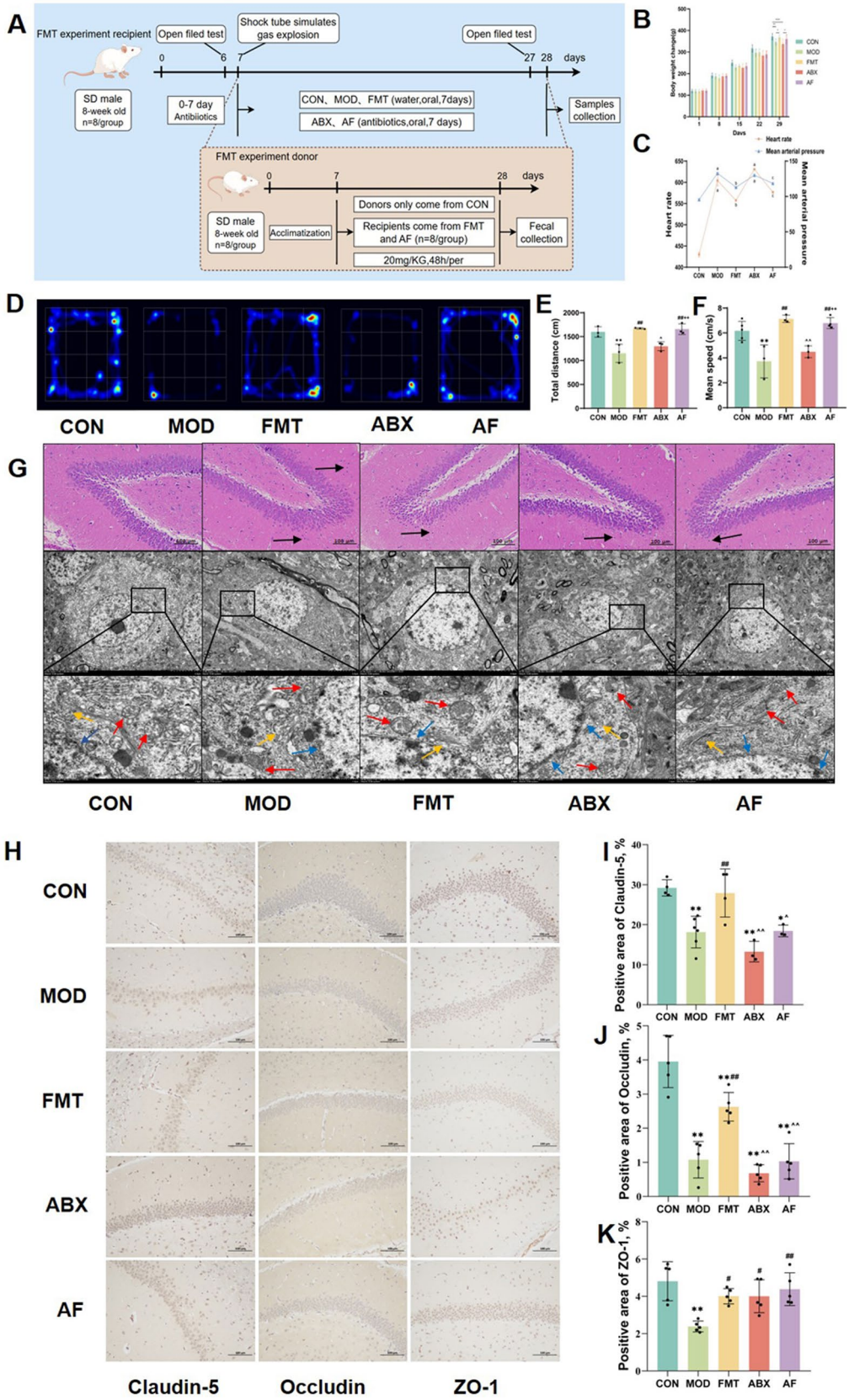

FIGURE 1
FMT facilitates the amelioration of cognitive impairment and blood–brain barrier injury induced by GE. The study included five groups: control group (CON), GE model group (MOD), GE+ fecal microbiota transplantation group (FMT), antibiotic clearance + GE group (ABX) and antibiotic removal + GE + fecal microbiota transplantation group (AF). **(A)** Flow chart depicting the fecal microbiota transfer experiment. **(B)** Body weight change observed

(Continued)

FIGURE 1 (Continued)
in each group. **(C)** Heart rate and mean arterial pressure measured in each group. **(D)** Heatmap illustrating open field test results for each group. **(E,F)** Total distance traveled and mean speed of each group. **(G)** H&E staining and TEM scanning of dentate gyrus of the hippocampus conducted. **(H)** The tight junction proteins in striatum were detected by IHC ($n = 3$). **(I–K)** Positive area of junction proteins (Claudin-5, Occludin5, and ZO-1) in the striatum quantified using image analysis software. The data represent the mean ± SD, $p < 0.05$ was set as the threshold for significance by one-way and two-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons. * Indicates statistical significance compared to the CON group, * $p < 0.05$, ** $p < 0.01$, # indicates statistical significance compared to the MOD group, # $p < 0.05$, ## $p < 0.01$, ^ indicates statistical significance compared to the FMT group, ^ $p < 0.05$, ^^ $p < 0.01$, +indicates statistical significance compared to the ABX group, ++ $p < 0.01$. Black arrows point to the inflammatory cells, blue arrows point to the nuclear membrane structure, red arrows point to the mitochondria, yellow arrows point to the endoplasmic reticulum. The histological sections are from the same anatomical regions, but at completely different coordinates. OFT experiment repeated 3 times, IHC experiment repeated 5 times.

system regulation *in vivo*, we assessed changes in the immunohistochemical expression of regulatory T cell (Tregs)-related proteins in the striatum (Figures 2A–D). Compared to the control group, there was a significant increase in the positive area ratio of PD-1, Foxp3, and IL-10 proteins (Figures 2B–D) in the model and antibiotic-treated groups; following FMT administration, these ratios decreased in the FMT and antibiotic-free groups ($p < 0.001$). Furthermore, to examine tight junction protein and Tregs-related factor expression at the mRNA level, we analyzed their transcript levels (Figures 2E–J). The expression patterns of these six indicators were largely consistent with those observed at the protein level.

3.3 FMT facilitated the amelioration of gastrointestinal barrier injury induced by GE

To assess the impact of GE on gut health and to investigate the direct influence of gut microbiota, we conducted histological evaluation of colon injury using H&E staining and TEM scanning (Figure 3A). In the MOD and ABX groups, decreased number of intestinal epithelial cells, intestinal glands exhibited irregular curvature and disorganized arrangement, and there was incomplete connective tissue. Following FMT treatment, cellular structure and numbers in the FMT group showed recovery; however, restoration of intestinal glands and connective tissues was not pronounced. In the AF group, cellular structure and numbers improved but upper mucosal damage persisted with limited recovery of intestinal glands and connective tissues. TEM scanning revealed desquamation of intestinal villi, damaged boundaries, and severe disruption of cell tight junctions in the MOD and ABX groups. Post-FMT treatment, intestinal villi in both FMT and AF groups displayed regrowth with relatively intact intercellular connections; nevertheless, these connections remained somewhat blurred.

To further confirm intestinal injury, we evaluated alterations in the expression of tight junction proteins in intestinal cells (Figures 3B–E). Our findings revealed that compared with the CON group, the H-scores of Claudin-5, Occludin, and ZO-1 proteins (Figures 3C–E) were significantly reduced in the MOD and ABX groups ($p < 0.001$). Following FMT, the expression levels in the FMT and AF groups exhibited varying degrees of improvement. Our experiments have demonstrated that gastrointestinal endotoxemia can induce damage to the intestinal barrier, while fecal microbiota transplantation directly ameliorates this impairment.

3.4 FMT ameliorated GE-induced intestinal inflammation and enhanced the expression of regulatory T cell factors in the colon

To investigate whether the protective effect of FMT against intestinal barrier damage caused by GE is associated with the modulation of the intestinal immune system, we evaluated changes in the protein expression of Tregs-related factors (Foxp3, PD-1, IL-1β, Claudin-1, IL-10) in the colon (Figures 4A–F). Compared to the CON group, a significant increase was observed in the expressions of IL-1β in the MOD and ABX groups ($p < 0.01$), following FMT, there were varying degrees of decrease in the expressions of IL-1β in the FMT group and AF group ($p < 0.01$) (Figure 4E). Compared to the CON group, a significant increase was observed in the expressions of PD-1, Foxp3, and IL-10 in the MOD and ABX groups ($p < 0.01$). Following FMT, there were varying degrees of decrease in the expressions of PD-1, Foxp3, and IL-10 in the FMT group; no significant change was observed in the AF group (Figures 4B,D,F). Similar alterations were also noted for inflammatory factor IL-1β. Additionally, Claudin-1 expression decreased in MOD and ABX groups (Figure 4C) but increased after FMT ($p < 0.05$).

The expression of Tregs-related protein factors in the colon was evaluated through immunohistochemistry (Figure 4G; Supplementary Table S2), and the results were consistent with those obtained from Western blot analysis (Figures 4H–J). Subsequently, we investigated tight junction proteins and Tregs-related protein factors at the mRNA level, and the expression patterns were concordant with the aforementioned findings.

3.5 FMT influences the composition of gut microbiota in GE rats

To assess the impact of FMT on gut microbiota modulation, we conducted 16S rRNA gene sequencing analysis to examine bacterial composition following microbial treatment in rats. A total of 25 samples from five groups of rats ($n = 5$) were utilized to generate 16S rRNA gene profiles. The ASV/OTU numbers in CON, MOD, FMT, ABX and AF groups were 750, 626, 802, 724, and 739, respectively, (Supplementary Figure S1A). When the sequencing depth exceeded 5,000 for all samples, a plateau was observed in the rarefaction curve indicating sufficient sample diversity (Supplementary Figure S1B). Additionally, when sample abundance surpassed 200, the flat trendline suggested high uniformity between samples (Supplementary Figure S1C). Alpha diversity assessment revealed no significant differences in Chao1 and

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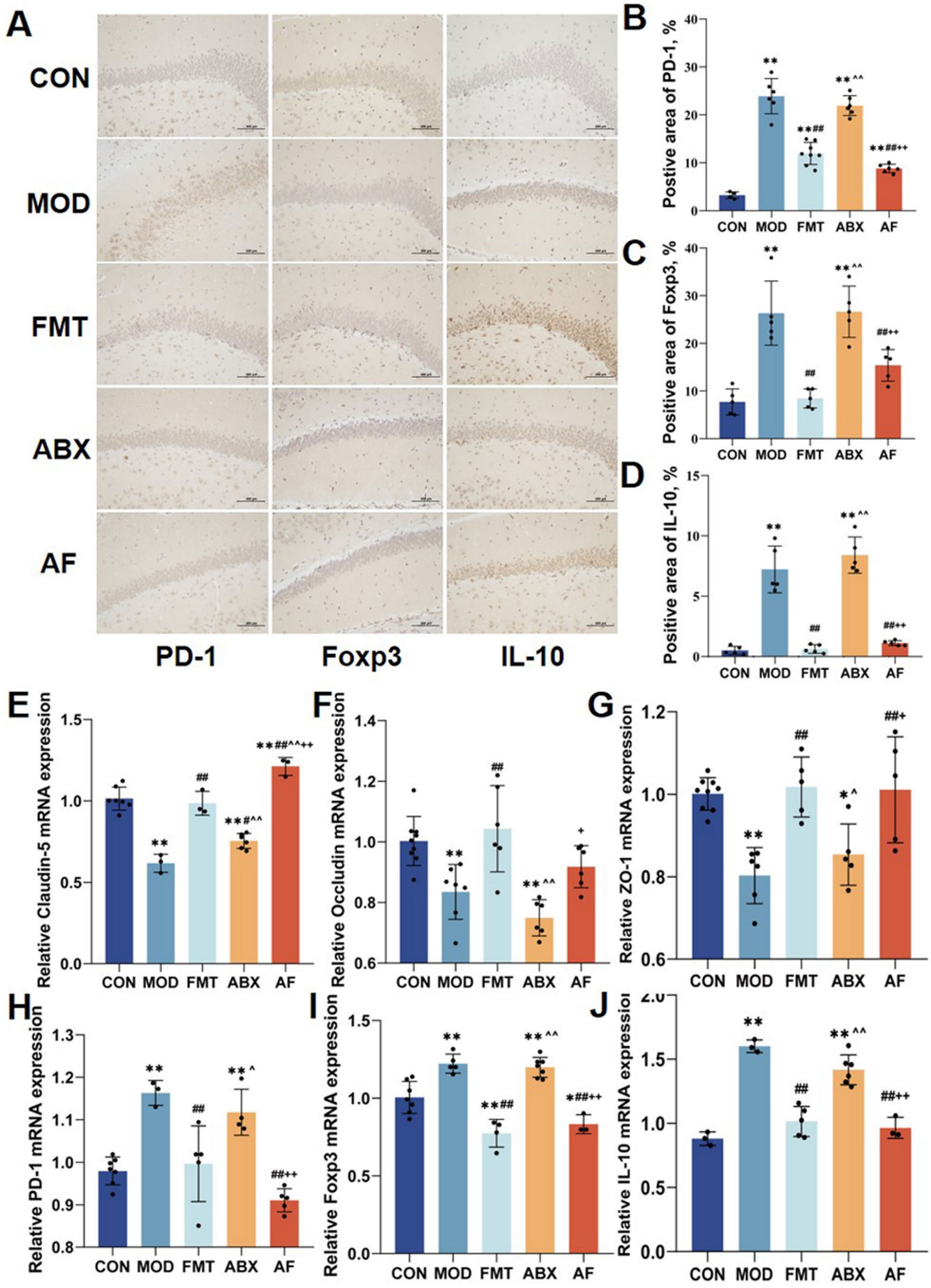

FIGURE 2
FMT prompted the striatum Tregs cell factor expression. There were five groups: control group (CON), GE model group (MOD), GE+ fecal microbiota transplantation group (FMT), antibiotic clearance + GE group (ABX) and antibiotic removal + GE + fecal microbiota transplantation group (AF). **(A)** Tregs associated factors proteins in striatum were detected by IHC ($n = 3$). **(B–D)** Positive area of Tregs-associated factors proteins (PD-1, Foxp3 and IL-10) in the striatum ($n = 3$). **(E–J)** Relative mRNA levels with β-actin of the junction proteins (Claudin-5, Occludin and ZO-1) and regs-associate factors (PD-1, Foxp3 and IL-10) in striatum ($n = 6$). The data represent the mean ± SD, $p < 0.05$ was set as the threshold for significance by one-way and two-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons. * Indicates statistical significance compared to the CON group, * $p < 0.05$, ** $p < 0.01$, # indicates statistical significance compared to the MOD group, # $p < 0.05$, ## $p < 0.01$, ^ indicates statistical significance compared to the FMT group, ^ $p < 0.05$, ^ ^ $p < 0.01$, +indicates statistical significance compared to the ABX group, + $p < 0.05$, ++ $p < 0.01$. IHC experiment repeated 5 times, RT-PCR experiment repeated more than 3 times.

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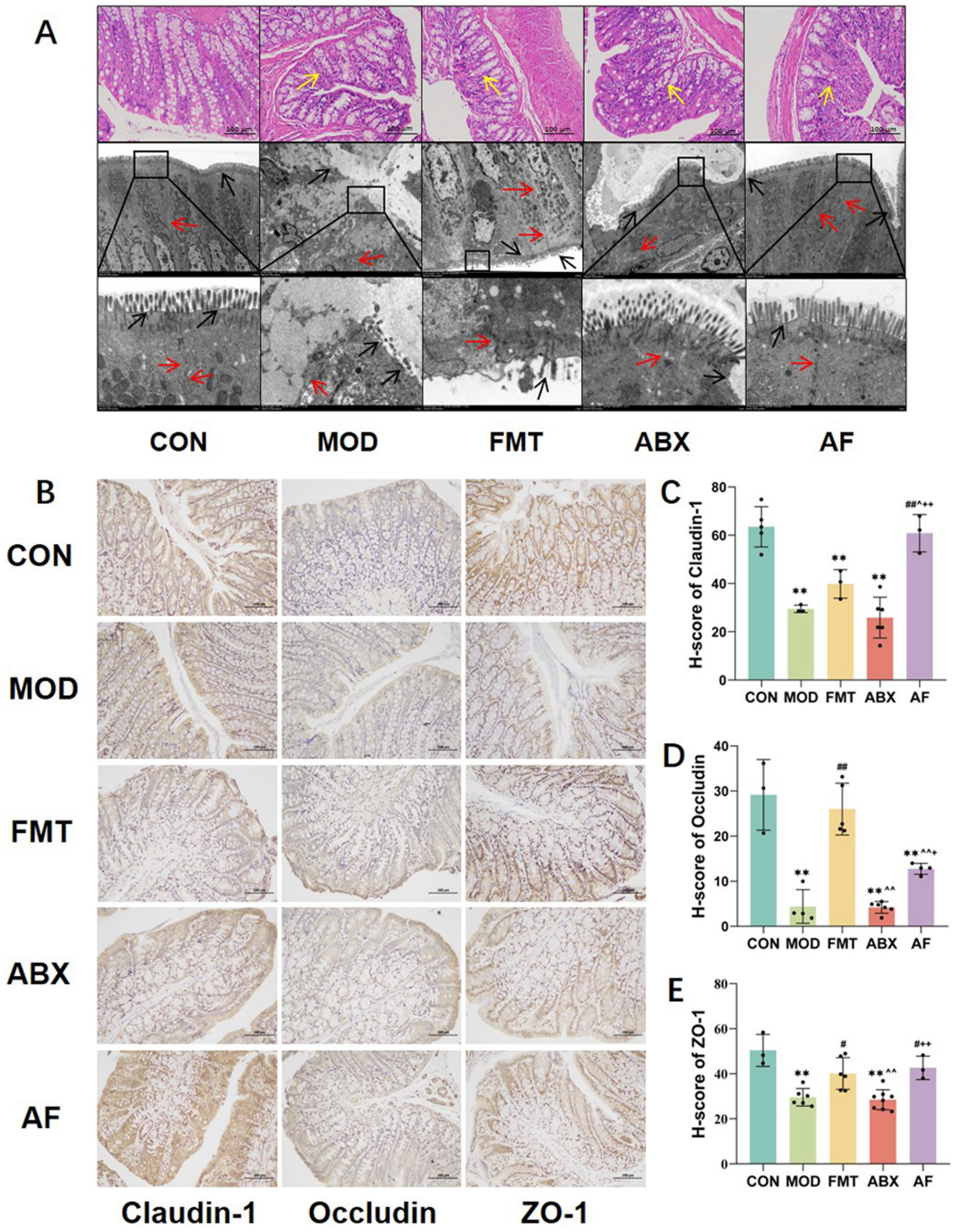

FIGURE 3
FMT facilitated the amelioration of intestinal barrier injury induced by GE. The study included five groups: control group (CON), GE model group (MOD), GE+ fecal microbiota transplantation group (FMT), antibiotic clearance +GE group (ABX), and antibiotic removal +GE+ fecal microbiota transplantation group (AF). **(A)** H&E staining and TEM scanning of the colon were performed. **(B)** Immunohistochemistry was used to detect the junction proteins in the colon ($n = 3$). **(C–E)** The H-score of the junction proteins, including Claudin-1, Occludin, and ZO-1, in the colon was calculated ($n = 3$). The data represent the mean ± SD, $p < 0.05$ was set as the threshold for significance by one-way and two-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons. * Indicates statistical significance compared to the CON group, ** $p < 0.01$, # indicates statistical significance compared to the MOD group, # $p < 0.05$, ## $p < 0.01$, ^ indicates statistical significance compared to the FMT group, ^ $p < 0.05$, ^^ $p < 0.01$, +indicates statistical significance compared to the ABX group, + $p < 0.05$, ++ $p < 0.01$. Yellow arrows point to intestinal glands, black arrows point to intestinal villous structures and red arrows point to cell junctions. The histological sections are from the same anatomical regions, but at completely different coordinates. IHC experiment repeated 5 times.

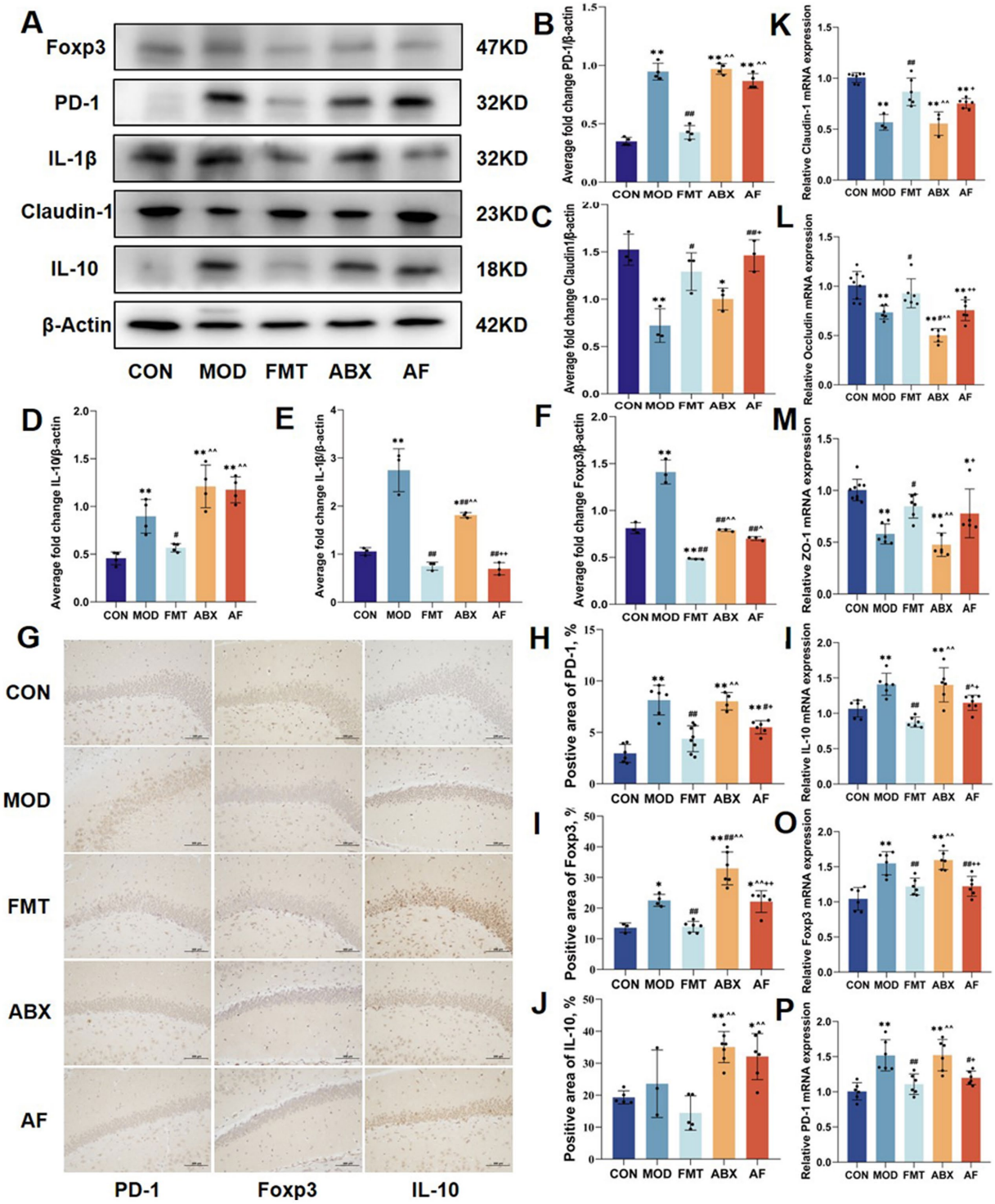

FIGURE 4
FMT enhanced GE induced by intestinal inflammation and stimulated the expression of colon Tregs cell factors. The study included five groups: control group (CON), GE model group (MOD), GE + fecal microbiota transplantation group (FMT), antibiotic clearance +GE group (ABX), and antibiotic removal +GE+ fecal microbiota transplantation group (AF). **(A–F)** Relative protein levels of PD-1, Claudin-1, IL-10, IL-1β and cleaved Foxp3 in the colon ($n = 5$). **(G)** Tregs related factors protein colon was detected by IHC ($n = 3$). **(H–J)** Positive area of Tregs-associated factors protein (PD-1, Foxp3 and IL-10) in the colon ($n = 3$). **(K–P)** Relative mRNA levels with β-actin of the junction proteins (Claudin-1, Occludin and ZO-1) and Tregs-associate factors protein (PD-1, Foxp3 and IL-10) in colon ($n = 6$). The data represent the mean ± SD, $p < 0.05$ was set as the threshold for significance by one-way and two-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons. * Indicates statistical significance compared to the CON group, * $p < 0.05$, ** $p < 0.01$, # indicates statistical significance compared to the MOD group, # $p < 0.05$, ## $p < 0.01$, ^ indicates statistical significance compared to the FMT group, ^ $p < 0.05$, ^^ $p < 0.01$, +indicates statistical significance compared to the ABX group, + $p < 0.05$, ++ $p < 0.01$. WB experiment repeated more than three times, IHC experiment repeated 5 times, RT-PCR experiment repeated more than three times.

Shannon indices among the five groups ($p > 0.05$) which reflect microbiota richness and diversity (Figure 5A). To evaluate community similarity, Bray-Curtis PCoA was employed for β-diversity assessment revealing that principal components PCo1, PCo2, and PCo3 explained variation at rates of 44.1, 10.41, and 8%, respectively (Figures 5E,F). At phylum level, *Firmicutes*, *Verrucomicrobiota*, and *Actinobacteriota* were predominant (Figures 5B,C). At genus level, *Akkermansia, Romboutsia_B,* and *Allobaculum* exhibited dominance (Figure 5D; Supplementary Table S3). The heatmap displays the top 20 microbial flora abundance and correlations among different groups at the genus level (Figure 5G).

To identify the specific genus within each group, LEfSe and LDA were employed to pinpoint the core taxa most likely responsible for the observed differences between groups, as illustrated in Figures 6A,B. The abundance of *Firmicutes* was significantly higher in the ABX group compared to the CON group ($p = 0.025$, LDA = 3.268). The FMT group exhibited a higher abundance of *Clostridium_T* ($p = 0.042$, LDA = 4.438), while the AF group showed elevated levels of *Faecousia*, *Onthebecus*, and *Choladousin* (*Faecousia*, $p = 0.011$, LDA = 4.384; *Onthebecus*, $p = 0.024$, LDA = 4.297; *Choladousin*, $p = 0.006$, LDA = 3 0.886). Subsequently, Metagenome Seq analysis was conducted between these two groups to identify differential flora (Figures 6C–F). Compared with the CON group, the MOD group demonstrated a predominance of *Firmicutes* at genus level including *Clostridium_T* (LogFC = 3.445), *Clostridium_Q* (LogFC = 2.482), and *Lachnospiraceae* (LogFC = 3.3) (Figure 6C). In contrast, *Firmicutes* and *Proteobacteria* were dominant in the ABX group with *Clostridium_T* (LogFC = 5.404) *Ligilacttobacillus* (LogFC = 5.338) and *Eubacterium_F* (LogFC = 4.498) at the genus level (Figure 6D). Compared with MOD group, *Firmicutes* dominated in FMT group, among which *Clostridium_T* (LogFC = 4.095), *Ruminococcus_C* (LogFC = 3.908), and Lawsonibacter (LogFC = 3.277) dominated at genus level (Figure 6E). *Firmicutes* and *Bacteroidetes* were predominant in AF compared to ABX group, with *Blautia_A* (LogFC = 7.73), *Faecousia* (LogFC = 6.379), and Choladousia (LogFC = 4.877) dominating at genus level (Figure 6F). In metabolic

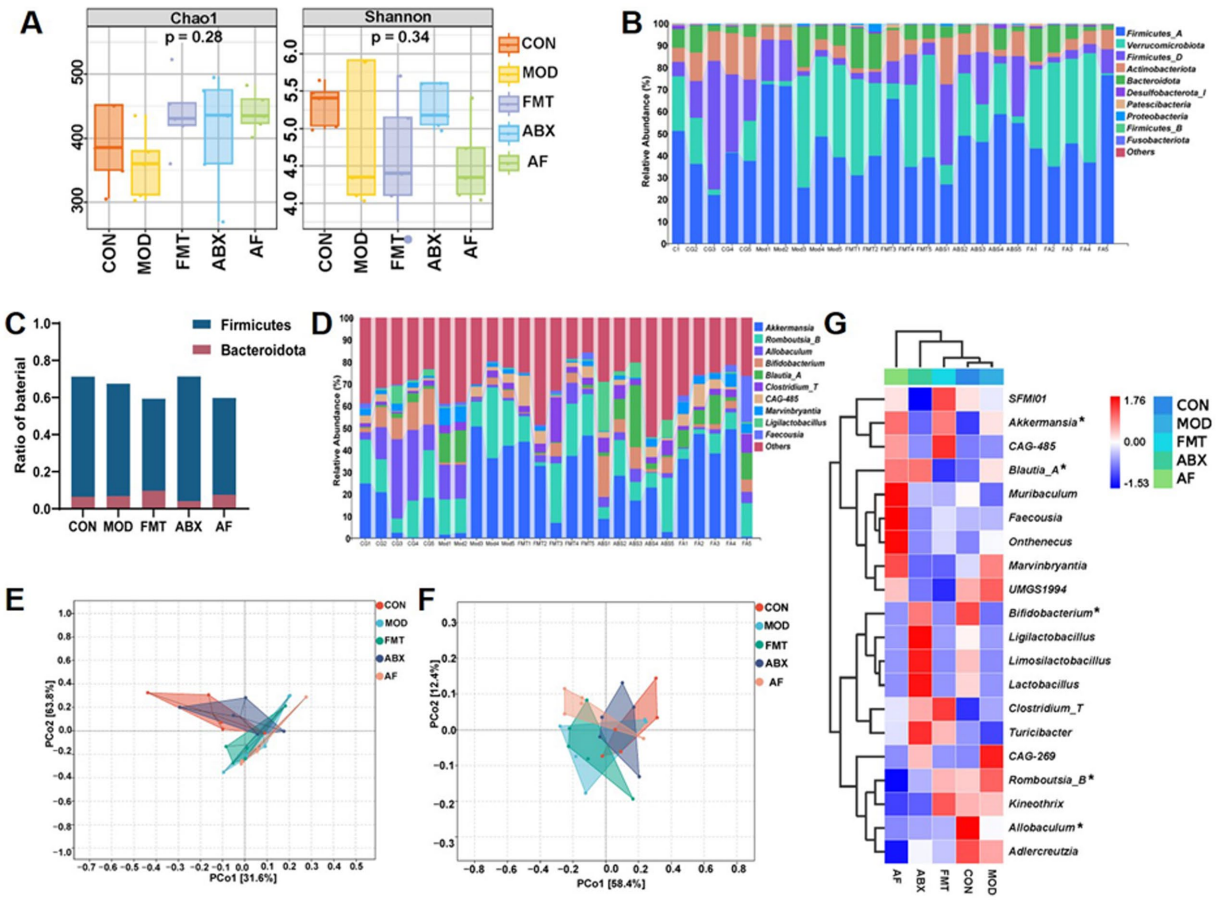

FIGURE 5
FMT intervention influences the gut microbiota composition in GE rats. The study included five groups: control group (CON), GE model group (MOD), GE+ fecal microbiota transplantation group (FMT), antibiotic clearance +GE group (ABX), and antibiotic removal +GE+ fecal microbiota transplantation group (AF). **(A)** Boxplot of alpha diversity indices ($n = 5$), the vertical axis represents the value of the corresponding alpha diversity index. **(B)** Taxonomic composition analysis at the phylum level for each sample set ($n = 5$). **(C)** Ratio of Firmicutes and Bacteroidetes in the phylum level. **(D)** Taxonomic composition analysis at the genus level for each sample set ($n = 5$). **(E)** PCA plot visualizing differences in species composition at the phylum level between samples across groups ($n = 5$). **(F)** PCA plot visualizing differences in species composition at the genus level between samples across groups ($n = 5$). **(G)** Heatmap displaying variations in species composition across groups ($n = 5$), asterisk indicate the bacterial genus with the highest enrichment at the genus level.

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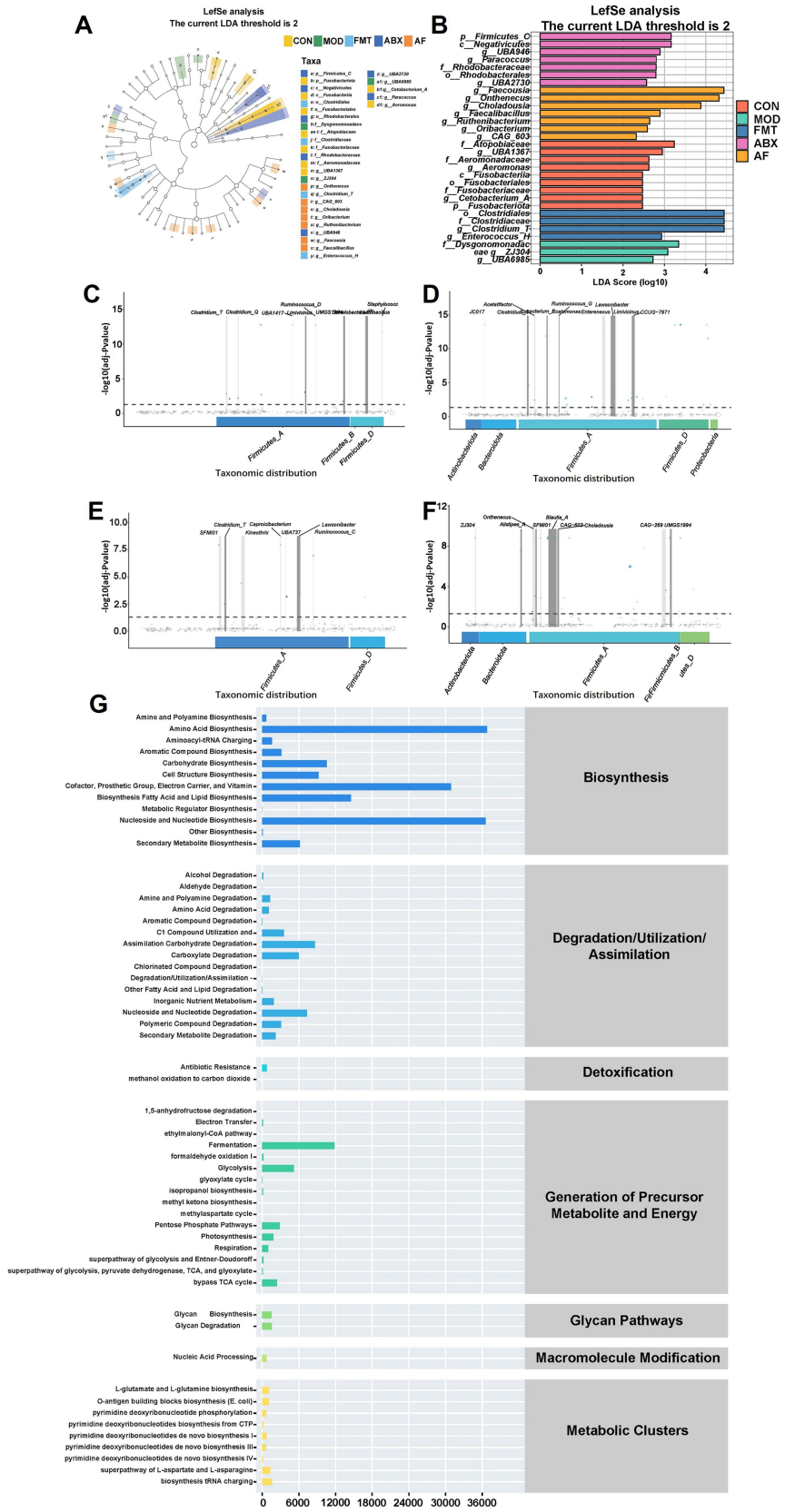

FIGURE 6
FMT modulates gut microbiota metabolite composition in GE rats. There were five groups: control group (CON), GE model group (MOD), GE+ fecal microbiota transplantation group (FMT), antibiotic clearance + GE group (ABX) and antibiotic removal + GE + fecal microbiota transplantation group (AF). **(A,B)** LDA effect size analysis of each group ($n = 5$), the vertical axis is the –log10 (adj-*P*value) value, and the more significant the difference, the higher the Y-axis position. **(C–F)** MetgenomeSeq analysis of species difference composition between sample groups (COM-MOD, CON-ABX, MOD-FMT, ABX-AF) ($n = 5$). **(G)** Statistics of metabolic pathways in bacterial genera ($n = 5$).

pathway statistics, compared with the MOD group, more differential genera were enriched in amino acid biosynthesis, nucleoside, and nucleotide biosynthesis, and fatty acid and lipid biosynthesis in the FMT group (Figure 6G).

3.6 FMT modulates the composition of gut microbiota metabolites in genetically engineered rats

To investigate the impact of gut microbiota on the host through metabolites, a total of 25 samples from five groups ($n = 5$) were subjected to metabolomics analysis. The Venn diagram illustrated distinct metabolic changes resulting from different treatments (Figures 7A,B). The specific number of unique metabolites in positive and negative ion modes is depicted in the figure (Figures 7C,D). A total of 1,381 metabolites were identified as significant differential metabolites based on VIP > 1 and $p < 0.05$ screening criteria. These metabolites were comprehensively categorized under positive and negative ion modes (Figures 7E,F). In order to further elucidate the distinctions among the groups, we conducted Partial Least Squares Discriminant Analysis (PLS-DA) for in-depth investigation. The PLS-DA model revealed limited separation between the five groups, with quality parameters R2X = 0.241, R2Y = 0.376, and Q2 = 0.117, indicating suboptimal reliability and predictability of the model (Figures 7J,G,H). Subsequently, KEGG enrichment analysis of differential metabolites was performed between the two groups, and the top 20 pathways exhibiting the lowest p value—representing the most significant enrichment—were selected for presentation (Figures 7I–L). In comparison with the CON group, the differentially expressed metabolites (DEMs) in the MOD group (Supplementary Table S4) exhibited enrichment in Cysteine and methionine metabolism, Metabolic pathways, and Purine metabolism (Figure 7I). In comparison with the CON group, the DEMs in the ABX group (Supplementary Table S5) showed enrichment in Arachidonic acid metabolism, Glutathione metabolism, and Biosynthesis of amino acids metabolic pathways (Figure 7J). Contrasted with the MOD group, the DEMs in the FMT group (Supplementary Table S6) demonstrated enrichment in Taurine and hypo taurine metabolism, Glycine, serine and threonine metabolism,

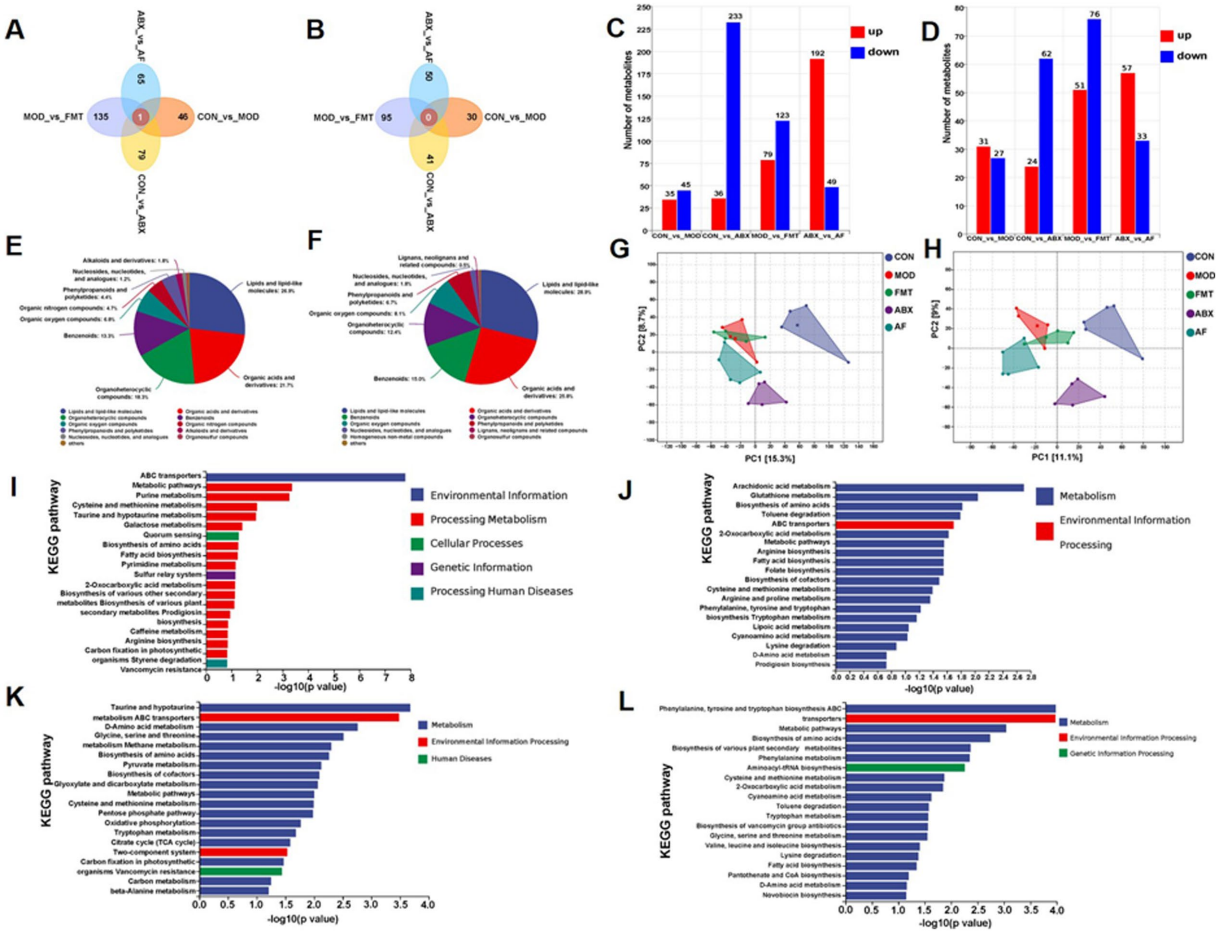

FIGURE 7
FMT modulates gut microbiota metabolite composition in GE rats. There were five groups: control group (CON), GE model group (MOD), GE+ fecal microbiota transplantation group (FMT), antibiotic clearance + GE group (ABX) and antibiotic removal + GE + fecal microbiota transplantation group (AF). **(A,B)** Venn graph of each group in positive and negative modes ($n = 5$). **(C,D)** Positive and negative modes comparison between groups of DEMs statistics ($n = 5$). **(E,F)** Positive and negative modes identification of metabolites ($n = 5$). **(G,H)** Partial Least Squares-Discriminate Analysis in positive and negative modes ($n = 5$). **(I–L)** Statistics of metabolic pathways between sample groups (COM-MOD, CON-ABX, MOD-FMT, ABX-AF) ($n = 5$).

as well as D-Amino acid metabolism (Figure 7K); Compared with the ABX group, The DEMs in the AF group (Supplementary Table S7) exhibited enrichment in pathways related to Phenylalanine, Tyrosine, and Tryptophan biosynthesis as well as Metabolic and amino acid biosynthesis pathways (Figure 7L).

3.7 Correlation between microbiome composition, differentially expressed metabolites, and phenotypic variables

In order to further investigate the mutual influence of microbiome composition, differentially expressed metabolites (DEMs), and phenotypic variables, we conducted pairwise correlation analysis involving two to three variables. Additionally, we performed correlation analysis to explore the interaction among microbiome composition, DEMs, and phenotypic variables through pairwise comparison of the three. We focused on the top 10 bacterial genera with the most significant differences at the genus level (Supplementary Table S3). The top 20 metabolites exhibiting the largest differences in various comparison groups (MOD vs. CON: Supplementary Table S4; ABX vs. CON: Supplementary Table S5; FMT vs. MOD: Supplementary Table S6; AF vs. ABX: Supplementary Table S7) were selected for correlation analysis (Figures 8A–D). In comparison with the CON group, *Allobaculum* and *Ligilactobacillus* in the MOD group exhibited significant correlations with differentially expressed metabolites (DEMs) (Figure 8A). In comparison with the CON group, *Allobaculum*, *Clostridium_T*, etc., displayed correlations with DEMs in the ABX group (Figure 8B). When compared to the MOD group, *Marvinbryantia* and *Blautia_A* demonstrated correlations with DEMs in the FMT group (Figure 8C). Conversely, compared to the ABX group, *Marvinbryantia*, *Faecousia*, etc., exhibited correlation with DEMs in the AF group (Figure 8D). The microbiota did not exhibit strong correlations with metabolites; therefore, we conducted a correlation analysis between the microbiota and tight junction proteins as well as Tregs-related factors (Figure 8E). Our findings revealed a positive correlation between the expression of tight junction proteins and *Ligilactobacillus* and *Blautia_A*. Conversely, Tregs-related factors exhibited a negative correlation with *Ligilactobacillus* and *Allobaculum*. Additionally, we examined the correlation between differentially expressed genes (DEGs) and the expression of brain barrier function indexes (Occludin, Claudin-1, and ZO-1) and immune function indexes (IL-10, Foxp3, and PD-1) (Figures 8F–I). The results indicated that, in comparison with the CON group, the MOD group exhibited positive correlations between poncirin and trihexyphenidyl, among others, and the index expression. Conversely, perphenazine and sulfoacetic acid showed negative correlations with the index expression (Figure 8F). In the ABX group, hc toxin displayed a positive correlation while zectran and cinchonidine, among others, exhibited negative correlations (Figure 8G). Furthermore, compared to the MOD group, the FMT group demonstrated positive correlations for trihexyphenidyl and simazine among others; however, oxybutynin showed a negative correlation (Figure 8H). Lastly, in comparison with the ABX group, hc toxin was positively correlated with the brain barrier function indexes (Occludin, Claudin-1, and ZO-1) and immune function indexes (IL-10, Foxp3, and PD-1) in the AF group, while zectran and pilocapine were negatively correlated with those aforementioned indexes (Figure 8I).

4 Discussion

Numerous studies have highlighted the significant role of the "gut-brain axis" in mediating communication between gut and brain tissues, with the amelioration of traumatic brain injury (TBI) being closely associated with the modulation of intestinal flora (Liu et al., 2020). Conversely, dysbiosis of intestinal flora may result in compromised brain function, this type of injury can lead to neuroinflammation and other injuries (Alam et al., 2018), and various pro-inflammatory factors can cause chronic neuroinflammatory symptoms, leading to cognitive impairment and disturbances in memory formation (Kim et al., 2021; Kim et al., 2020). Hence, we postulate that enhancing gut microbiota could mitigate gas explosion (GE) induced cognitive impairment by modulating brain tissue via the "gut-brain axis".

To investigate this hypothesis, we developed both a GE model and an FMT model to further validate the role of gut microbiota in cognitive impairment induced by GE. Additionally, we conducted an antibiotic clearance experiment to deplete the majority of gut microbiota and facilitate bacterial colonization (Jing et al., 2021). Our findings indicate that total moving distance and average moving speed in the open field box were restored in both FMT and AF rats following fecal bacteria transplantation from the CON group into recipient rats, suggesting that microbial dysbiosis caused by GE may lead to cognitive impairment. Furthermore, we observed a reduction in brain tissue cell count, mitochondrial cavitation, and loss of most cristae in MOD and ABX rats, all of which showed improvement after FMT treatment, consistent with recent research (Wang X. et al., 2023). Similarly, we observed that GE led to the down-regulation of tight junction proteins (Claudin-1, Occludin, and ZO-1) and the up-regulation of Tregs-related factors protein (IL-10, PD-1, and Foxp3), as well as pro-inflammatory cytokines (IL-1β) in the colon and striatum of MOD and ABX rats. Following the remodeling of intestinal flora in FMT experiment, all indices were restored. These findings indicate that GE induces both damage and inflammation in both the intestinal barrier and brain barrier of rats, which contributes to cognitive impairment and subsequent immune system activation. We posit that modulating intestinal flora directly contributes to ameliorating colon injury while working in conjunction with the immune system to facilitate repair of the blood–brain barrier and nervous system via the "gut-brain axis", thereby enhancing cognitive function.

The alpha diversity of the gut microbiota decreased after GE, indicating a reduction in species richness. In mammals, the decrease in gut bacterial species has been linked to an elevated risk of cognitive dysfunction through the "gut-brain axis" (Zheng et al., 2024). Furthermore, GE led to significant structural changes in the gut bacterial community of male rats, characterized by increased abundance of *Akkermansia*, *Romboutsia* and *Allobaculum* at the genus level, consistent with our previous findings (Dong et al., 2024). The gut microbiota of recipient rats following FMT exhibited similar behavior to that of the Control group. *Akkermansia* is a gram-negative anaerobic bacterium representing *Verrucomicrobia* and plays a key role as a mucin-degrading bacterium in the colon (Cani et al., 2022). Studies have indicated that *Akkermansia* may modulate TBI in the cerebral

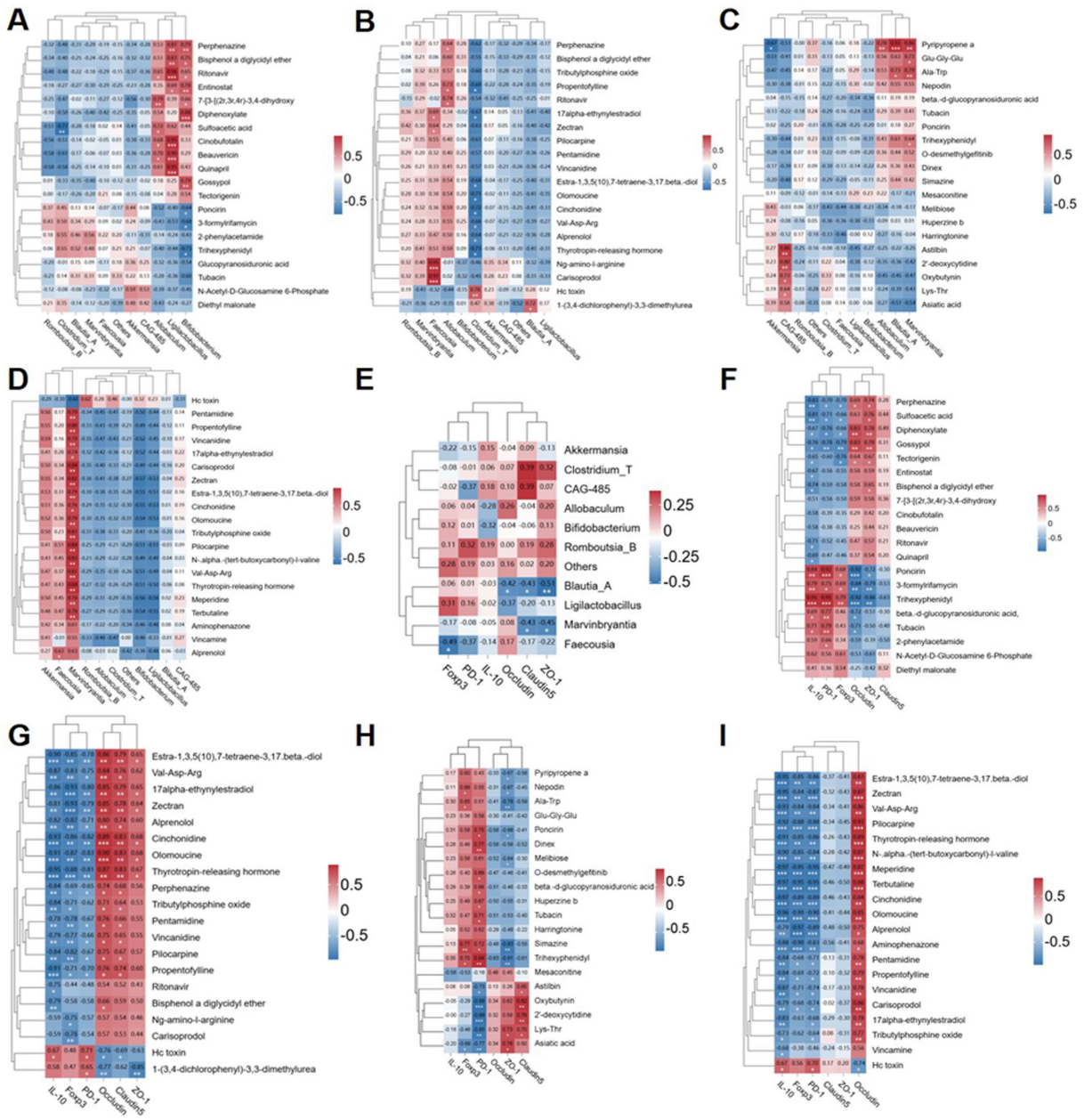

FIGURE 8
Correlation heatmap intergroup analysis was conducted to examine the relationships among microbiome composition, differentially expressed metabolites (DEMs), and phenotypic variables. **(A–D)** The top 20 DEMs were correlated with microbiome composition at the genus level in four groups (COM-MOD, CON-ABX, MOD-FMT, ABX-AF). **(E)** Correlation analysis was performed between the top 10 genera of microbiome composition and junction proteins (Claudin-1, Occludin and ZO-1), as well as Tregs-associated factors protein (PD-1, Foxp3 and IL-10) in the striatum. **(F–I)** Additionally, correlation analysis was carried out between the top 20 DEMs in the four groups and junction proteins (Claudin-1, Occludin and ZO-1), along with Tregs-associated factors protein (PD-1, Foxp3 and IL-10) in the striatum.

cortex by regulating NLRP3 inflammatory activation to ameliorate nerve inflammation and neurological damage (Chen et al., 2023). Furthermore, it can also restore mitochondrial structure and function to mitigate brain injury and intestinal barrier dysfunction by modulating mitochondrial homeostasis and metabolism (Lian et al., 2023). This aligns with our observations from H&E staining and TEM analysis. Additionally, *Akkermansia* is capable of producing butyric acid in the intestine (DeSana et al., 2024), which has been reported in studies to improve dopamine metabolism disorders and cognitive impairment (Wang et al., 2020). Meanwhile, studies have shown that with the enrichment of *Akkermansia*, the concentration of SCFAs also increases (Li et al., 2023), this also indicates the positive role of *Akkermansia* in the intestine. Current research suggests that butyric acid, as an intestinal metabolite, is among the most crucial short-chain fatty acids for TBI repair (Witkin et al., 2024). As a newly discovered genus, recent studies have indicated that an decrease in *Allobaculum* abundance in the colon may contribute to brain injury recovery (Yang et al., 2023), enhance the level of gut brain barrier proteins and alleviate

brain inflammation response (Yuan et al., 2022). There is also study indicating that as a gut probiotic, *Allobaculum* has the potential to reshape the gut brain axis as a mediator and repair nerve injury (Rahman et al., 2023). The two types of bacteria, which are the intestinal bacteria in the intestinal mucosa, play distinct roles in the composition and integrity of the intestinal barrier (van Muijlwijk et al., 2021). It is noteworthy that *C. butyricum* was also found to be one of the most abundant bacteria in the microbiota. Numerous studies have confirmed that *C. butyricum* metabolizes butyrate in the colon and exerts its effects on brain tissue through the "gut-brain axis" (Stoeva et al., 2021), *C. butyricum* can improve the integrity of the blood–brain barrier (Liu et al., 2024) and thus improve cognitive impairment (Wang J. et al., 2023). The three species mentioned above may contribute to our experimental results and validate our hypothesis. They recolonize the colon following FMT, ameliorating intestinal injury and TBI, consequently enhancing cognitive ability. Interestingly, LefSe analysis revealed an increase in relative abundance of *g_Clostridia_T* in both FMT and AF groups after FMT. Furthermore, elevated levels of *g_Faecalibacillus* and *g_Oribacterium* identified in our study were found to activate the immune system (Wang et al., 2024). Subsequently, we identified enriched metabolic pathways at the genus level of gut microbiota. The top three pathways enriched in bacterial genera were amino acid biosynthesis, nucleoside and nucleotide biosynthesis, and fatty acid and lipid biosynthesis. Previous studies have indicated that fatty acid biosynthesis can dynamically improve neurodegeneration and nerve regeneration, as well as help to ameliorate cognitive decline caused by TBI (Witkin et al., 2024). Meanwhile, nucleoside and nucleotide biosynthesis pathways have been associated with restoring hippocampal function and preventing or ameliorating schizophrenia-like behaviors (Hao et al., 2022). In addition, fatty acid and lipid biosynthesis play a crucial role in maintaining lipid balance in the body. Their oxidative stress can alleviate nerve inflammation and neurodegeneration by activating astrocytes and stimulating neurons through the IL-3 signaling pathways (Mi et al., 2023). The significant involvement of gut microbiota in CNS disease is undeniable. Conversely, gut microbes have the potential to reverse brain function deterioration. For instance, transplanting fecal microbes from young mice into older mice can delay aging-induced impairments in brain function, including cognitive-behavioral deficits (D'Amato et al., 2020). These findings underscore the substantial therapeutic potential for reversing TBI-related effects by modulating the gut microbiota (Choi and Cho, 2016). Therefore, it would be valuable to observe the behavioral phenotypes of rats treated with FMT in future studies to explore its exceptional therapeutic potential. Our study demonstrates that GE-induced gut microbiota disorder could contribute to increased proinflammatory cytokines in the brain. These results suggest that modulating the gut microbiota could serve as a therapeutic target for GE-induced TBI. For example, optimizing the gut microbiota through probiotic administration and FMT may be a prospective strategy for treating GE-induced TBI and subsequent long-term cognitive dysfunction.

In animal models, glyphosate exposure can induce chronic gut and systemic inflammation, often accompanied by dysbiosis of the gut microbiota (Dong et al., 2024). Our findings indicate that normal rats exposed to glyphosate may experience more severe inflammation than control rats, suggesting a potential exacerbation of inflammation by gut bacteria. The permeability of the gut epithelium is primarily regulated by a series of tight junction (TJ) proteins expressed by gut epithelial cells. Proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1β can disrupt intestinal TJ proteins, leading to increased permeability of the gut epithelium (Al-Sadi et al., 2009). TJ proteins restrict paracellular movement of molecules between adjacent epithelial cells and form a network regulatory mechanism that anchors neighboring cells while maintaining the physical barrier of the gut. ZO-1 belongs to a group of auxiliary TJ proteins that bind Occludin, a transmembrane TJ protein, to the cytoskeleton (Huang et al., 2020). The protein Claudin-1 is responsible for maintaining the integrity of the colon mucosal barrier and ensuring its functional stability (Huang et al., 2020). Our findings indicate that rats exposed to GE showed reduced expression of TJ proteins, which are crucial for protecting epithelial cells. These results suggest an increase in colon permeability in rats following exposure to GE. Furthermore, FMT confirmed that gut microbiota disorder induced by GE leads to gut barrier damage, potentially facilitating bacterial translocation.

To further investigate the potential mechanism of gut microbiota in ameliorating cognitive impairment induced by GE, we conducted untargeted metabolomics to monitor alterations in metabolites. We observed distinct changes in metabolites among different groups, mirroring microbial shifts. Following FMT treatment of GE model rats, we were surprised to discover an enrichment of more DEMs in Taurine and hypo taurine metabolism. Intestinal injury results in taurine metabolism dysregulation and inflammatory response, leading to intestinal barrier damage (Zhou et al., 2019). Studies have shown that taurine can mitigate inflammation and oxidative stress via the MAPK signaling pathway to alleviate LPS-induced injury (Zhang B. et al., 2022). LPS has been demonstrated to induce an inflammatory response by enhancing the permeability of the blood–brain barrier and translocating across the intestinal barrier into systemic circulation (Galea, 2021). LPS is recognized as a significant factor in disrupting the BBB, which restricts the entry of molecules from systemic circulation into brain tissue and is essential for maintaining CNS stability (Banks and Robinson, 2010). Elevated levels of LPS in brain circulation led to increased BBB permeability and subsequent brain damage. The BBB is a highly specialized structure formed by brain micro vessels through TJ proteins, such as ZO-1 and Occludin, that link adjacent cells (Cardoso et al., 2010). Reduced expression or altered distribution of TJ proteins in brain endothelium can result in BBB impairment, leading to neurovirulence, neuroinflammation, and potentially contributing to cognitive dysfunction. Our study revealed that gut microbiota imbalance induced by GE serves as a source of inflammatory response, potentially leading to elevated proinflammatory cytokines in the brain and reduced levels of brain TJ proteins.

Furthermore, a significant number of differentially expressed metabolites (DEMs) are involved in D-amino acid metabolism. Research has also demonstrated that the release of D-serine from glial cells can inhibit synaptic damage, restore nerve cell function, and consequently improve cognitive function (Tapanes et al., 2022). In this study, more metabolites were enriched in these two pathways, supporting our hypothesis that activating these pathways may improve cognitive impairment caused by GE via the "gut-brain axis." Importantly, fatty acid biosynthesis was also a key focus of DEMs enrichment. This pathway primarily generates new fatty acids in the gut, while SCFAs are products of intestinal flora and their levels are inversely related to inflammation (Opeyemi et al., 2021). Thus, the metabolite pathway enrichment results were consistent with our microbiome experiments, further confirming the importance of the "gut-brain axis" in improving

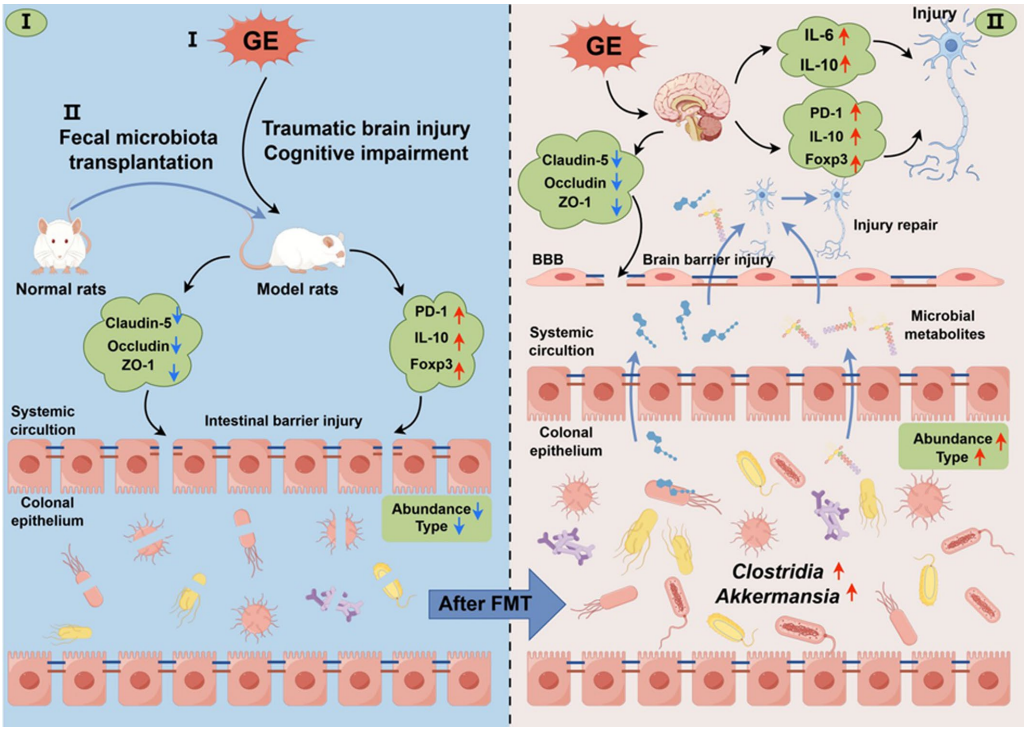

FIGURE 9
Schematic representation of fecal microbiota transplantation (FMT) intervention in the context of colon and brain barrier function injury/repair following traumatic brain injury (TBI) induced by GE (GE). GE induces dysbiosis of the abundance and type of gut microbiota in male TBI rats. Targeted supplementation of gut bacteria not only mitigated acute brain injury and cognitive dysfunction, but also significantly improved the immune microenvironment, the types and abundance of gut microbiota are increased after FMT, facilitating restoration of colon and brain barrier function after GE-induced TBI. These findings were corroborated by the application of FMT intervention post-TBI. Collectively, these results demonstrate that gut microbiota and their metabolites promote restorative activation of the "gut-brain axis" for neuroprotection, contributing to accelerated repair of colon and brain barriers as well as recovery of cognitive function after GE-induced TBI.

cognitive impairment. Gut bacteria play a significant role in this process. Additionally, Person correlation analysis revealed that the microbiota and DEMs enriched in these pathways correlated with the expression of tight junction proteins (ZO-1, Claudin-5, and Occludin) and Tregs-related factors (IL-10, PD-1, and Foxp3). These findings suggest that GE may induce inflammatory responses, which could be exacerbated by gut bacteria. Furthermore, FMT demonstrated that GE-induced gut microbiota dysbiosis and metabolic disorders could trigger increased brain proinflammatory cytokines. Therefore, it is important to consider that while gut bacteria are one cause of GE-induced TBI, they are not the only cause (Dong et al., 2020). Furthermore, the LPS-induced inflammatory response can result in abnormal behavior. For instance, brain inflammation triggered by LPS may contribute to GE-induced TBI development (Dong et al., 2024). however, behavioral changes (e.g., exploratory activity) are not always linked to brain inflammation (Fragopoulou et al., 2019). Therefore, behavioral experiments would help to extend our findings. In future studies on GE injury, more research should be performed on the relationship between gut microbiota, brain inflammation, and behavioral phenotypes.

Our research has provided new insights into understanding the injury mechanism of GE-induced TBI at a deeper level. However, while we used molecular experimental data to characterize brain inflammation, we did not conduct functional analysis. As a result, our comprehensive characterization of specific damage to the brain and barrier function was limited. Although FMT confirmed that GE-induced gut microbiota disorder may contribute to brain inflammation through the fatty acid biosynthesis pathway, it remains uncertain whether this is the sole contributing factor. We recommend additional intervention approaches such as cytokine antibody neutralization and *in vitro* experiments to confirm this association.

5 Conclusion

Gas explosion increases the risk of traumatic brain injury in miners. Currently, research on the pathogenic mechanism of GE-induced TBI is limited. However, our study provides new insights into addressing these unknowns. In summary, GE triggers dysbiosis of intestinal flora in male TBI rats. Targeted supplementation of gut bacteria not only reduced acute brain injury and cognitive dysfunction but also significantly improved the immune microenvironment, promoting restoration of colon and brain tight junctions after GE-induced TBI. These findings were further supported by administering an FMT intervention post-TBI. Collectively, these results demonstrate that specific gut microbiota such as *Clostridium_T* and *Allobaculum*, along with their metabolites, stimulate restorative "gut-brain axis" activation for neuroprotection, contributing to accelerated repair of the colon and brain as well as recovery of cognitive function after GE-induced TBI (Figure 9).

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 approved by the Animal Ethics Committee of the Xinxiang Medical University (No. XYLL-2020007), the animal disposal was performed according to the animal ethics standards. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XD: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. YS: Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft. ZL: Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft. CL: Formal analysis, Methodology, Writing – original draft. JG: Formal analysis, Methodology, Writing – original draft. XH: Methodology, Software, Validation, Writing – original draft. SY: Project administration, Resources, Supervision, Writing – original draft. WW: Data curation, Resources, Writing – review & editing. LT: Resources, Software, Writing – original draft. YB: Formal analysis, Methodology, Validation, Writing – original draft. GW: Resources, Supervision, Visualization, Writing – original draft. WR: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1485936/full#supplementary-material

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The gut-brain-metabolic axis: exploring the role of microbiota in insulin resistance and cognitive function

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The gut-brain-metabolic axis has emerged as a critical area of research, highlighting the intricate connections between the gut microbiome, metabolic processes, and cognitive function. This review article delves into the complex interplay between these interconnected systems, exploring their role in the development of insulin resistance and cognitive decline. The article emphasizes the pivotal influence of the gut microbiota on central nervous system (CNS) function, demonstrating how microbial colonization can program the hypothalamic–pituitary–adrenal (HPA) axis for stress response in mice. It further elucidates the mechanisms by which gut microbial carbohydrate metabolism contributes to insulin resistance, a key factor in the pathogenesis of metabolic disorders and cognitive impairment. Notably, the review highlights the therapeutic potential of targeting the gut-brain-metabolic axis through various interventions, such as dietary modifications, probiotics, prebiotics, and fecal microbiota transplantation (FMT). These approaches have shown promising results in improving insulin sensitivity and cognitive function in both animal models and human studies. The article also emphasizes the need for further research to elucidate the specific microbial species and metabolites involved in modulating the gut-brain axis, as well as the long-term effects and safety of these therapeutic interventions. Advances in metagenomics, metabolomics, and bioinformatics are expected to provide deeper insights into the complex interactions within the gut microbiota and their impact on host health. Overall, this comprehensive review underscores the significance of the gut-brain-metabolic axis in the pathogenesis and treatment of metabolic and cognitive disorders, offering a promising avenue for the development of novel therapeutic strategies targeting this intricate system.

KEYWORDS

brain-gut axis, microbiota, insulin resistance, cognition, hypothalamo-hypophyseal system, volatile fatty acids, neuroinflammatory diseases, neuronal plasticity

1 Introduction

The gut-brain-metabolic axis represents a complex and bidirectional communication network that integrates the gut microbiota, the central nervous system (CNS), and metabolic processes (Zhang Q. et al., 2022). Emerging evidence suggests that the gut microbiota plays a crucial role in modulating insulin resistance and cognitive function through various mechanisms (Zhang Q. et al., 2022; Takeuchi et al., 2023). Understanding the interplay between the gut microbiota, insulin resistance, and cognitive function is crucial for developing targeted therapeutic interventions to prevent and treat metabolic and neurodegenerative disorders (Fekete et al., 2024).

The gut microbiota can influence brain function and metabolic processes through neural, endocrine, and immune pathways (Makris et al., 2021). Microbial metabolites, particularly short-chain fatty acids (SCFAs), have been shown to modulate inflammation, enhance gut barrier integrity, and promote neurogenesis (Loh et al., 2024). Butyrate, a SCFA produced by gut bacteria, has been found to improve insulin sensitivity and protect against neuroinflammation (Li et al., 2022).

Insulin resistance in the brain, often referred to as brain insulin resistance, has been associated with cognitive decline and neurodegenerative diseases like Alzheimer's disease (Ye et al., 2022). Impaired insulin signaling in the brain leads to decreased glucose uptake, increased oxidative stress, and neuroinflammation, contributing to synaptic dysfunction and neuronal loss (Martinez-Montoro et al., 2022). Gut microbiota dysbiosis has been linked to increased intestinal permeability and systemic inflammation, which can exacerbate insulin resistance and cognitive impairment (Rubinstein et al., 2023).

Therapeutic interventions targeting the gut-brain-metabolic axis, such as probiotics, prebiotics, dietary modifications, and fecal microbiota transplantation (FMT), have shown promise in improving insulin sensitivity and cognitive function (Kolobaric et al., 2024). However, further research is needed to optimize these interventions, understand their long-term effects, and identify the most effective strategies for different populations (Zheng et al., 2023).

Despite of all of the knowledge in this field of study, the majority of the existing research has focused on the general population or specific disease states, such as metabolic disorders and neurodegenerative diseases (Matheson and Holsinger, 2023). However, the role of the gut-brain-metabolic axis in other conditions, including psychiatric disorders and neurological diseases, remains largely unexplored. Expanding the scope of research to investigate the broader implications of this axis could lead to the development of more personalized and effective therapeutic strategies (Loh et al., 2024).

Current research on the gut-brain-metabolic axis is diverse, focusing on various mechanisms through which gut microbiota influence insulin resistance and cognitive function (Fekete et al., 2024). For instance, it has demonstrated the role of SCFAs in modulating inflammatory pathways, while other research highlights the influence of gut-derived neurotransmitters on cognitive processes (Silva et al., 2020). However, the landscape of research remains fragmented, with varying methodologies and outcomes across different studies, leading to ongoing debates about the precise mechanisms at play. As such, it is essential to approach conclusions with caution and recognize the need for further investigation to build a comprehensive understanding of these interactions.

This review aims to provide a comprehensive analysis of the role of gut microbiota in insulin resistance and cognitive function, highlighting the underlying mechanisms and potential therapeutic targets. By elucidating the complex interactions within the gut-brain-metabolic axis, we can pave the way for personalized approaches to prevent and treat metabolic and neurodegenerative disorders.

2 Gut-brain axis: mechanisms of interaction

2.1 Gut microbiota and the central nervous system

The gut-brain axis facilitates bidirectional communication between the gut microbiota and the CNS through neural, endocrine, and immune pathways (Forsythe and Kunze, 2013).

2.1.1 Neural pathways

The vagus nerve plays a pivotal role in the neural pathway, serving as the primary conduit for communication between the gut and the brain (Breit et al., 2018). Through afferent sensory fibers, the vagus nerve detects changes in the gut environment, such as microbial composition and nutrient availability, and transmits these signals to the brainstem (Gershon and Margolis, 2021). Studies have shown that activation of the vagus nerve can modulate mood and behavior, influencing anxiety and depression (Breit et al., 2018). Additionally, gut-derived metabolites, such as SCFAs, can influence vagal afferent signaling, further linking gut microbiota to neural pathways (Silva et al., 2020).

2.1.2 Endocrine pathways

The hypothalamic–pituitary–adrenal (HPA) axis is central to the endocrine communication between the gut and brain (Rusch et al., 2023). Stress-induced activation of the HPA axis leads to the release of cortisol, which can affect gut permeability and alter microbial composition (Rusch et al., 2023). In turn, certain gut microbes can modulate the release of hormones like ghrelin and leptin, which regulate appetite, energy homeostasis, and even cognitive function (Han et al., 2021). For example, microbial metabolites such as SCFAs and bile acids can influence hormone secretion by interacting with enteroendocrine cells in the gut (Masse and Lu, 2023).

2.1.3 Immune pathways

Immune signaling is another crucial component of gut-brain communication (Kasarello et al., 2023). The gut-associated lymphoid tissue (GALT) plays a key role in immune surveillance and responds to microbial antigens (Kasarello et al., 2023). Cytokines released by immune cells in the gut can cross the blood–brain barrier or signal through the vagus nerve, influencing neuroinflammation and CNS function (Kasarello et al., 2023). Dysbiosis, or microbial imbalance, has been linked to an increase in pro-inflammatory cytokines, which can contribute to neuroinflammatory conditions such as Alzheimer's disease and depression (Leblhuber et al., 2021).

The vagus nerve is a primary conduit for these signals, transmitting information from the gut to the brain and vice versa.

Gut microbiota can influence brain function by producing neurotransmitters such as serotonin, dopamine, and gamma-aminobutyric acid (GABA), which can cross the blood–brain barrier and affect mood, cognition, and behavior (Cryan and Dinan, 2012).

Gut microbiota also affects the HPA axis, a major stress response system (Rusch et al., 2023). Dysbiosis can lead to altered HPA axis activity, resulting in changes in cortisol levels that impact both metabolic and cognitive functions (Misiak et al., 2020). Changes in HPA axis activity can be assessed through various methods, such as measuring cortisol levels in saliva, blood, or hair samples (Wright et al., 2015). These assessments provide insight into the relationship between dysbiosis and stress response, highlighting how disruptions in gut microbiota can influence cortisol production, which in turn affects metabolic and cognitive health (Rogers et al., 2016). Long-term alterations in cortisol levels due to dysbiosis may contribute to insulin resistance, inflammation, and impaired cognitive function (Rogers et al., 2016). Additionally, microbial metabolites such as SCFAs play a critical role in modulating inflammation and maintaining the integrity of the blood–brain barrier, which is crucial for preventing neuroinflammation and preserving cognitive function (Braniste et al., 2014).

The bidirectional communication between the gut microbiota and the host involves complex neural, endocrine, and immune pathways, each playing distinct roles in modulating metabolic and cognitive functions:

2.1.4 Neural pathways

The gut-brain axis facilitates communication between the gut microbiota and the central nervous system (CNS) through the vagus nerve (Carabotti et al., 2015). This pathway enables the transmission of signals, such as neurotransmitters (e.g., serotonin and dopamine), produced by gut microbiota, which can influence mood, cognition, and behavior (Chen et al., 2021). Additionally, gut microbiota can affect the release of neurotrophic factors that support neuronal health and synaptic plasticity, essential for learning and memory (Glinert et al., 2022).

2.1.5 Endocrine pathways

Gut microbiota interact with the endocrine system by influencing the secretion of various gut hormones, including glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Zeng et al., 2024). These hormones not only regulate appetite and glucose homeostasis but also have neuroprotective effects that can enhance cognitive function (Suzuki et al., 2011). Dysregulation of these hormones due to altered gut microbiota composition has been linked to insulin resistance and cognitive decline (Patra et al., 2023).

2.1.6 Immune pathways

The gut microbiota plays a crucial role in modulating the immune system, particularly through the production of SCFAs that promote anti-inflammatory responses (Wu and Wu, 2012). These metabolites can influence systemic inflammation, which is a contributing factor in both insulin resistance and neuroinflammation (Vinuesa et al., 2021). Furthermore, the immune response triggered by gut microbiota can affect the permeability of the blood–brain barrier, thereby impacting the communication between the gut and the CNS (Tang et al., 2020).

By elucidating these pathways, we can better understand the mechanisms through which gut microbiota influence both metabolic and cognitive health. Future research should focus on the interplay between these pathways to develop targeted interventions that can optimize health outcomes.

2.2 Role of microbial metabolites

Microbial metabolites, particularly SCFAs like acetate, propionate, and butyrate, are produced through the fermentation of dietary fibers by gut bacteria (Abdelhalim, 2024). SCFAs have significant effects on host metabolism and brain function (Qian et al., 2022). Butyrate, for instance, has anti-inflammatory properties and can enhance insulin sensitivity by activating peroxisome proliferator-activated receptor gamma (PPAR-γ) and inhibiting histone deacetylases (HDACs), which regulate gene expression involved in glucose and lipid metabolism (Matheus et al., 2017).

Butyrate also promotes neurogenesis and protects against neuroinflammation by inhibiting nuclear factor-kappa B (NF-κB) signaling, a key pathway in inflammatory responses (Alpino et al., 2024). This neuroprotective effect is crucial in maintaining cognitive function and preventing neurodegenerative diseases. Additionally, SCFAs can influence gut-brain signaling through the gut hormone system, including the release of peptides like glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which regulate appetite and glucose homeostasis (Lai et al., 2024).

3 Dysbiosis and insulin resistance

3.1 Mechanisms of dysbiosis-induced insulin resistance

Dysbiosis, characterized by a reduced diversity and altered composition of gut microbiota, has been strongly associated with the development of insulin resistance (Abildinova et al., 2024). For example, a study demonstrated that individuals with type 2 diabetes had significantly lower levels of Firmicutes and increased Bacteroidetes, suggesting a microbial imbalance linked to insulin resistance (Craciun et al., 2022). Another study identified specific gut microbial markers associated with insulin resistance and type 2 diabetes, further supporting the link between dysbiosis and metabolic dysfunction (Wu et al., 2023). One primary mechanism involves increased intestinal permeability, often referred to as "leaky gut" (Vanuytsel et al., 2021). Dysbiosis can disrupt the gut barrier function, allowing the translocation of lipopolysaccharides (LPS) from gram-negative bacteria into the bloodstream, leading to metabolic endotoxemia (Violi et al., 2023). This endotoxemia triggers systemic inflammation, which impairs insulin signaling and promotes insulin resistance (Huang et al., 2021; Figure 1).

Inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) play a crucial role in this process by activating serine kinases that phosphorylate insulin receptor substrates, thereby inhibiting their ability to transmit insulin signals (Amabebe et al., 2020). In addition to TNF-α and IL-6, other pro-inflammatory cytokines such as interleukin-1β (IL-1β) also contribute to insulin resistance by exacerbating inflammatory

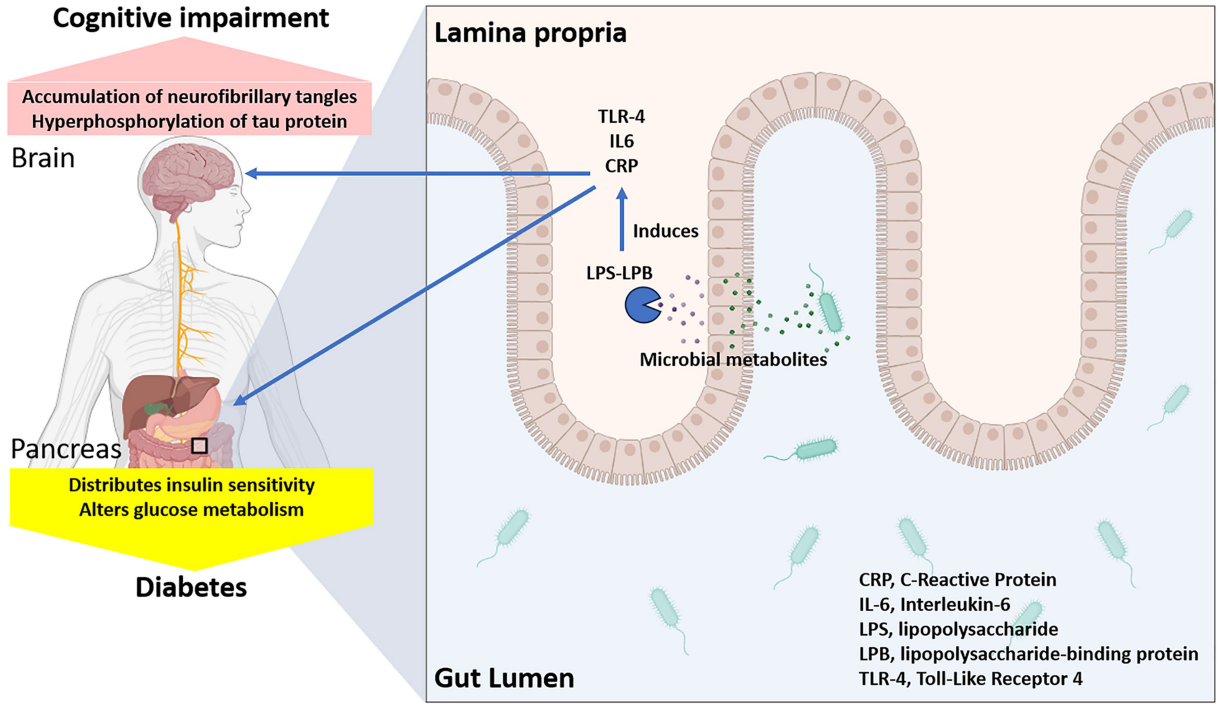

FIGURE 1
Gut dysbiosis and increased intestinal permeability leading to disrupted glucose metabolism and cognitive decline.

pathways (Al-Mansoori et al., 2022). These cytokines interact in a complex network, amplifying each other's effects through feedback loops that further promote inflammation (Megha et al., 2021). For instance, TNF-α can induce the production of IL-6 and IL-1β, which in turn perpetuate inflammation by activating downstream signaling pathways such as nuclear factor-kappa B (NF-κB) (Brasier, 2010). This persistent inflammatory state not only disrupts insulin signaling but also contributes to systemic metabolic dysfunction, highlighting the intricate and multifaceted role of cytokines in insulin resistance (Rehman and Akash, 2016). Moreover, dysbiosis-induced alterations in bile acid metabolism can influence insulin sensitivity (Patra et al., 2023). Bile acids act as signaling molecules that modulate metabolic processes through their receptors, such as farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (TGR5) (de Aguiar Vallim et al., 2013). Dysbiosis can disrupt bile acid homeostasis, leading to impaired insulin signaling and glucose metabolism.

3.2 Impact of gut microbiota on glucose metabolism

Gut microbiota influence glucose metabolism through various pathways, including the regulation of gluconeogenesis and glycolysis in the liver (Utzschneider et al., 2016). Certain gut bacteria, such as *Akkermansia muciniphila*, have been shown to improve glucose tolerance and insulin sensitivity by modulating mucin production and enhancing gut barrier integrity (Xu et al., 2020). Additionally, gut microbiota can affect the expression of glucose transporters in the intestinal epithelium, influencing glucose absorption and utilization (Ridaura et al., 2013).

Studies have demonstrated that FMT from lean donors to individuals with metabolic syndrome can improve insulin sensitivity, highlighting the potential of gut microbiota manipulation as a therapeutic strategy for insulin resistance (Kootte et al., 2017). Furthermore, dietary interventions that promote beneficial gut bacteria, such as high-fiber diets, have been shown to improve glycemic control and reduce the risk of type 2 diabetes (Deehan and Walter, 2016).

4 Insulin resistance and cognitive function

4.1 Insulin signaling in the brain

Insulin plays a crucial role in brain function, influencing synaptic plasticity, neurotransmitter release, and neuronal survival (Kleinridders et al., 2014). Insulin signaling in the brain is mediated through the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R), which activate downstream pathways such as phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) (Ferreira et al., 2018). These pathways are essential for maintaining cognitive functions, including learning and memory.

Impaired insulin signaling in the brain, often referred to as brain insulin resistance, has been associated with cognitive decline and neurodegenerative diseases like Alzheimer's disease (Escobar et al., 2022). Brain insulin resistance leads to decreased glucose uptake and utilization, increased oxidative stress, and neuroinflammation, all of which contribute to synaptic dysfunction and neuronal loss (Arnold et al., 2018).

4.2 Impact of insulin resistance on cognitive function

Insulin resistance is linked to cognitive impairment through several mechanisms (Tyagi and Pugazhenthi, 2021). Hyperinsulinemia, a compensatory response to peripheral insulin resistance, can lead to reduced insulin transport across the blood–brain barrier, resulting in decreased insulin availability in the brain (Vogelsang et al., 2018). This deficiency impairs glucose metabolism and energy production in neurons, contributing to cognitive deficits.

Additionally, insulin resistance is associated with increased amyloid-beta (Aβ) production and decreased clearance, a hallmark of Alzheimer's disease pathology (Escobar et al., 2022). Insulin can modulate the activity of enzymes involved in Aβ production and degradation, and impaired insulin signaling exacerbates Aβ accumulation and neurotoxicity. Moreover, insulin resistance is linked to tau hyperphosphorylation, another key feature of Alzheimer's disease, through the dysregulation of glycogen synthase kinase-3β (GSK-3β) (Rodriguez-Rodriguez et al., 2017).

5 Microbial metabolites and cognitive function

5.1 Role of SCFAs

SCFAs produced by gut microbiota have been shown to influence cognitive function through multiple pathways (Qian et al., 2022). SCFAs, such as acetate, propionate, and butyrate, exert their influence on cognitive function through several mechanisms (Qian et al., 2022). These include modulating the production of neurotransmitters like serotonin, gamma-aminobutyric acid (GABA), and dopamine, which are crucial for mood regulation and cognitive processes (Gasmi et al., 2022). SCFAs stimulate the release of gut hormones, such as glucagon-like peptide-1 (GLP-1), which can cross the blood–brain barrier and affect neuronal signaling (Moţăţăianu et al., 2023). Additionally, butyrate has been shown to inhibit histone deacetylase, enhancing gene expression linked to neuroprotection and cognitive health (Jaworska et al., 2017). Butyrate, in particular, enhances synaptic plasticity and memory formation by increasing the expression of brain-derived neurotrophic factor (BDNF) and promoting histone acetylation, which facilitates gene transcription involved in neurogenesis (Li et al., 2022). Butyrate also has anti-inflammatory properties, reducing neuroinflammation and protecting against cognitive decline (Matt et al., 2018; Figure 2).

SCFAs can also modulate the gut-brain axis by influencing the production of gut hormones such as GLP-1 and PYY, which have neuroprotective effects and regulate appetite and energy homeostasis, as previously described (Lai et al., 2024). Furthermore, SCFAs can cross the blood–brain barrier and directly impact brain function by interacting with G-protein-coupled receptors (GPCRs) expressed in the CNS (Frost et al., 2014).

5.2 Other microbial metabolites

In addition to SCFAs, other microbial metabolites such as tryptophan metabolites, bile acids, and neurotransmitters play significant roles in cognitive function (Figure 3). Tryptophan metabolites, including indole and its derivatives, can modulate the gut-brain axis by interacting with the aryl hydrocarbon receptor (AhR) and influencing immune responses and neuroinflammation (O'Mahony et al., 2015). Alterations in tryptophan metabolism have been linked to depression and cognitive impairment, highlighting the importance of gut microbiota in mental health.

Bile acids, traditionally known for their role in digestion, also function as signaling molecules that can influence brain function. Altered bile acid metabolism has been associated with cognitive decline and neurodegenerative diseases (Baloni et al., 2020). Bile acids can activate TGR5 and FXR receptors in the brain, regulating inflammation and synaptic plasticity (de Aguiar Vallim et al., 2013).

Neurotransmitters produced by gut microbiota, such as gamma-aminobutyric acid (GABA) and serotonin, also significantly impact cognitive function. GABA, an inhibitory neurotransmitter, can help regulate mood and anxiety levels, while serotonin is crucial for mood stabilization and is linked to various cognitive processes. The communication between gut-derived neurotransmitters and the central nervous system may occur through multiple pathways, including direct neural connections and the bloodstream. Recent studies suggest that dysregulation of these neurotransmitter levels can be associated with mood disorders and cognitive impairments, further emphasizing the intricate relationship between gut microbiota and mental health.

6 Therapeutic interventions

6.1 Probiotics and prebiotics

Probiotics, live microorganisms that confer health benefits to the host, have shown promise in improving insulin sensitivity and cognitive function. Specific strains of probiotics, such as *Lactobacillus* and *Bifidobacterium*, have been shown to reduce inflammation, enhance gut barrier integrity, and modulate metabolic pathways (Wallace and Milev, 2017). Probiotic supplementation can improve glycemic control and reduce markers of insulin resistance in individuals with metabolic disorders (Kootte et al., 2017; Salles et al., 2020).

Prebiotics, nondigestible dietary fibers that promote the growth of beneficial gut bacteria, also have significant potential in modulating the gut-brain-metabolic axis. Prebiotics such as inulin and fructooligosaccharides (FOS) can increase SCFA production, improve gut barrier function, and reduce systemic inflammation (Bindels et al., 2015; Costa et al., 2022). Dietary interventions that incorporate prebiotics can enhance insulin sensitivity and cognitive function by promoting a healthy gut microbiota composition (Deehan and Walter, 2016; Zhang et al., 2019).

6.2 Dietary interventions

Diet plays a crucial role in shaping the gut microbiota and influencing the gut-brain axis. High-fiber diets, rich in fruits, vegetables, and whole grains, promote the growth of beneficial gut bacteria and increase SCFA production, which can improve metabolic and cognitive health (Patterson et al., 2016). Conversely, diets high in

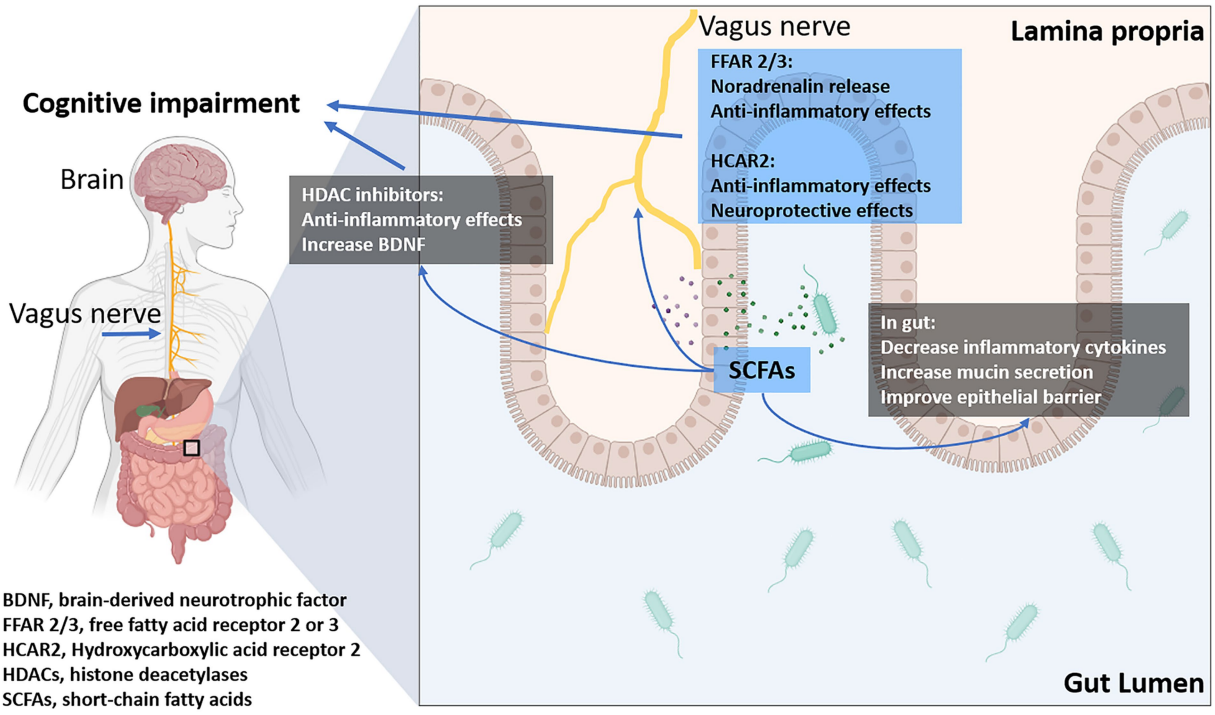

FIGURE 2
The influence of short-chain fatty acids (SCFAs) on cognitive impairment: SCFAs modulate intestinal inflammation and epithelial barrier function, indirectly affecting disease progression. Additionally, they directly influence the CNS by regulating energy metabolism, neuroinflammation through their receptors, transporters, and histone deacetylases (HDACs).

saturated fats and refined sugars can lead to dysbiosis, increased intestinal permeability, and systemic inflammation, contributing to insulin resistance and cognitive decline (Olson et al., 2018; Baspakova et al., 2024).

Intermittent fasting (IF) has emerged as a dietary intervention with potential benefits for the gut-brain-metabolic axis. IF involves cycles of eating and fasting, which can enhance gut microbiota diversity, reduce inflammation, and improve insulin sensitivity (Mattson et al., 2014; Guzzetta et al., 2022). IF has also been shown to promote neurogenesis and protect against cognitive decline by activating adaptive stress response pathways and increasing BDNF levels (Mattson et al., 2014).

6.3 FMT

FMT involves the transfer of gut microbiota from a healthy donor to a recipient with dysbiosis. FMT has shown promise in treating metabolic disorders and improving insulin sensitivity by restoring a healthy gut microbiota composition (Kootte et al., 2017). Emerging evidence suggests that FMT can also improve cognitive function in individuals with neurodegenerative diseases such as Alzheimer's disease (Baunwall et al., 2020; Tixier et al., 2022).

The therapeutic potential of FMT lies in its ability to reestablish a balanced gut microbiota, reduce systemic inflammation, and enhance gut barrier integrity. However, further research is needed to understand the long-term effects and optimize the protocols for FMT in treating metabolic and cognitive disorders (Baunwall et al., 2020; Tixier et al., 2022).

7 Discussion

The interplay between the gut microbiota, insulin resistance, and cognitive function represents a rapidly evolving field with significant implications for health and disease (Kossowska et al., 2024). Future research should focus on longitudinal studies to better understand the causal relationships between gut microbiota composition and changes in insulin sensitivity and cognitive outcomes. Investigating the effects of dietary interventions on gut microbiota, insulin resistance, and cognitive function could provide insights into potential therapeutic strategies. Additionally, exploring the mechanisms by which gut-derived metabolites influence brain function may open new avenues for addressing cognitive decline associated with metabolic disorders.

Key mechanisms involve neural, endocrine, immune, and metabolic pathways (Dinan and Cryan, 2017). The gut microbiota modulates the production of neurotransmitters such as serotonin and dopamine, influences gut-derived hormones like GLP-1, regulates systemic inflammation, and produces metabolites like SCFAs that enhance insulin sensitivity and influence cognitive function (Dicks, 2022). This review highlights the multifaceted mechanisms through which gut microbiota influence metabolic and cognitive processes.

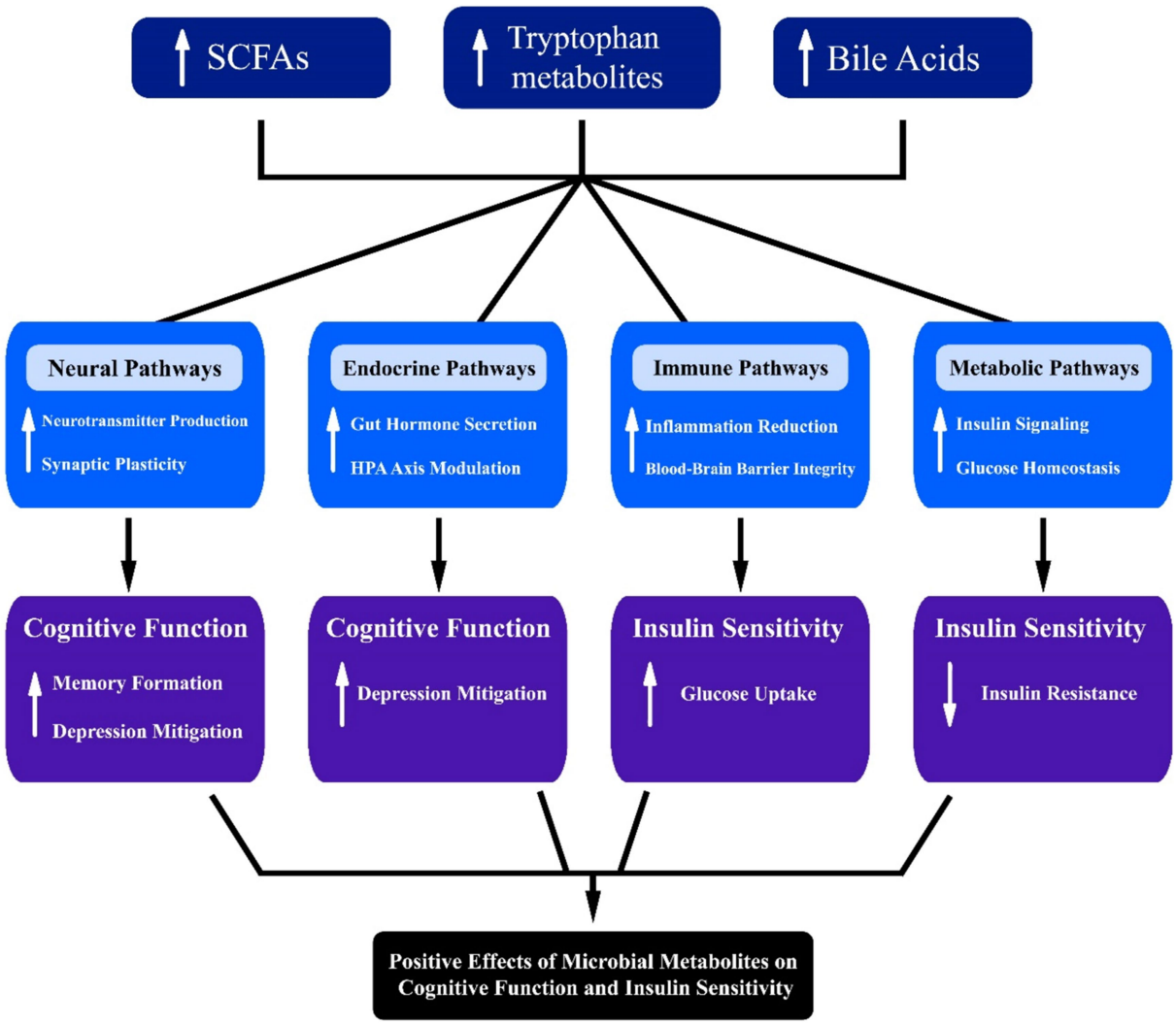

FIGURE 3
Interconnected pathways illustrating the influence of microbial metabolites, including short-chain fatty acids (SCFAs), tryptophan, and bile acids, on neural, endocrine, immune, and metabolic systems. Arrows denote directional interactions, highlighting potential implications for cognitive function, insulin sensitivity, and metabolic health.

7.1 Importance and rationale of this review

This comprehensive review aims to address the aforementioned deficiencies by providing a detailed analysis of the current understanding of the gut-brain-metabolic axis and its implications for insulin resistance and cognitive function. By synthesizing the latest research from various disciplines, including microbiology, neuroscience, and metabolic health, this review was offered a holistic perspective on the complex interplay between the gut microbiota, insulin resistance, and cognitive processes.

The findings of this review will be instrumental in guiding future research directions and informing the development of novel therapeutic interventions. For instance, future studies could explore the potential of targeting specific gut microbial species or their metabolites to improve insulin sensitivity and cognitive function. Additionally, research could focus on designing clinical trials that assess the efficacy of dietary modifications or probiotics in enhancing cognitive health in individuals with insulin resistance. By elucidating these pathways, we aim to pave the way for targeted interventions that leverage the gut-brain axis to mitigate cognitive decline. By elucidating the specific mechanisms underlying the gut-brain-metabolic axis, researchers and clinicians can work toward personalized approaches to prevent and treat metabolic and neurodegenerative disorders, ultimately improving the quality of life for affected individuals.

7.2 Novel hypotheses on gut-brain-metabolic axis

7.2.1 Hypothesis 1: multi-strain probiotic synergy

7.2.1.1 Rationale

Current research often focuses on individual probiotic strains. However, considering the complexity of the gut-brain-metabolic axis, a combination of multiple probiotic strains might have synergistic effects (Kwoji et al., 2021).

7.2.1.2 Hypothesis

Administering a multi-strain probiotic cocktail can synergistically enhance SCFA production, improve gut barrier integrity, and modulate neurotransmitter production more effectively than single-strain probiotics, leading to improved insulin sensitivity and cognitive function (Kwoji et al., 2021; Dias et al., 2022).

7.2.1.3 Pathways involved

7.2.1.3.1 Neural

Multi-strain probiotics promote the production of neurotransmitters like serotonin and dopamine via gut microbiota metabolites such as SCFAs. These neurotransmitters are crucial for cognitive processes, including mood regulation and memory function. SCFAs also influence neuroinflammation and the integrity of the blood–brain barrier, impacting cognitive function.

7.2.1.3.2 Endocrine

Increased production of gut hormones (GLP-1, PYY).

7.2.1.3.3 Immune

Reduced inflammation through enhanced SCFA production.

7.2.1.3.4 Metabolic

Improved insulin signaling and glucose homeostasis.

7.2.2 Hypothesis 2: personalized nutrition and microbiota modulation

7.2.2.1 Rationale

Individual variations in gut microbiota composition and metabolic health suggest that personalized dietary interventions could be more effective (Li, 2023).

7.2.2.2 Hypothesis

Personalized nutrition plans based on individual gut microbiota profiles can more precisely modulate gut microbiota composition, enhance SCFA production, and improve cognitive function and metabolic health compared to generalized dietary guidelines (Li, 2023; Kallapura et al., 2024).

7.2.2.3 Pathways involved

7.2.2.3.1 Neural

Personalized diets that enhance precursor availability for neurotransmitters like tryptophan (for serotonin synthesis) can improve gut-brain signaling. Individualized nutrition can also modulate SCFA production, which in turn influences the release of neurotransmitters crucial for cognition.

7.2.2.3.2 Endocrine

Diet-induced modulation of gut hormone levels.

7.2.2.3.3 Immune

Personalized nutrition reducing individual-specific inflammatory responses.

7.2.2.3.4 Metabolic

Enhanced insulin sensitivity through targeted dietary components.

7.2.3 Hypothesis 3: microbiota-derived exosome therapy

7.2.3.1 Rationale

Exosomes are extracellular vesicles that play a crucial role in intercellular communication. Gut microbiota-derived exosomes could carry bioactive compounds that influence distant organs, including the brain (Manzaneque-López et al., 2023; Díez-Sainz et al., 2021; Zhang B. et al., 2022; Zhao et al., 2021).

7.2.3.2 Hypothesis

Microbiota-derived exosome therapy can be developed to deliver specific microbial metabolites directly to the brain, enhancing cognitive function and insulin sensitivity by modulating the gut-brain-metabolic axis (Manzaneque-López et al., 2023; Díez-Sainz et al., 2021; Zhang B. et al., 2022; Zhao et al., 2021). However, several technical and safety challenges must be addressed to realize this therapeutic potential.

To overcome technical challenges, researchers should focus on optimizing exosome isolation and characterization methods to ensure purity and functionality. Advanced techniques such as ultrafiltration and size-exclusion chromatography can improve the yield and quality of exosomes. Moreover, developing robust delivery systems that protect exosomes from degradation and facilitate their transport across the blood–brain barrier is crucial.

Regarding safety challenges, comprehensive toxicological studies are essential to assess the biocompatibility and potential immunogenicity of microbiota-derived exosomes. Conducting preclinical trials in relevant animal models will help evaluate the therapeutic efficacy and safety profile before progressing to clinical trials. By addressing these challenges, microbiota-derived exosome therapy may become a viable strategy for enhancing cognitive function and metabolic health.

7.2.3.3 Pathways involved

7.2.3.3.1 Neural

Exosomes from microbiota can carry bioactive compounds, including neurotransmitters, peptides, and other neuroprotective molecules, across the gut-brain axis. This could influence brain function by modulating synaptic plasticity, reducing oxidative stress, and supporting neurogenesis, ultimately improving cognitive function and insulin sensitivity.

7.2.3.3.2 Endocrine

Regulation of HPA axis via exosome-contained signaling molecules.

7.2.3.3.3 Immune

Exosomes reducing systemic inflammation by inhibiting pro-inflammatory cytokine production, thereby improving blood–brain barrier integrity and preventing neuroinflammation.

7.2.3.3.4 Metabolic

Enhanced insulin signaling and metabolic regulation through targeted exosome delivery.

7.2.4 Hypothesis 4: microbiota-gut-brain peptide modulation

7.2.4.1 Rationale

Peptides produced by gut microbiota can influence gut-brain communication and metabolic processes (Lai et al., 2024; Sun et al., 2020).

7.2.4.2 Hypothesis

Enhancing the production of specific gut-derived peptides through dietary or probiotic interventions can improve gut-brain communication, reduce neuroinflammation, and enhance insulin sensitivity and cognitive function (Lai et al., 2024; Sun et al., 2020).

7.2.4.3 Pathways involved

7.2.4.3.1 Neural

Gut-derived peptides can enhance the release of neurotransmitters and support synaptic plasticity, crucial for learning and memory. For example, the gut peptide ghrelin has been shown to influence hippocampal function and cognitive performance, while other microbiota-derived peptides may reduce neuroinflammation.

7.2.4.3.2 Endocrine

Modulation of gut-brain peptides impacting hormone release.

7.2.4.3.3 Immune

Anti-inflammatory peptides reducing systemic and neuroinflammation.

7.2.4.3.4 Metabolic

Peptides enhancing insulin signaling pathways.

7.3 Therapeutic potential

Therapeutic interventions such as probiotics, prebiotics, dietary modifications, and FMT offer promising avenues for modulating the gut-brain-metabolic axis. Probiotics have been shown to increase the production of SCFAs, such as butyrate, which improve gut barrier integrity, reduce neuroinflammation, and enhance insulin sensitivity. SCFAs also cross the blood–brain barrier, impacting neurotransmitter synthesis and modulating cognitive function. Prebiotics selectively stimulate the growth of beneficial bacteria, thereby influencing metabolic pathways related to glucose homeostasis and neuroprotection. FMT, by restoring a balanced microbiota, can reduce systemic inflammation, restore insulin signaling, and improve cognitive performance by promoting the production of neuroactive metabolites. The efficacy of these interventions in improving insulin sensitivity and cognitive function underscores the potential for personalized approaches to prevent and treat metabolic and neurodegenerative diseases (Kootte et al., 2017; Matt et al., 2018). However, further research is needed to optimize these interventions, understand their long-term effects, and identify the most effective strategies for different populations.

7.4 Limitations of current therapeutic interventions

While therapeutic interventions targeting the gut-brain-metabolic axis, such as probiotics, prebiotics, dietary modifications, and FMT (Contarino et al., 2023), show promise in regulating insulin resistance and cognitive function, several limitations and unresolved issues warrant attention:

7.4.1 Variability in individual response

One of the significant challenges is the variability in individual responses to these interventions (Almeida et al., 2022). Factors such as baseline gut microbiota composition, genetic predispositions, dietary habits, and lifestyle can influence how individuals respond to therapeutic strategies (Strasser et al., 2021). This variability complicates the predictability of outcomes, making it difficult to generalize findings across populations.

7.4.2 Lack of standardization

Many probiotic products on the market lack standardization concerning strain composition, dosage, and delivery methods (Zawistowska-Rojek et al., 2022). This inconsistency can lead to varying efficacy and safety profiles across different studies, limiting the ability to draw definitive conclusions about their effectiveness in modulating the gut-brain-metabolic axis (Ullah et al., 2021).

7.4.3 Short-term study duration

Most clinical trials assessing the efficacy of therapeutic interventions are relatively short-term, often ranging from a few weeks to a few months (Biazzo and Deidda, 2022). This limited duration raises concerns about the sustainability of benefits observed and the potential emergence of adverse effects over longer periods of use (Biazzo and Deidda, 2022).

7.4.4 Mechanistic insights

There is still a lack of clear mechanistic understanding of how specific interventions exert their effects on the gut-brain-metabolic axis (O'Riordan et al., 2022). Without a robust understanding of the underlying biological pathways, it is challenging to optimize these therapies for maximum efficacy and safety (Chakrabarti et al., 2022).

7.4.5 Safety concerns

The long-term safety of interventions, particularly FMT, remains an unresolved issue (Dang et al., 2020). Concerns about the transfer of pathogenic microorganisms, alterations in gut microbiota composition, and unintended metabolic effects necessitate further investigation to ensure patient safety (Matzaras et al., 2022).

7.4.6 Regulatory challenges

Regulatory frameworks for probiotics and other microbiota-targeted therapies can be inconsistent, leading to disparities in product quality and safety (Merenstein et al., 2023). This inconsistency can hinder the advancement of effective therapies in clinical settings.

By addressing these limitations, future research can focus on developing more effective, personalized, and safer therapeutic interventions targeting the gut-brain-metabolic axis. Further studies

should aim to elucidate the specific microbial species and metabolites involved, evaluate long-term effects, and establish standardized protocols to enhance the clinical applicability of these interventions.

7.5 Challenges and controversies in researching the gut-brain-metabolic axis

Despite the promising findings regarding the role of gut microbiota in regulating insulin resistance and cognitive function, several challenges and controversies remain in this field:

7.5.1 Variability in microbiota composition

Individual variations in gut microbiota composition can lead to inconsistent results across studies. Factors such as diet, genetics, environment, and lifestyle can significantly influence microbial diversity, making it difficult to establish universal conclusions.

7.5.2 Causality vs. correlation

Much of the current research highlights correlations between gut microbiota and metabolic/cognitive outcomes. However, establishing causality remains a challenge, as it is unclear whether changes in gut microbiota directly cause metabolic and cognitive changes or if they are simply a consequence of these conditions.

7.5.3 Methodological differences

The diverse methodologies employed in studies, including variations in sampling techniques, analytical methods, and experimental designs, can lead to disparate findings. This lack of standardization complicates the interpretation and comparison of results.

7.5.4 Mechanistic understanding

While there are hypotheses about the mechanisms through which gut microbiota affect insulin resistance and cognitive function, more research is needed to elucidate these pathways fully. Understanding the specific microbial species and metabolites involved in these processes is crucial for advancing therapeutic approaches.

7.5.5 Safety and efficacy of interventions

As therapeutic strategies targeting gut microbiota, such as probiotics and FMT, gain attention, concerns about their long-term safety and efficacy need to be addressed. More rigorous clinical trials are necessary to evaluate the potential risks and benefits of these interventions.

7.6 Future directions

Future research should focus on elucidating the specific microbial species and metabolites involved in modulating insulin resistance and cognitive function. To achieve this goal, researchers could employ high-throughput sequencing techniques to identify and characterize gut microbiota in diverse populations, particularly those with varying degrees of insulin resistance and cognitive impairment. Additionally, metabolomic analyses can be utilized to profile metabolites produced by these microbial communities. Integrating these approaches with clinical studies will allow for the exploration of causal relationships and the identification of potential biomarkers for early intervention. Understanding these specific interactions will be crucial for developing targeted therapies aimed at improving metabolic and cognitive health.

Advances in metagenomics, metabolomics, and bioinformatics will enable a deeper understanding of the complex interactions within the gut microbiota and their impact on host health (Zhang Q. et al., 2022; Takeuchi et al., 2023). Additionally, exploring the role of the gut-brain-metabolic axis in various disease states, including neurodegenerative diseases, psychiatric disorders, and metabolic syndromes, will provide valuable insights into the underlying mechanisms and potential therapeutic targets (Ridaura et al., 2013; Kootte et al., 2017).

Moreover, the long-term effects and safety of therapeutic interventions targeting the gut-brain-metabolic axis, such as probiotics, prebiotics, and FMT, are not well-established. Longitudinal studies are necessary to evaluate the sustainability and potential adverse effects of these interventions (Fekete et al., 2024; Makris et al., 2021).

7.7 Future prospective

The gut-brain-metabolic axis represents a promising area of research with significant implications for understanding and treating metabolic and cognitive disorders. Emerging evidence suggests that targeting the gut microbiota through dietary interventions, probiotics, prebiotics, and FMT can improve insulin sensitivity and cognitive function, offering new avenues for therapeutic strategies.

Future research should focus on identifying the specific microbial species and metabolites that play critical roles in modulating the gut-brain axis and their impact on metabolic and cognitive health. Advances in sequencing technologies and bioinformatics will enable a more comprehensive understanding of the gut microbiota and its interactions with the host.

Additionally, personalized approaches that consider individual variations in gut microbiota composition, genetics, and lifestyle factors will be crucial for optimizing therapeutic interventions. Understanding the long-term effects and safety of these interventions, particularly FMT, will be essential for their clinical application (Kootte et al., 2017; Mattson et al., 2014).

Exploring the role of the gut-brain-metabolic axis in various disease states, including neurodegenerative diseases, psychiatric disorders, and metabolic syndromes, will provide valuable insights into the underlying mechanisms and potential therapeutic targets. Ultimately, a better understanding of the gut-brain-metabolic axis will pave the way for novel strategies to promote metabolic and cognitive health, improving the quality of life for individuals affected by these interconnected disorders (Arnold et al., 2018).

8 Conclusion

The gut-brain-metabolic axis is a promising area of research with significant implications for understanding and treating metabolic and cognitive disorders. This review uniquely synthesizes the latest findings on the interplay between gut microbiota, insulin resistance, and cognitive function, providing a comprehensive overview of the mechanisms involved. Unlike previous studies, this review not only highlights key microbial species and metabolites but also identifies

gaps in current knowledge and proposes targeted research directions. By integrating insights from multiple disciplines, we aim to facilitate the development of novel therapeutic strategies that leverage the gut-brain axis to improve metabolic and cognitive health. Our findings underscore the necessity for future studies to explore the clinical applications of these insights, ultimately contributing to the advancement of personalized medicine in metabolic and cognitive disorders. Targeting the gut microbiota through dietary, probiotic, and fecal transplant interventions has the potential to improve insulin sensitivity and cognitive function. Future studies should focus on identifying the key microbial species and metabolites involved, evaluating long-term intervention effects, and exploring the axis' role in diverse disease states. Personalized approaches considering individual variations will be crucial. Ultimately, this research can lead to novel strategies to promote metabolic and cognitive health.

Author contributions

GA: Conceptualization, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. VB: Conceptualization, Data curation, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. TV: Conceptualization, Methodology, Project administration, Software, Writing – review & editing. AA: Conceptualization, Methodology, Visualization, Writing – review & editing. NM: Investigation, Methodology, Validation, Writing – review & editing. AK: Investigation, Methodology, Resources, Validation, Writing – review & editing. ZZ: Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. AT: Conceptualization, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

Authors Alireza Afshar and Amin Tamadon were employed by PerciaVista R&D Co.

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

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Total Alkaloids of Rhizoma Corydalis regulates gut microbiota and restores gut immune barrier to ameliorate cognitive dysfunction in diabetic rats

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Background and objectives: Given the widespread dysbiosis of gut microbiota in patients with T2DM, it has been found that the microbiota-gut-brain axis plays an influential regulatory role in diabetic cognitive dysfunction, and improving gut dysbiosis may be a potential strategy for treating diabetic cognitive dysfunction. Total Alkaloids of Rhizoma Corydalis (TAC) is the main active component extracted from Rhizoma Corydalis. Pharmacological studies have demonstrated its significant pharmacological effects on the cardiovascular and cerebrovascular systems, and berberine, the main component of TAC, has a certain regulatory effect on gut microbiota.

Materials and methods: Rats were randomly divided into Control group, Model group, TAC-low group, TAC-mid group and TAC-high group. Cognitive function of diabetic rats was evaluated through behavioral testing using the Morris water maze experiment. The relative abundance of gut bacteria in rat feces was determined via 16S rRNA analysis. IHC and Western blot techniques were employed to assess IL-22, IL-23, Reg3g, ZO-1, occludin 1 expression in the colon tissue; GPX4, xCT, NLRP3, Caspase-1 p20, GSDMD-N were detected in the hippocampus.

Results: The cognitive function of diabetic rats decreased significantly. TAC demonstrated a significant reduction in inflammatory factors in serum, hippocampus, and colon, thus alleviating inflammation. Additionally, it effectively decreased ferroptosis induced by NLRP3 and reduced pathological damage in the hippocampus of diabetic rats. After treatment, the differential microbiota such as *Lachnoclotridium* and *Bacteroides*. TAC improved gut barrier permeability and integrity in rats while remodeling gut mucosal homeostasis. Moreover, pyroptosis and ferroptosis caused by the inflammatory cascade in the rat hippocampus were also significantly inhibited.

Conclusion: The combination of high lipid and high glucose with STZ can result in gut microbiota disturbance, damage gut immune barrier, decreased gut mucosal permeability and integrity, aggravated gut inflammation, further spread inflammatory factors to brain tissue, cause inflammatory cascade reaction of encephalopathy, and ultimately resulting in neuronal ferroptosis and cognitive dysfunction in diabetes mellitus. Our study suggests that TAC may regulate gut microbiota, restore gut immune homeostasis, improve gut barrier

permeability and integrity, inhibit brain tissue inflammatory cascade, reduce neuronal ferroptosis, and thus improve diabetes. This provides new targets for its treatment strategy.

KEYWORDS

Total Alkaloids of Rhizoma Corydalis, gut microbiota, cognitive dysfunction, diabetes, rats

1 Introduction

The incidence of type 2 diabetes mellitus (T2DM) is gradually increasing due to the aging population and the rise in obesity. Cognitive dysfunction is one of the most serious complications of diabetes (Xue et al., 2019), and the literature has shown that patients with T2DM have a 1.5–2.5 times greater risk of developing dementia than the general population (Biessels et al., 2006). Experimental studies have found that the etiology and progression of T2DM to diabetes cognitive dysfunction may be significantly mediated by the gut microbiota (Huang et al., 2023). The connection between the gut microbiota and brain is tightly intertwined through a bidirectional communication system called the microbiota-gut-brain axis, in which inflammation plays an essential role (Agirman et al., 2021). The occurrence and progression of diabetes are accompanied by the disorder of gut microbiota (Liu et al., 2020). Consumption of a high-fat and high-sugar diet can disrupt the intestinal immune barrier through its effects on gut microbiota, leading to chronic low-level activation of the inflammatory system. This inflammation may eventually propagate from peripheral tissues to the brain, triggering an inflammatory cascade in the brain and resulting in cognitive dysfunction (Solas et al., 2017). In this process, we have discovered a close association between chronic inflammation and ferroptosis, which is a newly identified form of cell death characterized by intracellular iron accumulation and lipid peroxidation. Recent studies have shown that ferroptosis is related to a variety of neurological diseases, including cognitive dysfunction and neurodegenerative diseases (Ratan, 2020). In the inflammatory environment, the imbalance of iron metabolism can lead to neuronal ferroptosis (Lee and Hyun, 2023), and studies have proved that NLRP3, the classical pathway of pyroptosis, can promote the occurrence and development of ferroptosis (Li Z. et al., 2023). Therefore, it is hypothesized that gut microbiota may compromise the immune barrier, triggering an inflammatory cascade that activates the NLRP3 pyroptosis signaling pathway. This activation could promote neuronal ferroptosis and result in cognitive dysfunction in diabetic rats (Bi et al., 2021).

The treatment of diabetic cognitive dysfunction in modern medicine mainly refers to the clinical medication of MCI and AD, but it has not shown obvious clinical efficacy (Xiong et al., 2021). Commonly used hypoglycemic drugs such as metformin and glibenclamide have limited effects on improving diabetic cognitive dysfunction (Launer et al., 2011). Rhizoma Corydalis is a dried rhizome derived from Papaveraceae and is a traditional Chinese medicine commonly used in clinical practice, especially for cardiovascular and nervous systems (Xu et al., 2021). Tetrahydropalma is the principal component of Total Alkaloids of Rhizoma Corydalis, with a content of 50%. Other subordinate components include Corydaline, Protopine, Corydalis, Corydalis H, Glaucine and Palmatine, which are present in a total content of 30% (Zhang et al., 2016). The previous research conducted by our research group has demonstrated that TAC exhibits pharmacological effects such as anti-inflammatory properties, inhibition of apoptosis and pyroptosis, which have the potential to significantly improve conditions such as epilepsy (Qi et al., 2023), cerebral ischemia (Li J. et al., 2023), etc.; A number of *in vivo* and *in vitro* related experiments have also verified that TAC has anti-depressant and anti-anxiety pharmacological effects in addition to anti-inflammatory, antibacterial, and analgesic effects (Alhassen et al., 2021); The main component of the TAC, berberine, can not only promote insulin release (Zhao et al., 2021) and reduce insulin resistance (Zhang et al., 2010), but also improve gut microbiota imbalance, and relieve anxiety (Fang et al., 2021). Collectively, these findings indicate promising prospects for further research and development of TAC.

In this study, T2DM rats were fed with high-lipid diet for 16 weeks and received intraperitoneal injection of STZ. Morris water maze test was used to evaluate cognitive dysfunction, 16S rRNA was used to detect the changes of gut microbiota structure, IHC and Western blot were used to detect gut immune barrier function, brain inflammation and ferroptosis-related proteins. Whether TAC can improve cognitive dysfunction by regulating gut microbiota to inhibit ferroptosis in diabetic rats was observed.

2 Materials and methods

2.1 Animals and medicines

Eight-week-old SPF male SD rats, weighing 180 ± 20 g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd. The experimental animal license number is SCXK (Liao) 2020-0001. The housing conditions maintained a temperature range of 20–25°C and relative humidity at 50–60%, with *ad libitum* access to food and water. Animal experiments were approved by the Experimental Animal Welfare Ethics Committee of Heilongjiang University of Traditional Chinese Medicine, project number: 2023112907. TAC was prepared in accordance with the previous methods of the research group. Specifically, the extraction method was acid water percolation. The percolation endpoint was determined by the detection of alkaloid precipitation reagents. Tetrahydropalmatine was used as the standard substance, and the content of TAC was determined by the acid dye colorimetric method. Considering various factors, it was determined that the optimal percolation volume was 12 times the mass of the medicinal materials, resulting in an extraction rate of TAC reaching 85% (Wang and Tian, 2015).

2.2 Laboratory apparatus

Morris water maze behavioral analysis system was provided by Shanghai Xinruan Information Technology Co., Ltd.; DNM -9602 microplate reader was obtained from Beijing Perlong New Technology Co., Ltd.; Upright fluorescence microscope FR-4A is from Shanghai Optical Instrument Factory; YD-1508B tissue slicer is manufactured by Zhejiang Jinhua Yidi Medical Equipment Factory; MoticBA400 microscopic photography and imaging system is a product of Motic Inc., United States; iCEN-24R tabletop high-speed refrigerated centrifuge was purchased from Hangzhou Aosheng Instrument Co., Ltd.; Electrophoresis equipment including Tanon EPS-300 electrophoresis apparatus, Tanon VE-186 electrophoresis tank, and Tanon VE-180B transfer tank were supplied by Shanghai Tianneng Technology Co., Ltd. Gel imaging was performed using the Tanon-4600 gel imaging system also from Shanghai Tianneng Technology Co., Ltd. Gut microbiota sequencing was entrusted to Shanghai Majorbio Biomedical Technology Co., Ltd.

2.3 Experimental design and administration

Grouping was conducted using a completely random method. The 35 rats were numbered from 1 to 35. Thirty five random numbers were randomly obtained from the random number table in the same direction. The random numbers were divided by the number of groups (5) to obtain the remainders. The groups were constantly adjusted until there were 7 rats in each group. Four randomly selected groups of rats were fed a high-fat diet (78.8% basal diet +15% lard +1% cholesterol +5% sucrose +0.2% sodium cholate) for 16 weeks in combination with two intraperitoneal injections of 30 $mg{\cdot}kg^{-1}$ streptozotocin to establish a T2DM rat model, with a 1-week interval. Seven days after the last STZ injection, fasting blood glucose greater than 16.5 $mmol{\cdot}L^{-1}$ indicated that a total of 24 rat models of diabetes were successfully established. The 4 groups of rats were randomly divided into the model group and the low-, medium-, and high-dose TAC groups. One healthy rat was randomly excluded from the control group, with 6 rats in each group. The administration groups were gavaged with the corresponding doses of drugs (low, medium, and high doses were 7, 11, and 14 $g{\cdot}kg^{-1}$, respectively) for 4 consecutive weeks.

2.4 Index collection and detection

2.4.1 Morris water maze experiment

Each group of rats were tested with the Morris water maze experiment. (1) Place navigation test: Rats in each group were positioned facing the pool wall at four different quadrant markers, with a 1-h interval between each training session. The latency period, which is the time taken for the rats to locate the platform within 60 s, was recorded. For those rats that did not find the platform within 60 s, they were re-placed on the platform for 10 s before being removed from the maze, and their latency period was recorded as 60 s. Taking the average of 4 latency period as the final result, this training was conducted continuously for 4 days. (2) Spatial probe test: On the fifth day, the platform was removed, and the rats were placed in the water facing the wall of the pool in the opposite quadrant of the platform; their latent time and number of times crossing over where it had been located were then recorded.

2.4.2 Fasting blood glucose test

For the FBG test, animals were fasted for 9 h and tested on blood glucose test strips measured by a glucose meter before tail blood was collected (Roche, United States). FBG was measured weekly before STZ injection, after STZ injection (weeks 1, 2), and during TAC treatment (weeks 1, 2, 3, 4). Each blood glucose measurement was performed three times for each animal at every time point, and the average value was recorded.

2.4.3 16S rRNA test

Bacterial genomic DNA was extracted from feces using a reagent kit, and a gene library was constructed. OTU clustering of sequences at 97% similarity was performed using bioinformatics methods with USEARCH software. The sequences were then annotated for taxonomic classification against the Silva 16S rRNA gene database (v138) with a confidence threshold of 70%, and the community composition of each sample was statistically analyzed at different taxonomic levels. Mothur was utilized for calculating Alpha diversity indices such as Simpson, Shannon, and for conducting Wilcoxon rank-sum tests to assess inter-group differences in Alpha diversity. Beta diversity analysis was employed to evaluate the similarity and dissimilarity of samples from different groups in terms of community structure, utilizing multivariate statistical analysis methods such as PcoA to analyze the results. The Kruskal-Wallis rank sum test was employed for multi-group difference tests to assess the intergroup differences among the three groups. Lefse multi-level discriminant analysis of species difference (multi-level: phylum, class, order, family, genus) was used to test the difference at multiple levels, analyze the multi-level differential species and screen the differential microbiota. The heat map of the correlation coefficient between dominant microorganisms and pharmacodynamic indexes was drawn, and the key genera and species affecting diabetic cognitive dysfunction were screened out.

2.4.4 Detection of insulin in rat serum, and the contents of IL-1β, IL-18, IL-6, and TNF-α in serum, hippocampus and colon using enzyme-linked immunosorbent assay

The blood, colon and tissue supernatant of rats in each group were extracted and detected using ELISA kits for IL-1β, IL-18, IL-6, and TNF-α according to the manufacturer's instructions (Andy gene, Beijing, China).

2.4.5 Observation of pathological changes in hippocampus and colon of rats in each group using hematoxylin-eosin staining

Before performing HE staining, the brain tissue and colon of rats from each group were fixed in 4% paraformaldehyde solution for 24 h, then embedded in paraffin wax, and then cut into slices with a thickness of 5 μm. The pathological changes in hippocampus and colon of rats from each group were then observed under microscope.

2.4.6 PAS staining of rat colon tissue

Paraffin sections were routinely dehydrated, stained with periodic acid Schiff (PAS) at room temperature for 30 min, counterstained with

hematoxylin, dehydrated, and then sealed with neutral gum; The changes of glycogen content in colon tissue of each group were observed under microscope, and the statistical analysis was performed using ImageJ.

2.4.7 Assessment of ferroptosis

Iron deposition, iron content, and SOD, MDA, 4-HNE, GSH, and GSH-PX levels were measured in rat brain hippocampal neurons to assess ferroptosis. Iron deposits in the brain were determined by Perls staining using the Perls staining kit (LEAGENE Biotechnology Co., Ltd., Anhui, China) according to the instructions. Iron content in serum and brain tissue of rats was measured using serum iron assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and tissue iron assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. The contents of SOD, MDA, GSH, GSH-PX, and 4-HNE were determined by SOD kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), MDA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), GSH kit (Nanjing Jiancheng, Nanjing, China), GSH-PX kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and 4-HNE Elisa kit (Beijing Biotoppeed Technology Co., Ltd., Beijing, China), respectively.

2.4.8 NLRP3, Caspase-1p20, GSDMD-N, GPX4, xCT in hippocampus, IL-22, IL-23, Reg3g, ZO-1, occludin1 protein in colon were detected by immunohistochemistry

Paraffin sections were routinely dewaxed, incubated with 10% H2O2 for 10 min and repaired by microwave; Endogenous enzymes were inactivated and washed with primary antibodies. The primary antibodies used were NLRP3 (1: 125, Servicebio, Wuhan, China), Caspase-1p20 (1: 100, Bioss, Beijing, China), GSDMD-N (1: 125, Affinity Biosciences, Jiangsu, China), GPX4 (1: 75, FineTest, Wuhan, China), xCT (1:50, ABclonal, Wuhan, China), IL-22 (1: 150, Wanleibio, Shenyang, China), IL-23 (1: 125, Bioss, Beijing, China), Reg3g (1: 125, Bioss, Beijing, China), ZO-1 (1: 150, Wanleibio, Shenyang, China), occludin1 (1: 150, Wanleibio, Shenyang, China), with a ambient temperature of 4°C overnight. Subsequently, the secondary antibody was added dropwise and incubated at 37°C for 30 min; The nuclei were counterstained with hematoxylin after DAB development; Seal after dehydration and transparency. Six cases from each group were selected for detection, and images were collected under a microscope at 200 fold magnification of the Motic3000 microphotography imaging system. Three different fields were randomly selected in each slice, and analyzed using Image-pro plus6.0 image analysis software to represent protein expression level as mean integrated optical density (IOD).

2.4.9 Western blot was used to detect NLRP3, Caspase-1p20, GSDMD-N, GPX4, xCT in hippocampus, IL-22, IL-23, Reg3g, ZO-1, occludin1 in colon

Proteins were extracted from the hippocampus and colon of rats, denatured, and then loaded into sample wells for electrophoresis separation. Following transfer and blocking for 2 h, primary antibodies (Servicebio, Wuhan, China), Caspase-1p20 (Bioss, Beijing, China), GSDMD-N (Affinity Biosciences, Jiangsu, China), GPX4 (FineTest, Wuhan, China), xCT (ABclonal, Wuhan, China), IL-22 (Wanleibio, Shenyang, China), IL-23 (Bioss, Beijing, China), Reg3g (Bioss, Beijing, China), ZO-1 (Wanleibio, Shenyang, China), occludin1 (Wanleibio, Shenyang, China). All at a dilution ratio of 1:1,000 were incubated overnight. After incubation at room temperature for 1 h, ECL chemiluminescence was developed uniformly. The captured images were analyzed using ImageJ software to calculate the grayscale value ratio of the target protein bands to the internal reference bands in each group to determine the expression level of the target protein.

2.4.10 Statistical treatment

The data were analyzed using SPSS25.0, and normally distributed data were expressed as mean ± SD. One-way ANOVA was used to analyze the experimental data analysis, and the LSD-t was used for pairwise sample tests. A significance level of $p < 0.05$ was considered statistically significant.

3 Results

3.1 TAC can ameliorate learning and memory impairment in STZ-induced diabetic rats

Morris water maze test was used to detect spatial learning and memory of rats in each group. The results showed that over the initial 4 days, as training duration increased, escape latency in locating the platform gradually decreased across all groups. The Model group exhibited a significant increase in escape latency compared to the Control group; however, TAC at various doses led to a significant reduction in escape latency compared to the Model group (Figure 1A). Following removal of the platform, analysis of day 5 crossings indicated a reduced number of platform crossings and significantly prolonged latency period for the Model group compared to the Control group. In contrast, each dose group increased the number of platform crossings and significantly shortened latency period compared to the Model group (Figures 1B,C). According to the judgment of the positioning trajectory map on the fifth day, it was found that the rats in the Model group could not quickly find the quadrant where the platform was located, and rotated around the wall in 4 quadrants. Platforms were easier to find across dose groups, and memory was also significantly improved in rats (Figure 1D). These data suggest that TAC has potential for mitigating diabetes-induced impairments in learning and memory while positively impacting cognitive function.

3.2 The changes of body weight, blood glucose and serum insulin in each group

Body weight (Figure 2A), blood glucose (Figure 2B), and serum insulin (Figure 2C) in rats were measured. Compared with the Control group, there was a significant decrease in body weight and a significant increase in blood glucose and serum insulin levels. In contrast, rats treated with TAC exhibited a significant increase in body weight and a significant decrease in serum insulin and blood glucose levels compared to the Control group.

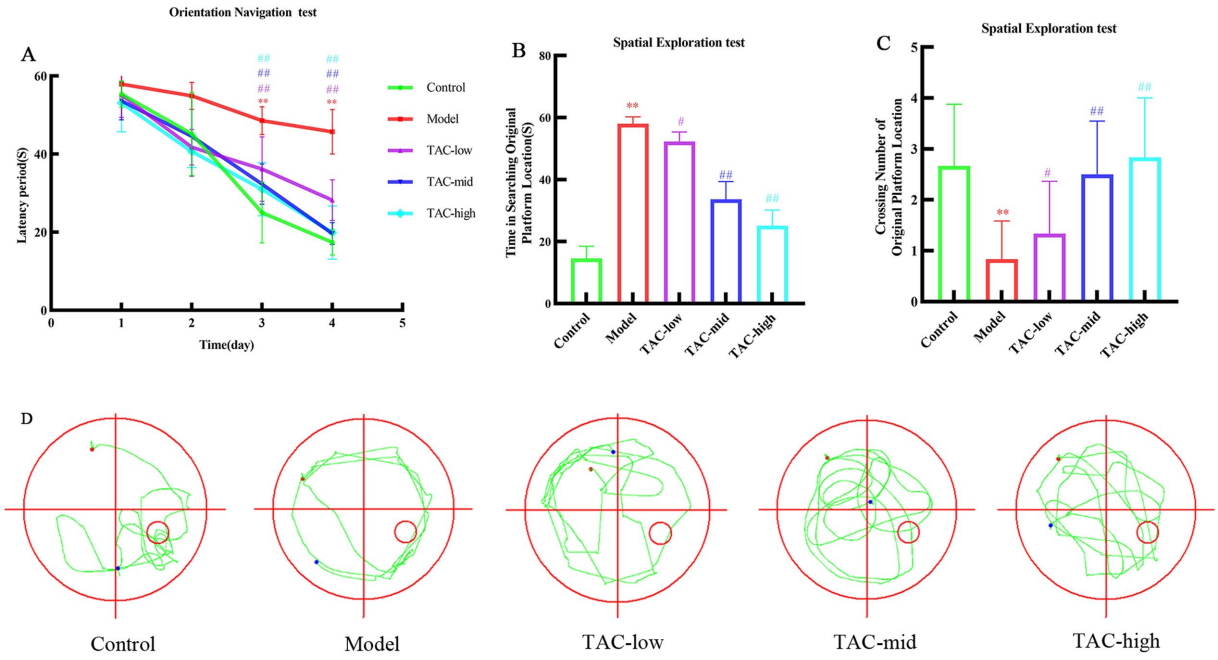

FIGURE 1
Effect of TAC on learning and memory performance in diabetic rats. **(A)** Escape latency period during the 4-day training of the place navigation test. **(B–D)** In the spatial probe test, rats escaped latency period **(B)**, number of crossings at platform positions **(C)**, representative swimming paths **(D)** mean ± SD. $n = 6$. Statistical analysis was performed using one-way ANOVA $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$ vs. Model Group. $^{\#\#}p < 0.01$ vs. Model Group.

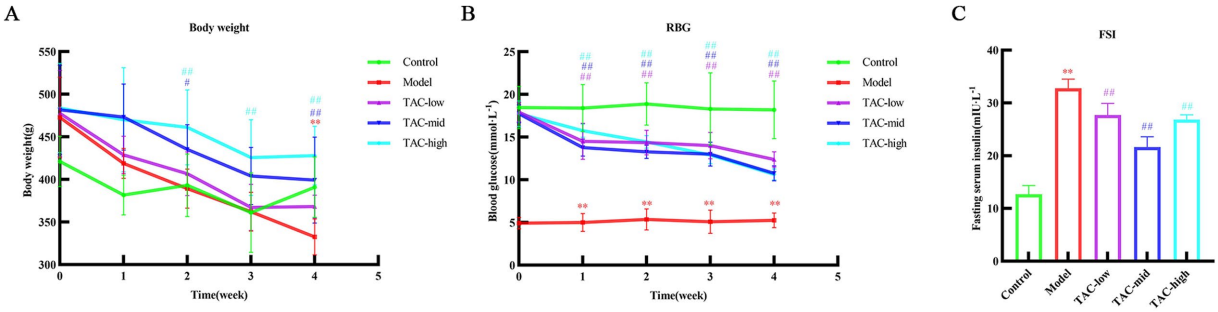

FIGURE 2
Effect of TAC on body weight, blood glucose and serum insulin in diabetic rats. Body weight **(A)**, blood glucose **(B)**, and serum insulin **(C)** mean ± SD. $n = 6$. Statistical analysis using one-way ANOVA $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$ vs. Model Group. $^{\#\#}p < 0.01$ vs. Model Group.

3.3 Effects of TAC on gut microbiota in diabetic rats

Alpha diversity of gut microbiota was analyzed by Simpson and Shannon. Among them, Simpson (Figure 3A) and Shannon (Figure 3B) in the Control group were lower than those in the Model and TAC groups. Furthermore, PCoA (Figure 3C) was employed to assess beta diversity. In this study, there was a clear separation between the 3 groups, suggesting that there may be different compositions of the intestinal flora among these 3 groups. To determine the target differential bacteria, differential bacteria between the 3 groups were evaluated. The Venn diagram results of this study show that at the gate level, there are 11 OTUs in the Control group and TAC group, and 12 OTUs in the Model group and TAC group. At the genus level, there were 179 OTUs in the Control and TAC groups, and 175 OTUs in the Model and TAC groups (Figures 3D,G). We found that *Firmicutes* were the predominant flora. Compared with the Control group, the abundance of *Bacteroidota* increased and the abundance of *Actinobacteriota* decreased in the Model group; The abundance of *Bacteroidota* increased significantly and the abundance of *Firmicutes* decreased in the TAC group compared with the Model group (Figures 3E,H). These are heat maps at the Phylum and Genus levels (Figures 3F,I). The Kruskal-Wallis rank-sum test and Lefse multilevel species differences were used for discriminant analysis. *Bacteroidota* and *Verrucomicrobiota* were found to be significantly different between the three groups at the phylum level by significance analysis (Figures 3J,L); At the genus level, the abundance of *Bacteroides* and *Lachnoclotridium* in the Model group was higher than that in the Control group, and the abundance of *Blautia*, *Akkermansia* and

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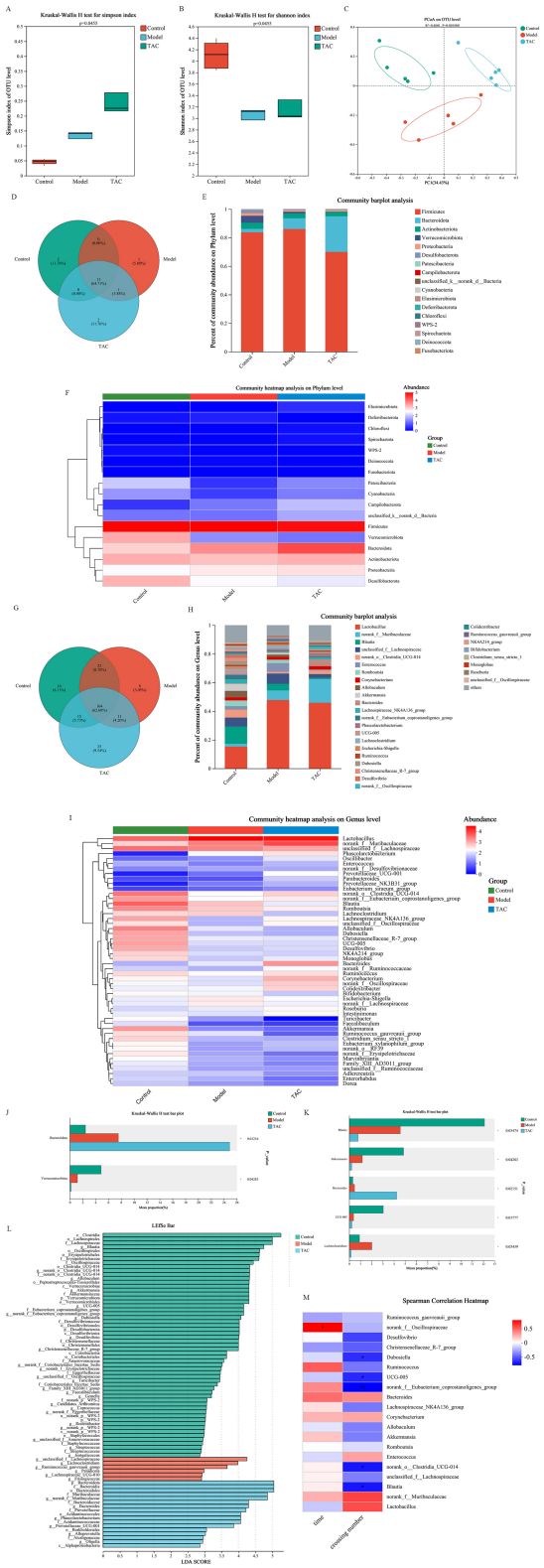

FIGURE 3
Effect of TAC on alpha and beta diversity indices in colonic microbiota. Alpha diversity was analyzed by Simpson **(A)** and Shannon **(B)**, and beta diversity was analyzed by PCoA **(C)**, with each point representing an individual sample. **(D)** Venn diagram of gut microbiota at the phylum level. **(E,F)** Composition of gut microbiota at the phylum level. **(G)** Venn diagram of gut microbiota at the genus level. **(H,I)** Composition of gut microbiota at the genus level. The **(J,K)** Kruskal-Wallis rank sum test assessed the differences between the three groups. **(L)** Lefse multilevel discriminant analysis of species differences **(M)** Heat map of correlation coefficients between dominant microorganisms and pharmacodynamic indexes. *$p < 0.05$, **$p < 0.01$.

UCG-005 was significantly reduced. Meanwhile, TAC treatment significantly increased the abundance of *Bacteroides* and decreased the abundance of *Blautia*, *Akkermansia*, *UCG-005*, and *Lachnoclotridium* (Figures 3K,L). The heat map shows the correlation coefficients between the abundance of 20 genera and the water maze latency and the number of platforms crossed in diabetic cognitively dysfunctional rats (Figure 3M). These findings suggest that the intestinal flora is involved in cognitive function in diabetic rats.

3.4 TAC attenuates inflammatory response in diabetic rats

By detecting the levels of inflammatory factors IL-1β, IL-6, IL-18, and TNF-α in the serum of rats in each group (Figure 4), the results showed that compared with the Control group, the levels of various inflammatory factors in the Model group were increased; Compared with the Model Group, the levels of inflammatory factors in each dose group were reduced. It is demonstrated that TAC can effectively alleviate the systemic chronic inflammatory response caused by diabetes.

3.5 TAC can ameliorate the pathological changes of hippocampal and colonic barrier in diabetic rats

Hematoxylin and eosin (H & E) staining were used to evaluate the hippocampal and colonic pathological changes of diabetic rats before and after TAC treatment. In Control Group, the colonic structure was clear, the gut mucosa arranged regularly, the structure of gut gland cells was normal, the cytoplasm and nucleus stained clearly and evenly, and there was no inflammatory cell infiltration or capillary congestion. In contrast, the Model group showed severe pathological changes in the colonic tissue, gut mucosa was disorganized, gut villi fell off, epithelial cells were deeply stained, the number of goblet cells was reduced, telangiectasia in the submucosa, and inflammatory cells were infiltrated. Notably, TAC treatment relieved colonic epithelial morphology, with significant improvement in the aforementioned pathological changes, increased number of goblet cells, decreased inflammatory cells, and reduced telangiectasia compared to the Model group (Figure 5A). In the Control group, hippocampal tissue had no edema, neurons arranged regularly, the boundaries between structures were clear, and no large number of inflammatory cells were seen.

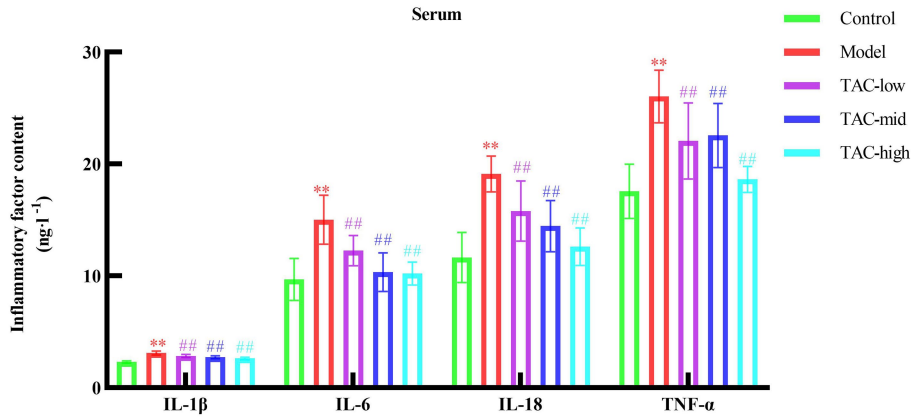

FIGURE 4
Effect of TAC on inflammatory factors IL-1β, IL-6, IL-18, TNF-α in serum of diabetic rats. Mean ± SD. n = 6. Statistical analysis using one-way ANOVA $^{**}p < 0.01$ vs. Control group; $^{\#\#}p < 0.01$ vs. Model Group.

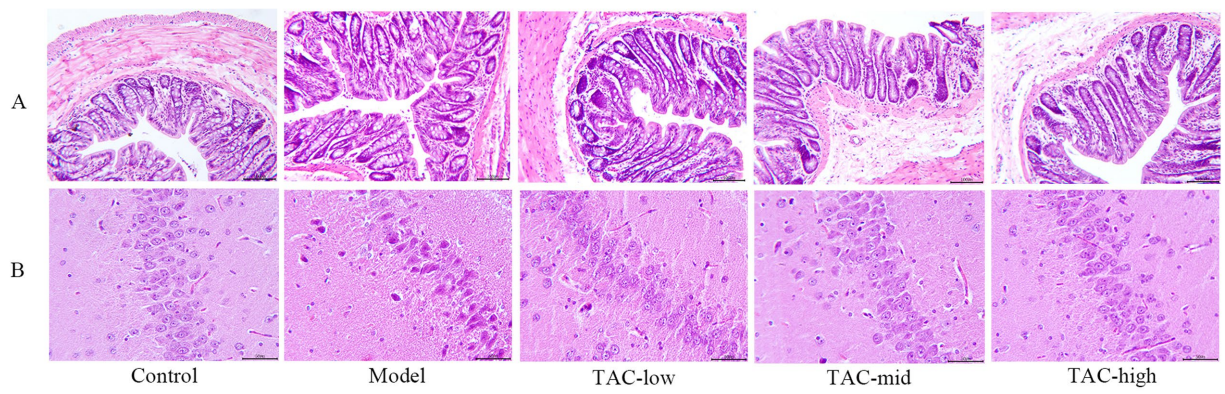

FIGURE 5
Changes of TAC on colon and hippocampus pathological morphology in diabetic rats. **(A)** HE × 100, scale bar = 50 μm, **(B)** HE × 200, scale bar = 50 μm.

Model group hippocampal tissue structure loose, inflammatory cell infiltration, karyopyknosis significantly increased, microglia significantly increased, vasodilation and congestion obvious. In each dose group, the hippocampal tissue was relatively tidy, the infiltration of inflammatory cells and microglia were significantly reduced, and the vasodilation and congestion were also relatively relieved (Figure 5B).

3.6 TAC can regulate the balance of gut immune function and reduce the damage of mucosal barrier

PAS staining results of the colon tissue (Figures 6A,B) showed that the glycogen content of the Model group was significantly increased compared with that of the Control group, and the differences in inflammatory factors such as IL-1β, IL-6, and TNF-α in the colon were also significant. TAC improved these indicators to a certain extent (Figure 6C). IHC and WB results on colonic IL-22, IL-23, and Reg3γ showed that compared with the Control group, the levels of IL-22, IL-23, and Reg3γ in the colon of diabetic rats were significantly reduced, while TAC could increase the expression of IL-22, IL-23, and Reg3γ in the colon of diabetic rats (Figures 6D–G). These results suggest that TAC can inhibit colonic inflammation and restore gut immune balance in diabetic rats. As shown in Figure 6F, ZO-1 and occludin1 expression was significantly reduced in diabetic rats compared to the Control group, indicating impaired gut mucosal barrier. However, compared to the Model group, the TAC-treated group showed a significant increase in ZO-1 and occludin1 protein expression, indicating that TAC is associated with the protective effect of the gut mucosal barrier.

3.7 TAC can inhibit inflammatory cascade in brain tissue

Inflammatory factors such as IL-1β, IL-18, and TNF-α were significantly different in the hippocampus (Figure 7A). The results of IHC and WB detection of NLRP3, Caspase-1p20 and GSDMD-N in the brain (Figures 7B–E) showed that the levels of NLRP3, Caspase-1p20 and GSDMD-N in the hippocampus of diabetic rats were significantly increased compared with Control group, while TAC could reduce the expression of NLRP3, Caspase-1p20 and GSDMD-N in the hippocampus of diabetic rats. It is suggested that TAC can alleviate pyroptosis caused by the inflammatory cascade mediated by the brain-gut axis.

3.8 TAC can inhibit ferroptosis in brain tissue

Prussian blue staining of brain tissue showed that iron deposition increased significantly in Model group, and decreased significantly after TAC treatment (Figures 8A,B). Consistently, the contents of iron (Figures 8C,D), MDA (Figure 8E), and 4-HNE (Figure 8F) were significantly regulated, while the levels of SOD (Figure 8G), GSH (Figure 8H), and GSH-PX (Figure 8I) were significantly upregulated in the brain of diabetic rats given TAC. IHC and WB were used to detect the expression levels of GPX4 and xCT protein in rats (Figures 8J–M). The results showed that TAC could significantly increase the expression levels of GPX4 and xCT protein. Collectively, these results suggest that TAC can reduce the content in the brain of diabetic rats and reduce lipid peroxidation, inhibiting the occurrence of ferroptosis in the brain.

4 Discussion

A considerable number of studies have pointed out that the gut microbiota participates in the energy metabolism process and is closely associated with the occurrence and development of diabetes. It is generally acknowledged that excessive dietary nutrition, such as excessive intake of salt, sugar, and fat, exerts a detrimental effect on the diversity and stability of the gut microbial flora, resulting in a decrease in beneficial microbiota and/or an increase in pathogenic microbial communities, inducing chronic low-grade inflammation in the intestine and thereby causing the onset of T2DM (Ma et al., 2019). Simultaneously, the gut microbiota can convey information to the central nervous system via multiple pathways including neural anatomical routes, the endocrine system, the immune system, and metabolism (Needham et al., 2022). The microbiota-gut-brain axis plays a significant role in various neurological disorders, and targeted regulation of the microbiota-gut-brain axis is potentially conducive to the improvement of cognitive impairment (Chen et al., 2017). In studies on T2DM rats with cognitive impairment, it was found that six types of bacteria such as *Firmicutes* (*Lactobacillus* and *Ruminococcus*) and *Bacteroidetes* (*Parabacteroides*, *Bacteroides*, *Butyricimonas*, and *Prevotella*) underwent dynamic alterations at the subspecies level during the occurrence and development of cognitive impairment (Bi et al., 2021). Moreover, experimental studies have demonstrated that restoring the gut microbiota can ameliorate cognitive dysfunction (Hazan, 2020). The results of this experiment demonstrated that the abundance of Bacteroides and Lachnoclotridium in diabetic rats were significantly higher than those in Control group, while that of *Blautia*, *Akkermansia*, and *UCG-005* were significantly lower than those in Control group. Numerous studies have revealed that the abundance of *Lachnoclotridium* in diabetic patients, especially those with type II diabetes, has changed significantly compared to healthy individuals (Goldenberg et al., 2017). This study also provides us with clues about the relationship between bacterial genera, including *Lachnoclostridium* strains, and diabetic cognitive dysfunction.

Recent studies have revealed that traditional Chinese medicines that modulate the microbiota-gut-brain axis possess remarkable potential in alleviating cognitive impairments. For instance, ginsenosides in ginseng and polysaccharides in Dioscorea opposita require the mediation of the gut microbiota to exert biological effects for improving cognitive functions (Bi et al., 2021). Research indicates that the active components of Corydalis have central nervous pharmacological effects such as inhibiting acetylcholinesterase activity (Xiao et al., 2011), activating opioid receptors (Kaserer et al., 2020), and antagonizing dopamine receptors (Zhang et al., 2014). Berberine in the total alkaloids of Corydalis can alleviate anxiety caused by ovariectomy by regulating the gut microbiota (Fang et al., 2021). Moreover, studies have demonstrated that Corydalis can promote insulin release, reduce insulin resistance, and regulate the gut

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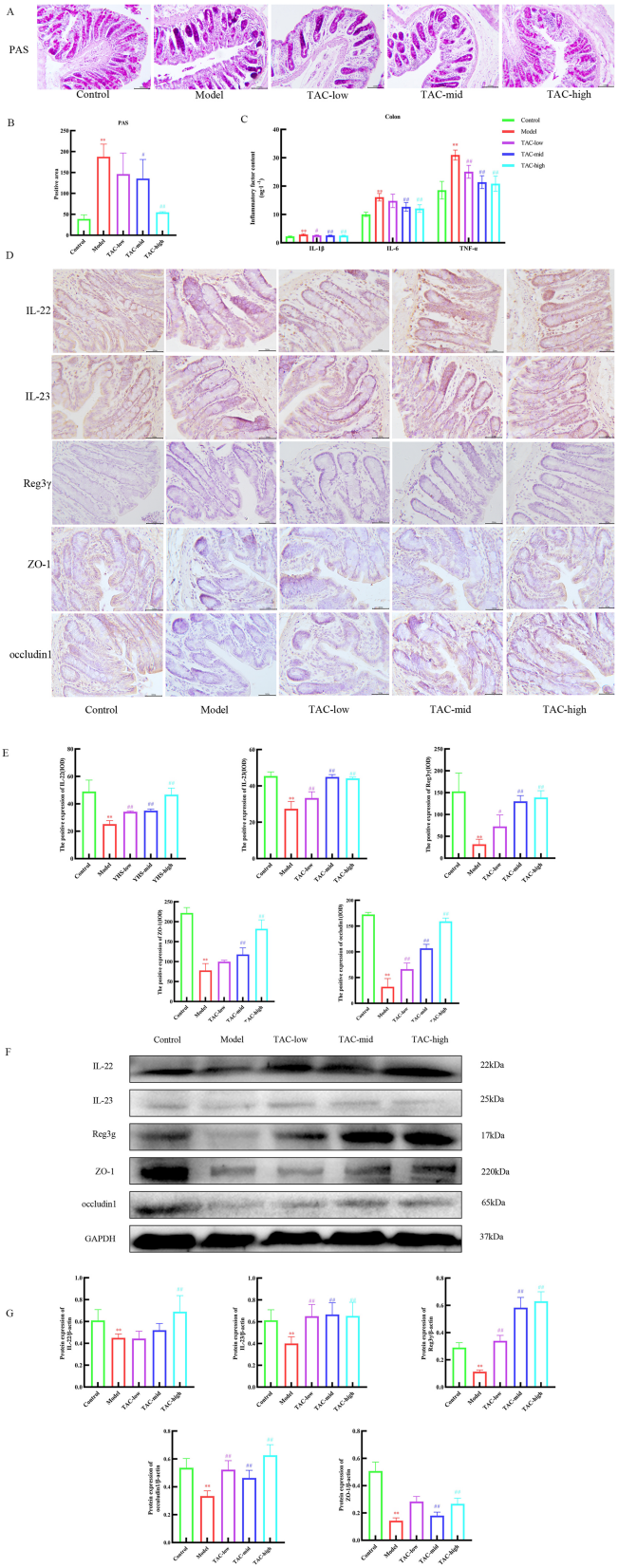

FIGURE 6
Effect of TAC on colonic immune homeostasis, mucosal barrier in diabetic rats. **(A,B)** Colon PAS staining **(C)** Effect of TAC on inflammatory factors IL-1β, IL-6, TNF-α in the colon of diabetic rats. **(D,E)** Immunohistochemical detection results of IL-22, IL-23, and Reg3γ in the colon. **(F,G)** Western blot results of IL-22, IL-23 and Reg3γ detection in the colon. Mean ± SD. $n = 3$. Statistical analysis using one-way ANOVA $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$ vs. Model Group. $^{\#\#}p < 0.01$ vs. Model Group.

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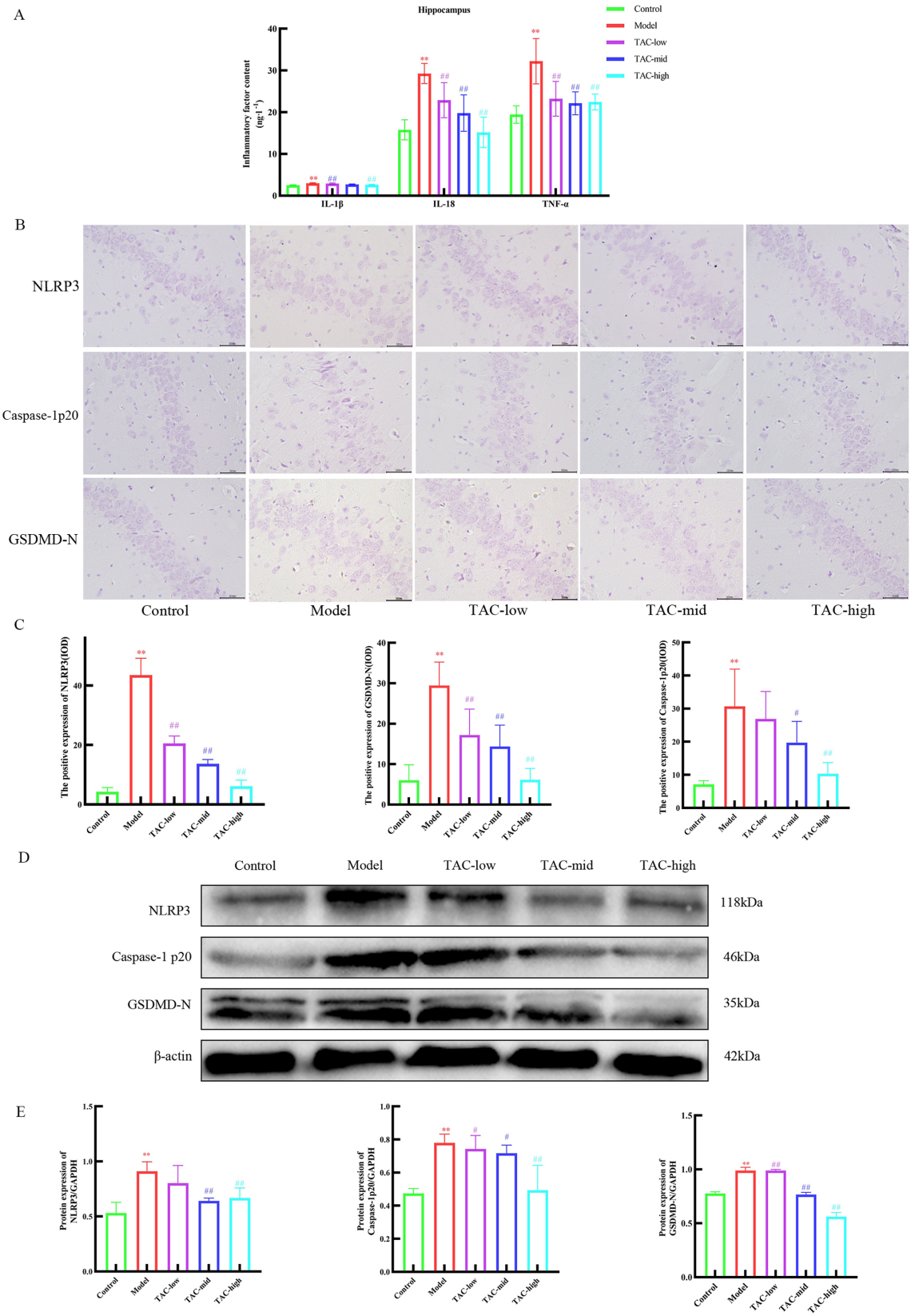

FIGURE 7
Effect of TAC on the inflammatory cascade in the brain tissue of diabetic rats. **(A)** Effects of TAC on inflammatory factors IL-1β, IL-18, TNF-α in hippocampus of diabetic rats. **(B,C)** Immunohistochemical detection results of NLRP3, Caspase-1p20 and GSDMD-N in hippocampus. **(D,E)** Western blot results of IL-22, IL-23, and Reg3γ in the colon. Mean ± SD. $n = 3$. Statistical analysis using one-way ANOVA $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$ vs. Model Group. $^{\#\#}p < 0.01$ vs. Model Group.

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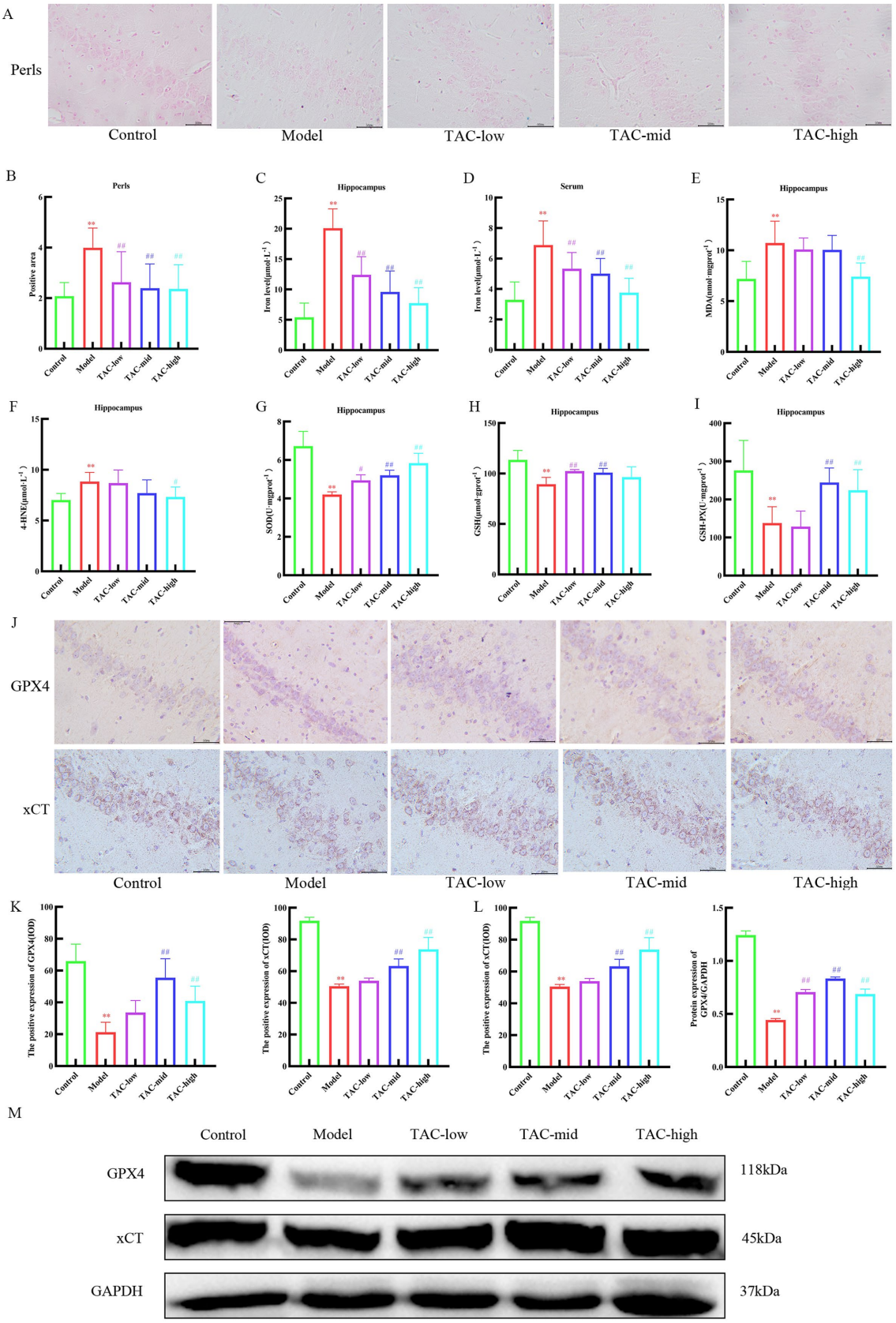

FIGURE 8
TAC on ferroptosis in the brain tissue of diabetic rats. **(A,B)** Prussian blue staining results of rats in each group. **(C–I)** Effects of TAC on hippocampal tissue iron **(C)**, serum iron **(D)** and SOD **(E)**, MDA **(F)**, 4-HNE **(G)**, GSH **(H)**, and GSH-PX **(I)** in diabetic rats **(J,K)** Results of immunohistochemical detection of GPX4 and xCT in hippocampus. **(L,M)** Results of GPX4 and Xct Western blot detection in hippocampus. Mean ± SD. $n = 3$. And statistical analysis using one-way ANOVA $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$ vs. Model Group. $^{\#\#}p < 0.01$ vs. Model Group.

microbiota, thereby improving diabetic complications (Feng et al., 2023). We discovered through the Morris water maze experiment that TAC could significantly improve the cognitive dysfunction of diabetic rats and also proved that TAC could regulate the gut microbiota, verifying the close relationship between the gut microbiota and the central nervous system. In terms of diabetes, after TAC treatment, the fasting blood glucose and serum insulin of diabetic model rats were significantly reduced, consistent with previous research results (Xie et al., 2022). Therefore, we suggest that TAC might improve the cognitive dysfunction of diabetic rats by regulating the gut microbiota. TAC significantly decreased the abundance of Blautia, Akkermansia, UCG-005, and Lachnoclotridium, while the abundance of Bacteroides increased significantly at the same time. Studies have found that the dysregulation of the gut microbiome in patients with Alzheimer's disease may be due to changes in the genus Bacteroides, which may be related to chronic low-grade inflammation (Vogt et al., 2017).

A considerable amount of evidence indicates that the gut microbiota plays a significant role in the occurrence and persistence of inflammation related to intestinal barrier dysfunction and bacterial translocation (Li et al., 2020). It has been reported that the dysregulation of the gut microbiota can disrupt the immune homeostasis of the intestinal barrier, which can lead to the downregulation of IL-22 expression and insufficient secretion of the antibacterial peptide Reg 3γ by epithelial cells, further damaging the intestinal mucosal permeability (Zong et al., 2020). Moreover, the deficiency of IL-22 or IL-23 can also cause dysbiosis of the gut microbiota in mice with a high-frequency diet (Fatkhullina et al., 2018). The results of this study show that the protein expressions of IL-22, IL-23, and Reg3γ in the colon tissues of diabetic model rats were significantly decreased, and the protein expressions of ZO-1 and occludin1 were also significantly reduced. This indicates that the dysbiosis of the gut microbiota in diabetic model rats disrupts the immune homeostasis of the intestinal barrier, thereby increasing the permeability of the intestinal mucosa.

The immune homeostasis of the intestinal barrier and dysbiosis of the microbiota are associated with central neural inflammation. The damage to the intestinal barrier and intestinal mucus enables external antigens to enter the host from the intestinal lumen. Some studies have found that the abundance of microbiota in the feces of patients with cognitive dysfunction is also highly related to the levels of inflammatory cytokines in the blood of patients (Cattaneo et al., 2017). For example, the intestinal mucosa of patients with Parkinson's disease shows increased permeability, inflammatory signs, and colonic flora invasion (Parker et al., 2020). The dysregulation of the intestinal microbiota caused by diabetes damages the integrity and permeability of the intestinal mucosal barrier, allowing intestinal microbiota and inflammatory factors to enter the central nervous system through multiple pathways such as blood circulation, generating a neuroinflammatory cascade reaction, thereby damaging brain neurons and leading to cognitive dysfunction. The results of this experiment show that the levels of inflammatory factors IL-1β, IL-6, IL-18, and TNF-α in the blood of diabetic model rats were significantly increased compared with the Control group, while the above indicators in the TAC group were decreased to varying degrees. Therefore, we believe that the improvement effect of TAC on cognitive dysfunction in diabetic model rats is closely related to this pathway. Particularly noteworthy is the NLRP3 inflammasome, which, as an intracellular pattern recognition receptor, is a intracellular multimeric protein complex formed by the combination of apoptosis-associated speck-like protein (ASC) and cysteine-containing aspartate proteolytic enzyme (Caspase-1). The NLRP3 inflammasome can be activated by inflammatory factors. After activation, it cleaves a large number of Caspase-1 precursors to form active Caspase fragments, further promoting the maturation of inflammatory factors such as IL-1β, IL-18, and TNF-α. Simultaneously, the activated Caspase-1 binds to GSDMD and cleaves it. Cytokines are released and secreted through protein pores formed at the N-terminus of GSDMD, accelerating the damage to brain neurons (Meng et al., 2014). Further research results show that the protein expressions of NLRP3, Caspase-1p20, and GSDMD-N in the brain tissue of model group rats were significantly increased, while the protein expressions of NLRP3, Caspase-1p20, and GSDMD-N in the brain tissue of TAC group rats were significantly decreased. This indicates that TAC can effectively inhibit the activation of the NLRP3 signaling pathway in the brain tissue of diabetic model rats and block the process of the central neural inflammatory cascade reaction. Therefore, inflammation may be a key hub connecting the microbiota-gut-brain axis, and regulating the microbiota-gut-brain axis through inflammation is a new therapeutic strategy for cognitive dysfunction in diabetes.

Ferroptosis is a novel concept of cell death characterized by iron overload and lipid peroxidation. In the central nervous system, iron homeostasis plays a vital role in enzyme catalysis, mitochondrial function, myelin formation, and synaptic plasticity. Dysregulation of iron homeostasis can cause oxidative stress and inflammation, leading to nerve cell damage and ultimately resulting in neurological diseases (Tang et al., 2022). Existing studies have proved that ferroptosis is involved in the occurrence and development of cognitive dysfunction in diabetes. Under the combined effects of factors such as oxidative stress, insulin resistance, and inflammatory responses, the intracellular iron accumulation in nerve cells increases (Liu et al., 2024), triggering ferroptosis to damage neurons and thereby causing the occurrence of cognitive dysfunction. In this study, through Prussian blue staining and the detection of Fe2+ in serum tissues, it was found that there was excessive accumulation of Fe2+ in the hippocampus of the model group rats, while the accumulation of Fe2+ in the hippocampus of the TAC group rats was significantly reduced. The influence of the xCT-GSH-GPX4 pathway on ferroptosis has been widely accepted (Dixon et al., 2012). The glutamate/cystine transporter system (system xc−) exchanges intracellular glutamate with extracellular cystine. Cystine is a precursor for glutathione synthesis, which can regulate the ratio of cysteine/glutathione and protect cells from oxidative damage (Tang et al., 2021). GSH is an important cofactor of GPX4 and can promote the reduction of phospholipid hydroperoxides mediated by GPX4 to alcohols, ultimately reducing ROS accumulation and iron-dependent cell death. Our detection of lipid peroxidation, oxidative stress and other indicators, as well as GSH, GSH-PX, xCT, and GPX4 in the hippocampus of rats, also verified the occurrence of ferroptosis in the hippocampus of diabetic rats.

A large number of studies have demonstrated a close relationship between NLRP3 and ferroptosis. The activation of the NLRP3 signaling pathway can facilitate the occurrence and development of ferroptosis. In the AKI model induced by pesticides, the inhibition of ferroptosis can alleviate the activation of the NLRP3 inflammasome (Zhang et al., 2023); in the acute lung injury model caused by sepsis, NLRP3 interacts with ferroptosis (Cao et al., 2022); in the macrophage model induced by PM, it was discovered that NLRP3 inflammasome inhibitors had an inhibitory

effect on the increase in intracellular free iron levels and the expression of iron-related proteins. Meanwhile, this experiment proved that the enhanced immune response induced by ferroptosis might trigger an excessive immune response, induce the activation of inflammasomes and pyroptosis of cells, and the increase in inflammatory cytokines caused by the activation of inflammasomes might contribute to the induction of ferroptosis (Beurel et al., 2020). Recently, it has been found that NLRP3 can regulate ferroptosis in LPS-induced S-AKI. The results of RNA sequencing of mouse kidney tissue were combined with the analysis of the FerrDb V2 database, revealing that the absence of NLRP3 can reduce renal ferroptosis, which is also confirmed by the expression of ferroptosis-driven genes and the detection of tissue iron (Li Z. et al., 2023). This study indicates that while NLRP3/Caspase-1 p20/GSDMS-N is significantly upregulated in the model group, xCT/GPX4 is significantly inhibited, resulting in a significant increase in the accumulation of Fe2+ in neurons. The inhibitory effect of TAC on NLRP3 is also manifested in the ferroptosis of nerve cells. NLRP3/Caspase-1 p20/GSDMS-N significantly decreases, xCT/GPX4 expression significantly increases, and lipid peroxidation and oxidative stress responses in hippocampal tissue are also significantly inhibited. In conclusion, ferroptosis and inflammasomes have established a positive feedback loop, influencing each other. This provides a novel mechanism targeting NLRP3 for diseases involving ferroptosis, which holds significant reference value.

Furthermore, a limitation of this study lies in the fact that only one animal model of diabetic cognitive impairment was selected for investigation, and no reverse validation was carried out. Further reverse validation of the application of TAC in diabetic cognitive dysfunction is required, for instance, through intestinal flora transplantation.

5 Conclusion

This study investigated the disruption of gut microbiota in diabetes mellitus, leading to damage of the gut mucosal immune barrier and subsequent initiation of gut inflammation spreading to brain tissue, resulting in inflammatory cascade reactions, neuronal ferroptosis, and cognitive dysfunction. Our study found that TAC can improve the integrity and permeability of the gut mucosa by regulating the gut microbiota, restore gut immune homeostasis, inhibit the spread of gut inflammation to the brain, improve the cascade of brain inflammation, and reduce the occurrence of brain tissue ferroptosis, thereby improving cognitive dysfunction in diabetic rats. These results strongly indicate that the gut microbiota may be an effective target for the treatment of cognitive dysfunction in diabetes, and TAC may be an effective candidate for alleviating cognitive dysfunction caused by diabetes.

Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) repository, accession number PRJNA1189586.

Ethics statement

The animal study was approved by Experimental Animal Welfare Ethics Committee of Heilongjiang University of Traditional Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YQ: Writing – original draft. JL: Writing – original draft. YT: Writing – original draft. RC: Writing – original draft. YG: Writing – original draft. QX: Writing – review & editing. YH: Writing – review & editing.

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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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Gut microbiota has important roles in the obstructive sleep apnea-induced inflammation and consequent neurocognitive impairment

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Obstructive sleep apnea (OSA) is a state of sleep disorder, characterized by repetitive episodes of apnea and chronic intermittent hypoxia. OSA has an extremely high prevalence worldwide and represents a serious challenge to public health, yet its severity is frequently underestimated. It is now well established that neurocognitive dysfunction, manifested as deficits in attention, memory, and executive functions, is a common complication observed in patients with OSA, whereas the specific pathogenesis remains poorly understood, despite the likelihood of involvement of inflammation. Here, we provide an overview of the current state of the art, demonstrating the intimacy of OSA with inflammation and cognitive impairment. Subsequently, we present the recent findings on the investigation of gut microbiota alteration in the OSA conditions, based on both patients-based clinical studies and animal models of OSA. We present an insightful discussion on the role of changes in the abundance of specific gut microbial members, including short-chain fatty acid (SCFA)-producers and/or microbes with pathogenic potential, in the pathogenesis of inflammation and further cognitive dysfunction. The transplantation of fecal microbiota from the mouse model of OSA can elicit inflammation and neurobehavioral disorders in naïve mice, thereby validating the causal relationship to inflammation and cognitive abnormality. This work calls for greater attention on OSA and the associated inflammation, which require timely and effective therapy to protect the brain from irreversible damage. This work also suggests that modification of the gut microbiota using prebiotics, probiotics or fecal microbiota transplantation may represent a potential adjuvant therapy for OSA.

KEYWORDS

obstructive sleep apnea, intermittent hypoxia, systematic inflammation, neurocognitive dysfunction, gut microbiota

Introduction

Obstructive sleep apnea (OSA) is a chronic sleep-related breathing disorder that is characterized by recurrent collapses in the upper airway during sleep, directly causing sleep fragmentation (SF) and chronic intermittent hypoxemia (IH) (Lévy et al., 2015). The development of OSA is largely attributed to a narrow, high-arched hard palate, or midface hypoplasia with retro-positioning of the maxilla and chin, or an enlarged pharynx, in the

majority of cases observed in individuals with obesity (Neelapu et al., 2017; Kubota et al., 2005). The structural disproportions would in turn bring the soft palate and tongue closer to the back of the throat, thus leading to partial or complete airway blockage. Apnea hypopnea index (AHI), which is defined as an average number of partial or full breathing stop events within an hour of sleep, is the most common used indicator for the OSA diagnosis and severity determination (Shahar, 2014; Pevernagie et al., 2020). A number of epidemiological studies based on AHI have revealed a high prevalence of OSA globally (Benjafield et al., 2019; Grote, 2019; Wei et al., 2022; Lv et al., 2023). Two consecutive works have demonstrated that the OSA incidence in the adult population of the USA is approximately 33% among males but lower among females (Benjafield et al., 2019). The overall prevalence of OSA among 38,000 Russian citizens is 48.9% (Khokhrina et al., 2020). Notably, studies covering China (Ding et al., 2022), Chile (Peñafiel et al., 2019), Canada (Dosman et al., 2022), Germany (Fietze et al., 2019), Switzerland (Heinzer et al., 2015), Singapore (Tan et al., 2016), and Japan (Nakayama-Ashida et al., 2008) revealed a higher incidence of OSA, all of them exceeding 50%. Therefore, these findings collectively indicate that OSA is the most prevalent disease diagnosed in the department of otorhinolaryngology. The typical symptoms of OSA often include snoring, breathing breaks, excessive daytime sleepiness, and dry mouth and headache upon waking (Veasey and Rosen, 2019; Gottlieb and Punjabi, 2020). More importantly, prolonged exposure of OSA patients to IH could activate systematic inflammation and impact central nervous system (CNS), which ultimately lead to brain structural injury and severe neurocognitive deficits. The precise mechanism by which inflammation is induced in the OSA condition remains poorly understood.

Herein, we provide an overview of the current state of the art, and discuss a hypothetical scenario in which OSA may directly alter the composition of the gut microbiota, elicit inflammatory responses, and consequently lead to neurocognitive impairment. This paper reviews progress from both clinical studies and animal models are included.

Neurocognitive dysfunction is prevalent in OSA

Recent decades have borne witness to an increasing clarity regarding the prevalence of neurocognitive dysfunction among patients diagnosed with OSA. This phenomenon is characterized by deficits in attention, memory, and executive functions (Vanek et al., 2020; Xia et al., 2023; Kloepfer et al., 2009; Bawden et al., 2011; Hrubos-Strøm et al., 2012; Shieu et al., 2022). Indeed, clinical studies focusing on the effects of OSA have found that three distinct types of memory, including verbal, procedural and working memory, significantly decayed in the patients with OSA (Cunningham et al., 2023; Teh et al., 2023; Kloepfer et al., 2009; Naëgelé et al., 2006). Furthermore, several studies employed a more comprehensive set of tools to systematically identify cognitive impairment relevant to OSA (Xia et al., 2023; Bawden et al., 2011; Gnoni et al., 2023). Reviews of high quality are recommended to be consulted for a more detailed account of the cognitive impairment caused by OSA. This relatively underdiagnosed syndrome affects approximately 1 billion people globally and represents a significant public health concern (Benjafield et al., 2019).

In alignment with the abnormal cognitive function, structural alterations in brain tissues or regions have been identified through the utilization of diverse imaging technologies such as resting-state functional magnetic resonance imaging (fMRI) and computed tomography (CT). The affected areas across studies are diverse and often involved with multiple subregions (Zimmerman and Aloia, 2006), including the integrity of the gray or white matter (Lee et al., 2022; Castronovo et al., 2014), hippocampus (Kheirandish-Gozal et al., 2018; Macey et al., 2018; Gale and Hopkins, 2004), frontal lobe (Bai et al., 2021; Shu et al., 2022), temporal lobe (Shu et al., 2022; Morrell et al., 2010), cerebellum (Shu et al., 2022, Morrell et al., 2010), corpus callosum (Kheirandish-Gozal et al., 2018), and insular cortex (Kheirandish-Gozal et al., 2018). Despite the complexity and diversity, most of these areas are responsible for neurocognitive performance, indicating that structural alteration is a probable underlying cause of the observed deterioration in neurocognitive performance. For example, changes in the hippocampal volume have been identified in multiple studies through the MRI-based imaging analysis, while the hippocampus apoptosis or atrophy can cause learning, mnemonic, attentional, and executive function deficits. An early study, which involved with 17 newly diagnosed, untreated OSA patients and 15 age-matched healthy control subjects found that neurocognitive impairments were linked with a reduction of gray matter volume in the left hippocampus (entorhinal cortex), left posterior parietal cortex, and right superior frontal gyrus (Canessa et al., 2011). In another similar investigation, researchers identified atrophy of the neocortex and cerebellum, as well as a reduction in the volume of the hippocampal dentate gyrus and cerebellar dentate nucleus in patients with OSA (Kim H. et al., 2016). Of particular interest, after the continuous positive airway pressure (CPAP) treatment was administered to the patient cohorts in both studies, the impaired brain structure was restored to normality together with improved cognitive function, indicative of the reversibility of cognition deficits.

The intimate association of OSA with neurocognitive dysfunction has been also observed in a range of animal models, including pigs (Lonergan et al., 1998), dogs (Hendricks et al., 1993; Hendricks et al., 1987), rabbits (Schiefer et al., 2020; Yu et al., 2014), cats (Neuzeret et al., 2011), rats (Nácher et al., 2007; Farré et al., 2003), and mice (Qiu et al., 2023; Puech et al., 2022; Veasey et al., 2013; Nair et al., 2011a; Zhu et al., 2007; Puech et al., 2023). The disease has been modeled using either sleep fragmentation (SF), or intermittent hypoxia (IH), or both. SF can be induced by sleep disruption with experimental devices (Nair et al., 2011b; Ramesh et al., 2012), while IH is triggered by repeated exposure to lower oxygen levels (Badran et al., 2020; Puech et al., 2022). Although adverse effects of IH and SF on cognitive function may differ, common outcomes include impaired sleep quality, abnormal behavior, reduced learning ability and impaired physical functioning. In a study based on a rat model of OSA, for example, exposure to IH resulted in deficits in spatial memory and learning performance as assessed by Morris water maze tasks, together with the hippocampal apoptosis (Gao et al., 2017).

Inflammation caused by OSA is very likely to induce cognitive deficits

The pathogenesis of cognitive impairment induced by OSA is believed to be complex and remains poorly elucidated (Lv et al., 2023;

Liu et al., 2020; Orrù et al., 2020). However, several lines of evidence from both clinical and animal model studies strongly support involvement of inflammation. Firstly, numerous studies have found that OSA can cause a systematic or local inflammation, as evidenced by increased levels of serum inflammatory cytokines are often observed in OSA patients (Liu et al., 2020; Nadeem et al., 2013; Bouloukaki et al., 2017; Bozic et al., 2018; Motamedi et al., 2018; Svatikova et al., 2003; Sozer et al., 2018). Tumor necrosis factor (TNF)-α and interleukin (IL)-6 are two representative biomarkers that are closely linked with OSA (Kheirandish-Gozal and Gozal, 2019), and more intriguingly both are also thought to contribute to neurocognitive dysfunction (Tegeler et al., 2016). Secondly, it is frequently observed that the activation of inflammatory processes and cognitive deficits occur concurrently in a considerable number of rodent models of OSA induced by IH (Liu et al., 2020; Dong et al., 2018; Sapin et al., 2015; Shi et al., 2018; Snyder et al., 2017; Darnall et al., 2017; Kim et al., 2013; Smith S. M. et al., 2013; Deng et al., 2015). Thirdly, in clinical studies, the magnitude of inflammation, as indicated by serum levels of various inflammatory cytokines, is frequently correlated with the severity of OSA (Nadeem et al., 2013; Sozer et al., 2018; Bouloukaki et al., 2017; Bozic et al., 2018; Motamedi et al., 2018). For instance, a recent analysis involving 858 OSA patients and 190 matched controls demonstrated that the serum levels of uric acid and high-sensitivity C-reactive protein (hsCRP), two markers of inflammation, were elevated in the severe group compared to the mild group (Bouloukaki et al., 2017). Fourthly, effective OSA therapy by CPAP can improve neurocognitive performance and also reduce/resolve inflammation (Tichanon et al., 2016; Lu et al., 2017; Ohga et al., 2003; Kuramoto et al., 2009; Wu et al., 2010; Jin et al., 2017; Yokoe et al., 2003; Steiropoulos et al., 2009; Schiza et al., 2010). Altogether, ample evidence from these works substantiates the pivotal role of OSA-induced neuroinflammation in the pathogenesis of neuronal injury and subsequent cognitive deficits.

The precise mechanistic pathway by which OSA triggers inflammation remains poorly understood. However, it has been postulated that HIF-1α, a critical transcription factor responsive to hypoxic conditions, is activated to increase ROS synthesis, which would in turn initiate oxidative stress and the inflammatory process (McGettrick and O'Neill, 2020). Furthermore, there is also evidence to show that chronic IH conditions observed in OSA patients stimulate leptin, an obesity biomarker in white adipose tissue (Pan and Kastin, 2014), while leptin can further promote production of proinflammatory cytokines (Berger and Polotsky, 2018). These changes may further lead to monocyte-endothelial cell adhesion, dysfunction of endothelial cells, breach of the blood–brain barrier, and finally the perfusion of inflammatory cytokines and macrophages into the central nervous system. Consequently, the excessive neuroinflammatory response results in the activation of glial cells, synaptic damage and loss, neuronal necrosis and apoptosis, and ultimately a significant exacerbation of neurocognitive deficits (Liu et al., 2020).

OSA altered gut microbiota

In addition to the aforementioned effects on inflammation and cognitive function, the impact of OSA can even extend to the gastrointestinal tract to modulate the oxygen concentrations and further the ecosystem, where at least 100 trillion bacteria colonize (Honda and Littman, 2016; Rooks and Garrett, 2016; Gomaa, 2020). In light of the existence of an oxygen concentration gradient in the range of 150–200 μm near the gut epithelium (Espey, 2013) and the susceptible responsiveness of gut microbiota to oxygen level change (Albenberg et al., 2014), it seems highly probable that chronic exposure to hypoxia would favor the survival of obligate anaerobes and therefore alter the bacterial diversity. Indeed, several mouse model-based studies have demonstrated that IH intervention can induce a periodic hypoxia pattern in the arterial blood and the lumen of the small intestine, as well as an increased abundance of obligate and facultative anaerobes (Moreno-Indias et al., 2015; Khalyfa et al., 2021; Lucking et al., 2018; Durgan et al., 2016), despite the possibility that IH-induced systemic immune responses may exert a modulatory effect on gut microbiota. Another important feature of OSA is nocturnal arousal due to sleep fragmentation. Interestingly exposure of mice to sleep fragmentation also caused notable change in gut microbiota, characterized by an increase in *Firmicutes* and a decrease in *Bacteroidetes* at the phylum level (Poroyko et al., 2016). It is noteworthy that a gut dysbiosis was also a prevalent trait in OSA patients. Two clinical studies investigating the OSA patients from disparate regions in China identified an altered gut microbiota profile (Ko et al., 2019; Wang et al., 2022). Furthermore, the composition of the gut microbiota was found to be significantly altered in pediatric patients with OSA in comparison to their age-matched healthy controls (Valentini et al., 2020; Collado et al., 2019). Consistently, an interesting single-armed study that investigated the responses of nine normal-weight men under two occasions, either with two nights of normal sleep or two nights of partial sleep deprivation. It revealed that sleep loss can directly induce an increased *Firmicutes* to *Bacteroides* (F/B) ratio in gut microbiota (Benedict et al., 2016).

Gut microbiota composition alteration associated with inflammatory activation in OSA patients

In light of the pivotal roles of gut microbiota in regulating human physiology, particularly immunity (Kamada et al., 2012; Donohoe et al., 2011; Zheng et al., 2020; Belkaid and Harrison, 2017; Albhaisi et al., 2020; Morais et al., 2021), a hypothesis was therefore proposed that the OSA-induced gut dysbiosis, often featured with a changed F/B ratio, might contribute to inflammation, and potentially the cognitive dysfunction. In the context of the microbiota-immune system interaction, multiple microbial metabolites and components, including short-chain fatty acids (SCFAs), lipopolysaccharide (LPS) and exotoxins, act as potent effectors, facilitating a bridge between gut microbiota and local or systematic immunity (Rooks and Garrett, 2016; Wang G. et al., 2019). Indeed, the immunological effects of these microbiota-derived molecules are manifold, encompassing both innate and adaptive immunity (Tang et al., 2021). SCFAs are primarily produced from indigestible oligosaccharides by some beneficial members of the *Bacteroidetes* phylum, including the families *Lactobacillaceae*, *Ruminococcaceae*, *Erysipelotrichaceae*, *Bifidobacteriaceae*, and *Clostridium* (den Besten et al., 2013). In addition to serving as a source of energy for intestinal epithelial cells

(IEC) (Rivière et al., 2016), SCFAs have pleotropic roles in the fortification of the gut barrier and maintenance of immune homeostasis. More specifically, these beneficial regulatory actions include stimulation of mucus production (Wrzosek et al., 2013), regulation of tight junction (TJ) proteins via multifaceted signaling pathways (Parada Venegas et al., 2019), polarization of anti-inflammatory macrophages (Ji et al., 2016), increased production of antimicrobial peptide (AMP) (Qiu et al., 2012), activation of NLR-family-pyrin-domain-containing-3 (NLRP3) inflammasomes and production of homeostatic cytokine interleukin-18 (IL-18) (Macia et al., 2015), modulation of B cell differentiation and Immunoglobulin A (IgA) secretion (Kim M. et al., 2016), reduced expression of T cell-activating molecules on antigen-presenting cells (Park et al., 2015), and increased number and function of colonic regulatory T (T_{regs}) cells (Smith P. M. et al., 2013). In contrast, several gut microbial members, including *Desulfovibrio, Prevotella, Lachnospiraceae,* and *Paraprevotella*, have been demonstrated to induce inflammatory responses and disrupt the structural integrity of the gut barrier. LPS is a well-known bacterial endotoxin with profound immunostimulatory and inflammatory capacity. Contrary to the beneficial effects of SCFAs, LPS has the potential to bind to its cognate toll-like-receptor (TLR4) and promote the activation of inflammatory macrophage (M1 polarization), which in turn leads to the production of an array of inflammatory cytokines, IL-1β, IL-6, IL-12, and TNF-α (Martinez et al., 2008). Through the same signal pathway, LPS can also compromise the integrity of the intestinal barrier (Guo et al., 2013). Furthermore, the prevalence of Prevotella and Desulfovibrio, which possess mucin-degrading capabilities, was found to be elevated in individuals with OSA, thereby exacerbating the gut permeability. An increase in those bacterial species will therefore cause the leakage of LPS and other bacterial components from the gut into the blood circulation, thus stimulating the release of inflammatory mediators and aggravating systemic inflammation.

Consistent with the above analysis, an investigation into the alteration of the gut microbiota in OSA patients indeed validated the abundance reduction in the abundance of bacteria associated with SCFA production, or alternatively an increase in the abundance of pathogenic ones, despite the fact that only a limited number of clinical studies in this direction existed. One study involving 93 OSA patients revealed that alterations in the gut microbiota were characterized by a decrease in the abundance of SCFA producers (including *Faecalibacterium, Bifidobacterium, Lactobacillus*, and *Bacteroides*), an increase for the pathogenic *Prevotella*, and concomitantly a reduction in the serum levels of IL-6 (Ko et al., 2019). Interestingly, this work did not identify a profound change in the F/B ratio between the OSA patients and healthy controls (Ko et al., 2019), suggesting that the F/B ratio alteration may not be a general OSA-associated rule, and that a more detailed analysis of specific bacterial taxa level should be necessary. In another observational study, the inflammation-related bacteria (*Megamonas, Lactobacillus, Megasphaera*, and *Coprococcus* at the genus level) were also enriched in OSA patients, while *Alistipes*, *Eubacterium coprostanoligenes, Blautia, Roseburia, Fusobacteria,* and *Ruminococcus gnavus* were found to be depleted. The pro-inflammatory cytokine IL-1β and TNF-α were elevated with OSA (Lu et al., 2022). A comparable pattern was identified among the pediatric OSA patients, whereby the relative abundances of several well-documented SCFA producers, including *Bacteroides, Bifidobacterium, Ruminococcus, Collinsella,* and *Faecalibacterium*, exhibited a decline (Valentini et al., 2020). The hypothesis was further validated in the two clinical trials, in which healthy human subjects were exposed to either a short-term or a long-term period of sleep deprivation, directly resulted in a reduction of the abovementioned species with SCFA-production capacity (Gao et al., 2023; Benedict et al., 2016).

Moreover, a comparable pattern was observed in multiple OSA animal model-based gut microfloral analyses, with an increase in pathogenic microbes being a particularly prevalent finding. For example, *Prevotella, Paraprevotella, Desulfovibrio*, and *Lachnospiraceae* were found to be enriched in a mouse model of OSA, induced by either SF or IH (Gao et al., 2023; Poroyko et al., 2016; Badran et al., 2020; Khalyfa et al., 2021; Badran et al., 2023; Yan et al., 2024). Interestingly, treatment of the sleep-deprived mice with butyrate, a type of SCFA, significantly ameliorated intestinal mucosa injury and inflammation response through the suppression of the HDAC3–GSK-3β–Nrf2–NF-κB signaling cascade (Gao et al., 2023). In another study, neonatal brain immaturity, white matter injury (WMI), reduced abundance of beneficial gut microbes *Bacteroides thetaiotaomicron* and *Parabacteroides distasonis* and accumulation of microbiota-derived cholic acid were identified in chronically hypoxic rats, whereas administration of *B. thetaiotaomicron* and *P. distasonis* reverted the cholic acid concentration and rescued the chronic hypoxia-induced WMI and inflammation. These findings suggest that the administration of SCFAs or probiotics may represent a potential strategy for alleviating the inflammation and neurocognitive deficits associated with OSA (Yan et al., 2024).

Fecal microbiota transplant (FMT) simply implies the transfer of stool samples from a donor's colon to a recipient's colon (Gupta and Khanna, 2017). FMT has demonstrated considerable promise in the treatment of intestinal infection, inflammatory bowel disease, hypertension, obesity, and diabetes mellitus (Kelly et al., 2015; Turnbaugh et al., 2006; Adnan et al., 2017; Wang H. et al., 2019). Although only a few studies have employed FMT in analyzing the basis of OSA, two recent works showed that FMT from the IH-mice can elicit inflammation and neurobehavioral disorders in naïve mice (Badran et al., 2020; Poroyko et al., 2016). This provides further robust evidence that gut microbiota dysbiosis is, at least partially, a causal basis of cognitive dysfunction.

Conclusions and perspectives

The prevalence of OSA is markedly high across countries and regions, and it is frequently complexed with a variety of comorbidities. With firm evidence from both clinical studies and animal models, the present review reveals a clear and compelling link of OSA with alterations in the gut microbiota, systematic inflammation, brain substructural changes and neurocognitive impairment, respectively. A hypothesis is therefore proposed to explain the pathogenesis of OSA that chronic IH or SF in OSA patients would trigger gut microbiota dysbiosis, which is often characterized by depletion of producers of beneficial microbial metabolites, or/and enrichment of microbes with potentials to impair mucosa and activate inflammation (Figure 1). This in turn results in the activation of systematic inflammation, with inflammatory cytokines breaching the blood–brain barrier,

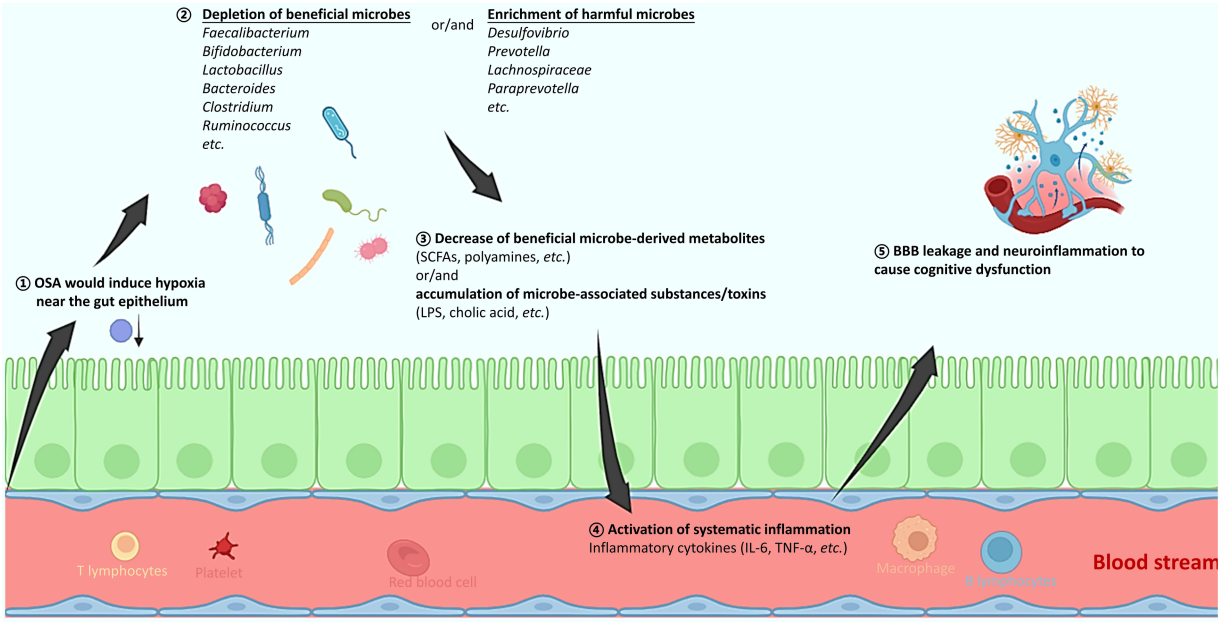

FIGURE 1
A schematic diagram explaining how obstructive sleep apnea (OSA) would alter the gut microbiota, activate systematic inflammation, and consequently induce brain tissue injury and cognitive dysfunction. (1) The OSA-induced hypoxia might alter the oxygen concentration near the gut epithelium. (2) The depleted oxygen radiation thus favors the increased abundance of obligate and facultative anaerobes, resulting in the gut microbiota change that is mostly featured with an increased ratio of *Firmicutes* to *Bacteroides* (F/B). Specifically, producers of beneficial metabolites such as SCFAs and polyamines decrease, while microbial members with pathogenic potentials increase. (3) The gut microbiota composition change further leads to accumulation of microbial toxins, and/or decrease of a wide spectrum of beneficial metabolites. (4) The gut barrier function might be compromised, and levels of various inflammatory cytokines are elevated, resulting in a systematic inflammation. (5) The inflammatory cytokines can breach the BBB, activate microglial cells, cause neuroinflammation, and consequently result in cognitive deficits. OSA, obstructive sleep apnea; SCFAs, short chain fatty acids; LPS, lipopolysaccharide; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; BBB, blood brain barrier.

activating microglial cells, and ultimately causing necrosis and apoptosis of neural cells and neurocognitive deficits. It is imperative to emphasize the importance of timely diagnosis and treatment of OSA and its associated inflammation, in order to prevent or alleviate the irreversible neurocognitive damage. Moreover, the modulation of gut microbiota using prebiotics (such as butyrate), or probiotics (SCFA producers) may represent a potential and effective adjuvant therapy for OSA. More future works in this direction are still needed.

Author contributions

MT: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing. YW: Data curation, Formal analysis, Writing – original draft. JL: Writing – original draft. SY: Data curation, Writing – original draft. ZH: Writing – original draft. JH: Writing – review & editing. QY: Writing – review & editing. FL: Writing – original draft. SL: Writing – original draft, Writing – review & editing.

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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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Influential articles in autism and gut microbiota: bibliometric profile and research trends

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Objective: Autism spectrum disorder (ASD) is a common neurodevelopmental disorder. Increasing evidence suggests that it is potentially related to gut microbiota, but no prior bibliometric analysis has been performed to explore the most influential works in the relationships between ASD and gut microbiota. In this study, we conducted an in-depth analysis of the most-cited articles in this field, aiming to provide insights to the existing body of research and guide future directions.

Methods: A search strategy was constructed and conducted in the Web of Science database to identify the 100 most-cited papers in ASD and gut microbiota. The Biblioshiny package in R was used to analyze and visualize the relevant information, including citation counts, country distributions, authors, journals, and thematic analysis. Correlation and comparison analyses were performed using SPSS software.

Results: The top 100 influential manuscripts were published between 2000 and 2021, with a total citation of 40,662. The average number of citations annually increased over the years and was significantly correlated to the year of publication (r = 0.481, $p < 0.01$, Spearman's rho test). The United States was involved in the highest number of publications (n = 42). The number of publications in the journal was not significantly related to the journal's latest impact factor (r = 0.016, $p > 0.05$, Spearman's rho test). Co-occurrence network and thematic analysis identified several important areas, such as microbial metabolites of short-chain fatty acids and overlaps with irritable bowel syndrome.

Conclusion: This bibliometric analysis provides the key information of the most influential studies in the area of ASD and gut microbiota, and suggests the hot topics and future directions. The findings of this study can serve as a valuable reference for researchers and policymakers, guiding the development and implementation of the scientific research strategies in this area.

KEYWORDS

bibliometric analysis, autism, gut microbiota, research trends, citations

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication and interaction, alongside the manifestation of repetitive and restrictive behavior patterns (APA, 2022). The global prevalence of ASD has been estimated to be around 1%, and the prevalence estimates have increased over time in various countries (Zeidan et al., 2022). Persons with ASD may have emotional and behavioral problems, such as self-harm, aggression, temper tantrums, and property destruction (Jang et al., 2011). They often have other psychiatric conditions, such as anxiety, depression, and psychosis (Dan et al., 2020). The economic costs of ASD are huge, and they include costs for healthcare services, special education, production loss for persons with ASD, lost productivity for caregivers, and respite care (Rogge and Janssen, 2019). In the United States, it has been reported that the average yearly expenditure for emergency room services is $15,929 for ASD, compared to $2,598 for non-ASD; and yearly expenditure for outpatient visits is $4,375 for ASD compared to $824 for non-ASD (Vohra et al., 2017). In the United Kingdom, it has been estimated that adolescents with ASD who need additional special education or residential schooling can cost £10,507 in 6 months (Barrett et al., 2015).

The composition of the gut microbiota has been reported to be associated with ASD. The gut microbiota has a very diverse composition and is composed of bacteria, as well as fungi, viruses and protists (Enaud et al., 2018). It has a bidirectional connection with the central nervous system. Millions of nerve cells in the gut form the enteric nervous system which is recognized as a second brain (Gershon, 1999). The microbiota-gut-brain axis has been studied and the bidirectional communication of this pathway occurs through various mechanisms, including enteric nervous system, autonomic nervous system, immune system, hormones, and neurotransmitters (Cryan and Dinan, 2012). Possible involvement of a microbial element in the pathogenesis of ASD was first reported in 1998, when Bolte (1998) introduced the hypothesis that *Clostridium tetani* neurotoxin was transported from the gastrointestinal tract to the central nervous system via the vagus nerve, causing symptoms of ASD. The link between gut microbiota and ASD has been studied in animal models. One study published in 2019 that transplanted gut microbiota from human ASD patients into germ-free mice revealed development of hallmark autistic behaviors in the recipient animals (Sharon et al., 2019). The association between gut microbiota and ASD has also been reported in human studies. For example a pyrosequencing study observed that *Bacteroidetes* were present at high levels in the persons with ASD, while *Firmicutes* were more abundant in the healthy control group (Finegold et al., 2010).

Given the rising trend of interest related to ASD and gut microbiota, it is worthwhile to identify the most influential scientific achievements amidst the abundance of literature in this research area. Bibliometric analysis is a widely used, rigorous approach for exploring extensive scientific datasets and extracting useful information, such as author names, total citations, and country distributions (Donthu et al., 2021). It can visualize the detailed results and help researchers to develop a thorough understanding of the research trajectory in the field and identify research hotspots and gaps. For example, a recent bibliometric analysis presents a comprehensive global overview of artificial intelligence in life science research and suggests that coordinated international research efforts are necessary to advance this research area (Schmallenbach et al., 2024). As bibliometric analysis offers both quantitative and qualitative insights into the influence and evolution of academic communication, it assists policymakers to track emerging trends and make informed choices about research funding and collaboration strategies (Hassan and Duarte, 2024).

To the best of our knowledge, no prior bibliometric analysis has been performed to explore the most influential works in the field of ASD and gut microbiota. This study seeks to fill this gap by conducting an in-depth analysis of the most-cited articles concerning the intersection of ASD and gut microbiota, with the goal of providing valuable insights to the existing body of research and guiding researchers and policymakers in evaluating and making informed decisions related to this field.

Materials and methods

Article selection

The Clarivate Analytics Web of Science database was used to identify relevant articles in this current bibliometric review. The Web of Science database has the capability to retrieve numerous articles with comprehensive details, including titles, author names, total download times, and total citations. It is an extensive repository which includes major journals across more than 170 subjects (Quan et al., 2024). In addition, it enhances coverage by including citations from scientific publications dating back to 1900 and encompasses all significant high-impact scientific journals (Martin-Martin et al., 2018; Tomova et al., 2015). Various studies, including those on gut microbiota and other diseases, have relied solely on the Web of Science database as their primary source for conducting bibliometric analyses (Chang et al., 2023; Ring et al., 2020; Wan et al., 2022; Ying et al., 2022).

To find pertinent articles, several recent systematic reviews related to ASD or gut microbiota were referenced to create search terms (Lewandowska-Pietruszka et al., 2023; Perna et al., 2023; Wang A. et al., 2023). Besides, an information specialist was consulted to help in further refinement of the search strategies and ensure the comprehensive retrieval of all relevant articles. The following search terminologies were used in this bibliometric review: TS = ("autism" OR "autistic" OR "Asperger*" OR "pervasive developmental disorder*") AND TS = ("microbiome*" OR "microbiota*" OR "flora*" OR "microbe*" OR "microflora*" OR "microbial"). The terminology TS denotes a search focused on the topic of interest.

Using this approach, the Web of Science database was systematically searched in January 2024. No restrictions were implemented in terms of the language of articles and the publication dates. The publications were ranked according to the number of citations, and they were then reviewed to identify the 100 most-cited papers. Studies were included if (1) one of their focuses was related to the topic of ASD and gut microbiota, (2) the type of the document was either Article or Review Article according to the Web of Science database. Other types of documents, such as Editorial Material, Meeting Abstract, and Book Chapters, were

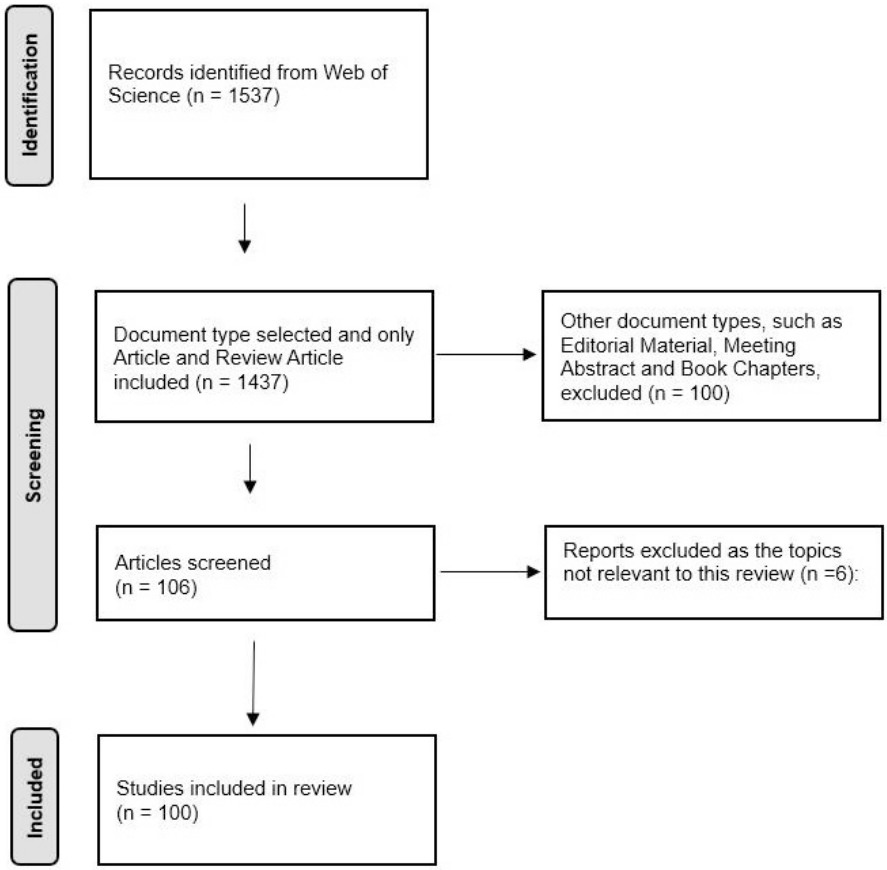

FIGURE 1
PRISMA flow diagram of the study selection process.

excluded. Two authors (JY and MZ) independently performed the selection of the top 100 papers with the most citations, based on the title and abstract and reading the full texts if needed. If any disagreement arose, a third author was consulted to achieve an agreement.

Data analysis

The bibliometric data analysis was performed using Biblioshiny package in R (Version 4.3.2) (Aria and Cuccurullo, 2017). The Biblioshiny package was previously utilized for this type of analysis in various areas, such as the application of deep learning in cancer (Wang R. et al., 2024), the use of monoclonal antibodies for atherosclerosis (Ma et al., 2023), and the global impact of metaproteomics research (Ascandari et al., 2023). All data were downloaded from the Web of Science database and imported into Biblioshiny, which could convert and analyze the information, including the authors, years of publication, number of citations, and distribution of countries/regions. The study type was categorized into three main groups: (1) animal studies, which incorporated animal models in their study design; (2) human studies, which involved persons with ASD; and (3) reviews, encompassing literature reviews or systematic reviews. The impact factor of each journal was extracted from the Clarivate Analytics Journal Citation Reports.

All statistical analysis was conducted using SPSS software (Version 25). The Shapiro–Wilk test was used to test the normality of the distribution of variables. Spearman's rho test was applied to assess the correlations between two variables. Mann–Whitney *U* test was performed to assess for any statistically significant differences between two groups, and the Kruskal–Wallis test was conducted to compare the differences between three or more groups. All *p* values were two-tailed, and a *p*-value of ≤ 0.05 was considered to indicate statistical significance.

Results

Overview

A total of 1,537 articles were retrieved from the Web of Science database on 10 January 2024, and the 100 most-cited papers were identified after screening. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) was used to describe the detailed screening process (Figure 1). General information of the selected articles is detailed in Table 1. The trends of the annual publications of the 100 most-cited articles are described in Figure 2.

TABLE 1 The 100 most-cited articles in autism spectrum disorder and gut microbiota.

No.	Reference	Journal	Journal impact factor at the year of publication	Journal impact factor in 2023	Article	Study type	Total citations	Annual citations
1	Hsiao et al., 2013	*Cell*	33.116	45.6	Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders	Animal study	2,081	173.42
2	Cryan et al., 2019	*Physiological Reviews*	25.588	33.4	The microbiota-gut-brain axis	Review	1,769	294.83
3	Carabotti et al., 2015	*Annals of Gastroenterology*	Nil	2.1	The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems	Review	1,412	141.20
4	Rinninella et al., 2019	*Microorganisms*	4.152	4.1	What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases	Review	1,395	232.50
5	Gilbert et al., 2018	*Nature Medicine*	30.641	58.7	Current understanding of the human microbiome	Review	1,133	161.86
6	Fung et al., 2017	*Nature Neuroscience*	19.912	21.3	Interactions between the microbiota, immune and nervous systems in health and disease	Review	1,027	128.38
7	Riviere et al., 2016	*Frontiers in Microbiology*	4.076	4.0	Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut	Review	928	103.11
8	Mayer et al., 2015	*Journal of Clinical Investigation*	12.575	13.3	Gut/brain axis and the microbiota	Review	847	84.70
9	Nguyen et al., 2015	*Disease Models & Mechanisms*	4.316	4.0	How informative is the mouse for human gut microbiota research?	Review	808	80.80
10	Sharon et al., 2016	*Cell*	30.41	45.6	The central nervous system and the gut microbiome	Review	787	87.44
11	Kang et al., 2017	*Microbiome*	9.133	13.8	Microbiota transfer therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study	Human study	722	90.25
12	Borre et al., 2014b	*Trends in Molecular Medicine*	9.453	12.8	Microbiota and neurodevelopmental windows: implications for brain disorders	Review	690	62.73
13	Buffington et al., 2016	*Cell*	30.41	45.6	Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring	Animal study	678	75.33
14	Sampson and Mazmanian, 2015	*Cell Host & Microbe*	12.552	20.6	Control of brain development, function, and behavior by the microbiome	Review	664	66.40
15	Morais et al., 2021	*Nature Reviews Microbiology*	78.297	69.2	The gut microbiota-brain axis in behaviour and brain disorders	Review	659	164.75
16	Finegold et al., 2010	*Anaerobe*	2.448	2.5	Pyrosequencing study of fecal microflora of autistic and control children	Human study	643	42.87
17	Adams et al., 2011	*BMC Gastroenterology*	2.422	2.5	Gastrointestinal flora and gastrointestinal status in children with autism-comparisons to typical children and correlation with autism severity	Human study	625	44.64

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10.3389/fmicb.2024.1401597

TABLE 1 (Continued)

No.	Reference	Journal	Journal impact factor at the year of publication	Journal impact factor in 2023	Article	Study type	Total citations	Annual citations
18	Kang et al., 2013	*PLoS One*	3.534	2.9	Reduced incidence of prevotella and other fermenters in intestinal microflora of autistic children	Human study	605	50.42
19	Mayer et al., 2014a	*Journal of Neuroscience*	6.344	4.4	Gut microbes and the brain: paradigm shift in neuroscience	Review	559	50.82
20	Sharon et al., 2019	*Cell*	38.637	45.6	Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice	Animal study	542	90.33
21	Strati et al., 2017	*Microbiome*	9.133	13.8	New evidences on the altered gut microbiota in autism spectrum disorders	Human study	539	67.38
22	Parracho et al., 2005	*Journal of Medical Microbiology*	2.318	2.4	Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children	Human study	527	26.35
23	Dinan and Cryan, 2017c	*Gastroenterology Clinics of North America*	3.265	2.9	The microbiome-gut-brain axis in health and disease	Review	526	65.75
24	De Angelis et al., 2013	*PLoS One*	3.534	2.9	Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified	Human study	526	43.83
25	Zhang et al., 2015	*International Journal of Molecular Sciences*	3.257	4.9	Impacts of gut bacteria on human health and diseases	Review	513	51.30
26	Kho and Lal, 2018	*Frontiers in Microbiology*	4.259	4.0	The human gut microbiome – a potential controller of wellness and disease	Review	511	73.00
27	Cryan et al., 2020	*Lancet Neurology*	44.182	46.6	The gut microbiome in neurological disorders	Review	503	100.60
28	Wang and Kasper, 2014	*Brain Behavior and Immunity*	5.889	8.8	The role of microbiome in central nervous system disorders	Review	500	45.45
29	Hills et al., 2019	*Nutrients*	4.546	4.8	Gut microbiome: profound implications for diet and disease	Review	488	81.33
30	Finegold et al., 2002	*Clinical Infectious Diseases*	Nil	8.2	Gastrointestinal microflora studies in late-onset autism	Human study	476	20.70
31	Dinan and Cryan, 2017b	*Journal of Physiology-London*	4.54	4.7	Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration	Review	431	53.88
32	Stilling et al., 2014	*Genes Brain and Behavior*	3.661	2.4	Microbial genes, brain & behaviour – epigenetic regulation of the gut-brain axis	Review	416	37.82
33	Sandler et al., 2000	*Journal of Child Neurology*	1.134	2.0	Short-term benefit from oral vancomycin treatment of regressive-onset autism	Human study	396	15.84
34	Sgritta et al., 2019	*Neuron*	14.415	14.7	Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder	Animal study	389	64.83

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10.3389/fmicb.2024.1401597

TABLE 1 (Continued)

No.	Reference	Journal	Journal impact factor at the year of publication	Journal impact factor in 2023	Article	Study type	Total citations	Annual citations
35	Song et al., 2004	*Applied and Environmental Microbiology*	3.81	3.9	Real-time PCR quantitation of clostridia in feces of autistic children	Human study	388	18.48
36	Kim et al., 2017	*Nature*	41.577	50.5	Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring	Animal study	385	48.13
37	Hoban et al., 2016	*Translational Psychiatry*	4.73	5.8	Regulation of prefrontal cortex myelination by the microbiota	Animal study	383	42.56
38	Tomova et al., 2015	*Physiology & Behavior*	2.461	2.4	Gastrointestinal microbiota in children with autism in Slovakia	Human study	372	37.20
39	Petra et al., 2015	*Clinical Therapeutics*	2.925	3.2	Gut-microbiota-brain axis and its effect on neuropsychiatric disorders with suspected immune dysregulation	Review	362	36.20
40	Akdis, 2021	*Nature Reviews Immunology*	108.555	67.7	Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions?	Review	356	89.00
41	Barko et al., 2018	*Journal of Veterinary Internal Medicine*	2.286	2.1	The gastrointestinal microbiome: a review	Review	347	49.57
42	Luczynski et al., 2016	*International Journal of Neuropsychopharmacology*	Nil	4.5	Growing up in a bubble: using germ-free animals to assess the influence of the gut microbiota on brain and behavior	Review	344	38.22
43	Kang et al., 2019	*Scientific Reports*	3.998	3.8	Long-term benefit of microbiota transfer therapy on autism symptoms and gut microbiota	Human study	337	56.17
44	Vuong and Hsiao, 2017	*Biological Psychiatry*	11.984	9.6	Emerging roles for the gut microbiome in autism spectrum disorder	Review	330	41.25
45	Ghaisas et al., 2016	*Pharmacology & Therapeutics*	11.127	12.0	Gut microbiome in health and disease: linking the microbiome-gut-brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases	Review	325	36.11
46	Vuong et al., 2017	*Annual Review of Neuroscience*	14.675	12.1	The microbiome and host behavior	Review	319	39.88
47	Dinan et al., 2015	*Journal Of Psychiatric Research*	4.465	3.7	Collective unconscious: how gut microbes shape human behavior	Review	319	31.90
48	Williams et al., 2011	*PLoS One*	4.092	2.9	Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances	Human study	313	22.36
49	de Theije et al., 2014	*Brain Behavior and Immunity*	5.889	8.8	Altered gut microbiota and activity in a murine model of autism spectrum disorders	Animal study	284	25.82
50	Wang et al., 2011	*Applied And Environmental Microbiology*	3.829	3.9	Low relative abundances of the mucolytic bacterium *Akkermansia muciniphila* and *Bifidobacterium* spp. In feces of children with autism	Human study	282	20.14

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10.3389/fmicb.2024.1401597

TABLE 1 (Continued)

No.	Reference	Journal	Journal impact factor at the year of publication	Journal impact factor in 2023	Article	Study type	Total citations	Annual citations
51	Williams et al., 2012	*mBio*	5.625	5.1	Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of *Sutterella* species in intestinal biopsy samples from children with autism and gastrointestinal disturbances	Human study	279	21.46
52	Moloney et al., 2014	*Mammalian Genome*	3.068	2.7	The microbiome: stress, health and disease	Review	274	24.91
53	Hoyles et al., 2018	*Microbiome*	10.465	13.8	Microbiome-host systems interactions: protective effects of propionate upon the blood-brain barrier	Human study	266	38.00
54	Wang et al., 2013	*Molecular Autism*	5.486	6.2	Increased abundance of *Sutterella* spp. and *Ruminococcus torques* in feces of children with autism spectrum disorder	Human study	261	21.75
55	Li et al., 2017	*Frontiers in Cellular Neuroscience*	4.3	4.2	The gut microbiota and autism spectrum disorders	Review	255	31.88
56	Wang et al., 2012	*Digestive Diseases and Sciences*	2.26	2.5	Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder	Human study	252	19.38
57	Mangiola et al., 2016	*World Journal of Gastroenterology*	3.365	4.3	Gut microbiota in autism and mood disorders	Review	237	26.33
58	Liu S. M. et al., 2019	*Scientific Reports*	3.998	3.8	Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder	Human study	235	39.17
59	Sherwin et al., 2019	*Science*	41.846	44.8	Microbiota and the social brain	Review	227	37.83
60	Yap et al., 2010	*Journal of Proteome Research*	5.113	3.8	Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls	Human study	224	14.93
61	Borre et al., 2014a	*Advances in Experimental Medicine and Biology*	1.958	3.65	The impact of microbiota on brain and behavior: mechanisms & therapeutic potential	Review	221	20.09
62	Lees et al., 2013	*Journal of Proteome Research*	5.001	3.8	Hippurate: the natural history of a mammalian-microbial cometabolite	Review	220	18.33
63	Pärtty et al., 2015	*Pediatric Research*	2.761	3.1	A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial	Human study	212	21.20
64	Cenit et al., 2017	*World Journal of Gastroenterology*	3.3	4.3	Influence of gut microbiota on neuropsychiatric disorders	Review	207	25.88
65	Cristofori et al., 2021	*Frontiers in Immunology*	8.787	5.7	Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body	Review	205	51.25
66	Wang et al., 2016	*Journal of Neurogastroenterology and Motility*	2.457	3.3	Effect of probiotics on central nervous system functions in animals and humans: a systematic review	Review	205	22.78

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TABLE 1 (Continued)

No.	Reference	Journal	Journal impact factor at the year of publication	Journal impact factor in 2023	Article	Study type	Total citations	Annual citations
67	Golubeva et al., 2017	*EBioMedicine*	6.183	9.7	Microbiota-related changes in bile acid & tryptophan metabolism are associated with gastrointestinal dysfunction in a mouse model of autism	Animal study	204	25.50
68	Mayer et al., 2014b	*BioEssays*	4.73	3.2	Altered brain-gut axis in autism: comorbidity or causative mechanisms?	Review	204	18.55
69	Knight et al., 2017	*Annual Review of Genomics and Human Genetics*	8.676	7.7	The microbiome and human biology	Review	203	25.38
70	Zhu et al., 2020	*Journal of Neuroinflammation*	8.322	9.3	The progress of gut microbiome research related to brain disorders	Review	199	39.80
71	Fattorusso et al., 2019	*Nutrients*	4.546	4.8	Autism spectrum disorders and the gut microbiota	Review	197	32.83
72	Diaz-Gerevini et al., 2016	*Nutrition*	3.42	3.2	Beneficial action of resveratrol: how and why?	Review	195	21.67
73	Evrensel and Ceylan, 2015	*Clinical Psychopharmacology and Neuroscience*	1.5	2.4	The gut-brain axis: the missing link in depression	Review	195	19.50
74	Altves et al., 2020	*Bioscience of Microbiota Food and Health*	3.121	2.5	Interaction of the microbiota with the human body in health and diseases	Review	193	38.60
75	Gacias et al., 2016	*eLife*	7.725	6.4	Microbiota-driven transcriptional changes in prefrontal cortex override genetic differences in social behavior	Animal study	190	21.11
76	Kang et al., 2018	*Anaerobe*	2.704	2.5	Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders	Human study	189	27.00
77	Luna et al., 2017	*Cellular and Molecular Gastroenterology and Hepatology*	Nil	7.1	Distinct microbiome-neuroimmune signatures correlate with functional abdominal pain in children with autism spectrum disorder	Human study	189	23.63
78	Sherwin et al., 2018	*Annals of the New York Academy of Sciences*	4.295	4.1	Recent developments in understanding the role of the gut microbiota in brain health and disease	Review	186	26.57
79	Vendrik et al., 2020	*Frontiers in Cellular and Infection Microbiology*	5.293	4.6	Fecal microbiota transplantation in neurological disorders	Review	183	36.60
80	Long-Smith et al., 2020	*Annual Review of Pharmacology and Toxicology*	13.82	11.2	Microbiota-gut-brain axis: new therapeutic opportunities	Review	183	36.60
81	Groer et al., 2014	*Microbiome*	Nil	13.8	Development of the preterm infant gut microbiome: a research priority	Review	183	16.64
82	Srikantha and Mohajeri, 2019	*International Journal of Molecular Sciences*	4.556	4.9	The possible role of the microbiota-gut-brain-axis in autism spectrum disorder	Review	179	29.83

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TABLE 1 (Continued)

No.	Reference	Journal	Journal impact factor at the year of publication	Journal impact factor in 2023	Article	Study type	Total citations	Annual citations
83	Hiippala et al., 2016	*Frontiers in Microbiology*	4.076	4.0	Mucosal prevalence and interactions with the epithelium indicate commensalism of *Sutterella* spp.	Human study	179	19.89
84	Nishino et al., 2013	*Neurogastroenterology and Motility*	3.424	3.5	Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods	Animal study	178	14.83
85	Grimaldi et al., 2018	*Microbiome*	10.465	13.8	A prebiotic intervention study in children with autism spectrum disorders	Human study	176	25.14
86	Dinan and Cryan, 2015	*Current Opinion in Clinical Nutrition and Metabolic Care*	4.033	3.0	The impact of gut microbiota on brain and behaviour: implications for psychiatry	Review	176	17.60
87	Mulle et al., 2013	*Current Psychiatry Reports*	3.054	5.5	The gut microbiome: a new frontier in autism research	Review	174	14.50
88	Burokas et al., 2015	*Advances in Applied Microbiology*	4.128	5.515	Microbiota regulation of the mammalian gut-brain axis	Review	173	17.30
89	Holmes et al., 2012	*Science Translational Medicine*	10.757	15.8	Therapeutic modulation of microbiota-host metabolic interactions	Review	171	13.15
90	Matta et al., 2019	*Brain Behavior and Immunity*	6.633	8.8	The influence of neuroinflammation in autism spectrum disorder	Review	170	28.33
91	Dinan and Cryan, 2017a	*Psychosomatic Medicine*	3.81	2.9	Brain-gut-microbiota axis and mental health	Review	170	21.25
92	Newell et al., 2016	*Molecular Autism*	4.833	6.2	Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder	Animal study	169	18.78
93	Sherwin et al., 2016	*CNS Drugs*	4.394	7.4	May the force be with you: the light and dark sides of the microbiota-gut-brain axis in neuropsychiatry	Review	168	18.67
94	Liu et al., 2015	*Journal of Agricultural and Food Chemistry*	Nil	5.7	Modulation of gut microbiota brain axis by probiotics, prebiotics, and diet	Review	167	16.70
95	Chen et al., 2021	*Nutrients*	6.706	4.8	Regulation of neurotransmitters by the gut microbiota and effects on cognition in neurological disorders	Review	165	41.25
96	Jyonouchi et al., 2002	*Neuropsychobiology*	2.065	2.3	Innate immunity associated with inflammatory responses and cytokine production against common dietary proteins in patients with autism spectrum disorder	Human study	164	7.13
97	Choi and Cho, 2016	*Clinical Endoscopy*	Nil	2.1	Fecal microbiota transplantation: current applications, effectiveness, and future perspectives	Review	163	18.11
98	Ming et al., 2012	*Journal of Proteome Research*	5.056	3.8	Metabolic perturbance in autism spectrum disorders: a metabolomics study	Human study	163	12.54
99	Liu F. T. et al., 2019	*Translational Psychiatry*	5.28	5.8	Altered composition and function of intestinal microbiota in autism spectrum disorders: a systematic review	Review	162	27.00
100	Spielman et al., 2018	*Neurochemistry International*	3.994	4.4	Unhealthy gut, unhealthy brain: the role of the intestinal microbiota in neurodegenerative diseases	Review	161	23.00

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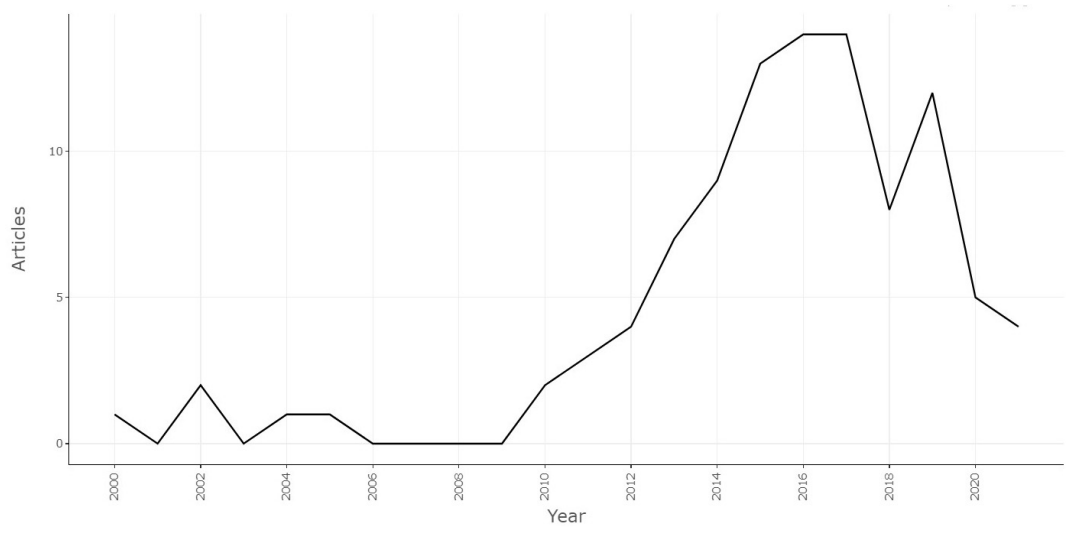

FIGURE 2
The trends of the annual publications of the 100 most-cited articles.

Citations

The total citation frequency for all the 100 selected articles was 40,662, with a median citation of 280.5. The number of citations for each article ranged from 161 to 2,081. The top article with the most total citations was "Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders" by Hsiao et al. (2013) (total citations of 2,081). The most-cited human study was "Microbiota transfer therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study" by Kang et al. (2017) (total citations of 722), while the most cited randomized clinical trial was "A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial" by Pärtty et al. (2015) (total citations of 212).

To exclude the effect of year on citation numbers, the annual citation rate was analyzed. Figure 3 demonstrates the trends of citations per year of the 100 selected articles. The annual citation rate was trending upward overall from 2000 to 2021. The annual citation was 15.8 in 2000, and it reached the peak in 2021 with annual citations of 86.6. The annual citation rate of each paper ranged from 7.13 to 294.83. The top article with the most annual citations was "The microbiota-gut-brain axis" by Cryan et al. (2019) (annual citations of 294.83).

To better understand the relationship between annual citations and annual publications, the average number of citations per article per year was calculated. Figure 4 presents this average citation count for each year. The trend was fluctuating prior to 2013, as there were fewer than five articles published each year. However, as the annual number of publications increased in recent years, the trend showed an overall upward trajectory.

The total citation rate of an article was not significantly correlated to the year of publication ($r = 0.097$, $p > 0.05$, Spearman's rho test). However, the annual citation rate of an article was significantly related to the year of publication ($r = 0.481$, $p < 0.01$, Spearman's rho test).

Among the 100 selected articles, there were 11 animal studies, 27 human studies, and 62 reviews. The total citation rate and annual citation rate were not significantly different between the three study types ($p > 0.05$, Kruskal–Wallis test). When combining animal and human studies into a single category of experimental studies, the citation rates for clinical studies were still not significantly different from those of review articles ($p > 0.05$, Mann–Whitney U test).

Countries

There were 28 countries involved in the 100 most-cited articles. The United States was involved in the highest number of publications ($n = 42$), followed by Ireland ($n = 20$), Italy ($n = 9$), England ($n = 8$), and China ($n = 8$). The details of the number of publications of each country are listed in Table 2.

There were 20 countries which had collaborations with others. Figure 5 displays the collaboration network between these countries. The color of the node in Figure 5 represents different collaboration cluster, the width of the curved line indicates the link strength, and the distance between the nodes denotes approximate relatedness among the nodes. The United States had the most collaborations with other countries, and worked closely with the United Kingdom, Ireland, and China.

Authors

Among the 491 authors involved in the 100 most-cited articles, 8 authors published 5 or more articles. John F. Cryan was the most productive author, with 20 articles, followed by Timonthy G. Dinan with 19 articles. Table 3 lists the top 10 authors with most published articles.

Most authors collaborated with others to publish their papers. However, Cezmi A. Akdis published a notable single-authored article titled "Does the epithelial barrier hypothesis explain the

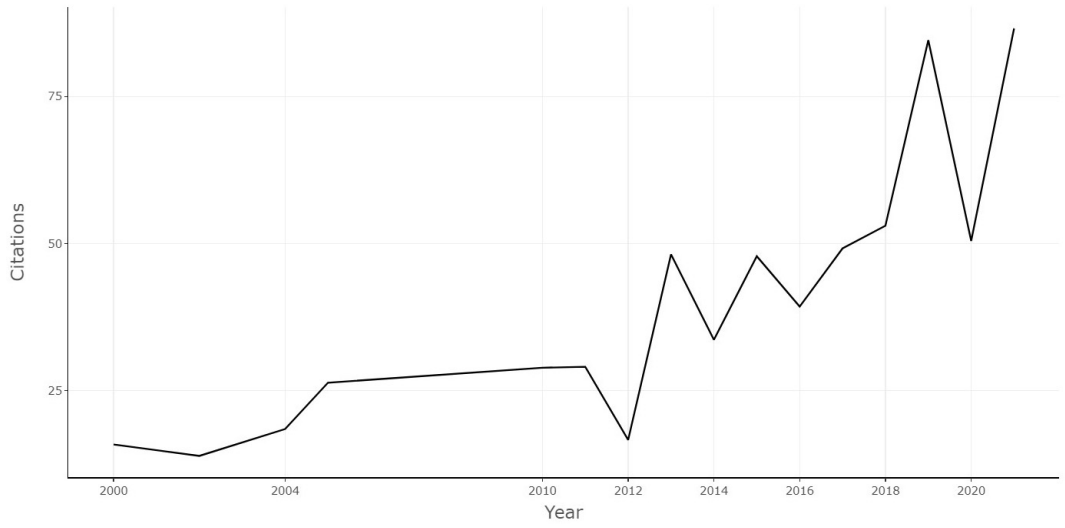

FIGURE 3
The trends of the annual citations of the 100 most-cited articles.

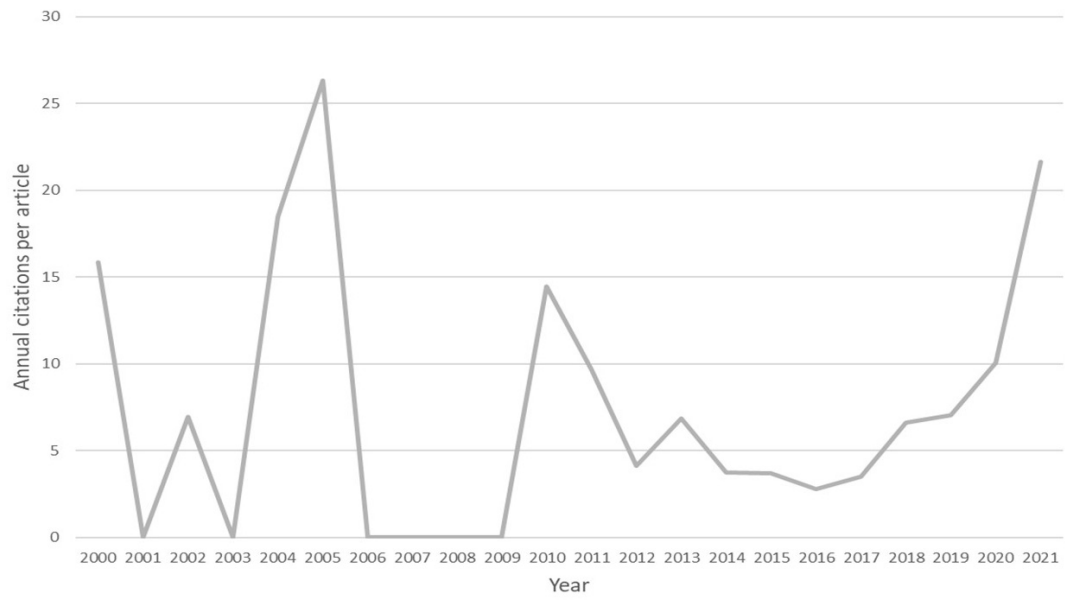

FIGURE 4
The trends of the annual citations per article of the 100 most-cited articles.

increase in allergy, autoimmunity and other chronic conditions?" in *Nature Reviews Immunology*. He is a professor in University of Zürich Medical Faculty and the Director of the Swiss Institute of Allergy and Asthma Research in Davos, Switzerland (Swiss Institute of Allergy and Asthma Research, 2024).

Among all the authors, Timothy G. Dinan and John F. Cryan collaborated most frequently. Timothy G. Dinan is a Professor of Psychiatry at University College Cork (University College Cork, 2024), while John F. Cryan is a Professor and Chair, Department of Anatomy and Neuroscience, University College Cork (APC Microbiome Ireland, 2024). Notably, many frequent collaborators are from the same institutions. For example, Emeran A. Mayer, the Director of the Gail and Gerald Oppenheimer Family Center for Neurobiology of Stress and a Professor of Psychology Medicine (UCLA Brain Research Institute, 2024), and Kirsten Tillisch, a Professor of Medicine and gastroenterologist with a clinical interest in chronic pain and functional gastrointestinal disorders (UCLA Health, 2024), are both based at the University of California, Los Angeles, United States. Likewise, Elaine Holmes and Jeremy K. Nicholson are affiliated with the Faculty of Medicine at Imperial College London, United Kingdom. Elaine Holmes is a Professor of Chemical Biology with research interests in discovering and developing metabolic biomarkers for disease in personalized healthcare and population studies (Imperial College London, 2024a). Jeremy K. Nicholson, an Emeritus Professor of Biological Chemistry, focuses on personalized healthcare through metabolic

TABLE 2 Number of publications of each country.

Country	Number of publications
United States	42
Ireland	20
Italy	9
England	8
China	8
Australia	6
Belgium	3
Canada	3
Finland	2
Japan	2
Netherlands	2
Sweden	2
Switzerland	2
Turkey	2
Argentina	1
Denmark	1
France	1
Germany	1
Greece	1
India	1
Israel	1
Malaysia	1
Philippines	1
Russia	1
Singapore	1
Slovakia	1
South Korea	1
Spain	1

phenotyping and systems medicine (Imperial College London, 2024b). All these researchers contributed to their collaborative works with their unique expertise and a shared focus on interactions between the brain, gut, and microbiome. The research area of autism and gut microbiota brought together experts from diverse disciplines, including psychiatry, neuroscience, gastroenterology, biological chemistry, and chemical biology.

Figure 6 illustrates the collaboration network between the authors who had at least three collaborations. The color of the node represents different collaboration cluster, the width of the curved line indicates the link strength, and the distance between the nodes denotes approximate relatedness among the nodes.

Journals

The 100 selected articles were published in 76 journals. The latest impact factors of the journals ranged from 2.0 to 69.2. *Nature Reviews Microbiology* was the journal with the highest impact factor, and published the article "The gut microbiota-brain axis in behaviour and brain disorders." *Journal of Child Neurology*, with the lowest impact factor (impact factor of 1.134 at the time of publication and impact factor of 2 in 2023), published the paper "Short-term benefit from oral vancomycin treatment of regressive-onset autism." Among the 76 journals, 14 published at least 2 of the selected articles. *Microbiome* was the most productive journal, with five articles, followed by *Cell* with four articles. *Brain Behavior and Immunity, Frontiers in Microbiology, Journal of Proteome Research, Nutrients,* and *PLoS One* all published three articles. The number of publications of the 100 most-cited articles in that journal was not significantly related to the journal's latest impact factor ($r = 0.016$, $p > 0.05$, Spearman's rho test). The list of journals with at least two publications is presented in Table 4.

KeyWords Plus

KeyWords Plus refer to indexed keywords derived from the titles of referenced articles that occur at least twice in the bibliography, and they offer valuable insights into research trends (Tomaszewski, 2023). Figure 7 provides an overview of the most-used KeyWords Plus. The most popular KeyWords Plus were "intestinal microbiota" ($n = 35$), "irritable bowel syndrome" ($n = 25$), and "chain fatty acids" ($n = 24$). To better understand the development of KeyWords Plus, the frequency of the original authors' keywords was analyzed. Figure 8 presents an overview of the frequency of authors' keywords, where larger font sizes indicate higher frequencies. In comparison to the authors' keywords, certain KeyWords Plus, such as "irritable bowel syndrome" and "chain fatty acids," appeared more frequently, highlighting emerging trends in this field. It was also observed that some KeyWords Plus, such as "obesity" and "probiotics" were used less frequently than the authors' keywords.

Figure 9 illustrates the co-occurrence network of KeyWords Plus. The size of the node is proportional to the frequency of the word, the color of the node represents different cluster, and the width of the curved line indicates the link strength. The most popular word "intestinal microbiota" was linked to many other words, such as "autism," "brain," "children," and "irritable bowel syndrome."

Thematic analysis

The thematic map analysis based on KeyWords Plus is illustrated in Figure 10. A thematic map allows four typologies of themes to be categorized based on their placement in specific quadrants. Themes in the upper-right quadrant are identified as motor themes, characterized by both high density and centrality, signifying their development and relevance in the research field. In the upper-left quadrant, themes are classified as niche themes, marked by high density but low centrality, indicating their isolated development. Themes in the lower-left quadrant have low centrality and density, suggesting they are weakly developed and marginal. In the lower-right quadrant are basic themes, featuring high centrality (relevance) and low density (less development). As illustrated in Figure 8, several pertinent themes are discernible in this

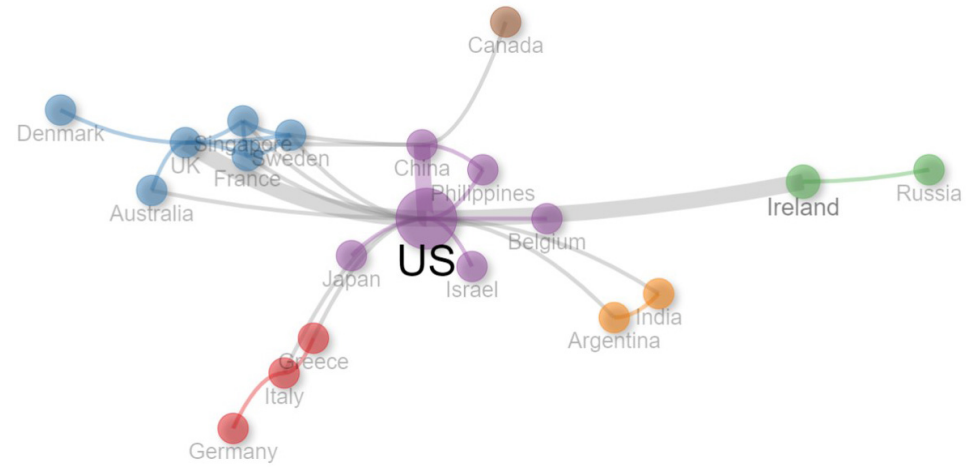

FIGURE 5
Collaboration network between countries. The color of the node represents different collaboration cluster, the width of the curved line indicates the link strength, and the distance between the nodes denotes approximate relatedness among the nodes.

TABLE 3 Number of publications of the top 10 authors.

Author	Number of publications
John F. Cryan	20
Timothy G. Dinan	19
Gerard Clarke	8
Sarkis K. Mazmanian	6
James B. Adams	5
Dae-Wook Kang	5
Rosa Krajmalnik-Brown	5
Eoin Sherwin	5
Elaine Y. Hsiao	4
Rob Knight	4

research field, such as chain-fatty acids, bacteria, and irritable bowel syndrome (IBS).

Discussion

General information

This study combined bibliometric analysis with network visualization to identify the first 100 highly impactful manuscripts in the field of ASD and gut microbiota, based on global citation frequency. It highlights the contributions that have driven substantial progress in this field, identifies the current research trends, and provides guidance for future research directions. Various aspects in this research domain were explored, including the top articles with most citations, correlations between citation rates and publication time, distribution of involved countries, contributions of key authors, impactful journals with most publications, and relevant themes in this field.

Among the 100 most-cited articles in the current review, the average number of citations annually increased over the years and was significantly correlated to the year of publication. However, there was no significant association between total number of citations and time of publication. These trends are consistent with bibliometric analyses in other areas, such as burns (Ring et al., 2020) and insomnia (Wan et al., 2022). This is likely due to the tendency of total citations to favor older publications, as more recent papers have a shorter duration to accumulation citations. The average number of citations annually, as different from the total citations, can eliminate the effect of time on citation numbers and provide a more accurate view of the immediate impact of the articles. Besides, as the association between autism and gut microbiota is a rapidly evolving field (Wang Q. et al., 2023), newer studies in this research area often receive high initial attention and are cited more frequently within the initial years, as they may represent cutting-edge findings or novel methodologies, leading to an increase in annual citation averages over time. The area in autism and gut microbiota may be different from foundational research which tends to accumulate citations consistently over long periods and has obscured direct association with the time of publication. Overall, the quantity of citations of an article is a useful proxy to indicate the significant of the study (Landreneau et al., 2020). It can be implied that the influence of research in autism and gut microbiota has been steadily increasing over the years.

In terms of the distribution of countries, the United States contributed to the largest volume of the publications, followed by other countries, such as Ireland, England, and China. These findings are similar to bibliometric reviews in other conditions, such as schizophrenia (Yang et al., 2022) and intellectual disability (Ying et al., 2022). The United States holds a competitive edge in this research domain and is likely to have a significant impact on the direction of research in this field and maintain the most robust global collaborations. The information of the distribution of countries can be valuable for researchers seeking to choosing the most suitable place for additional training or collaborative opportunities.

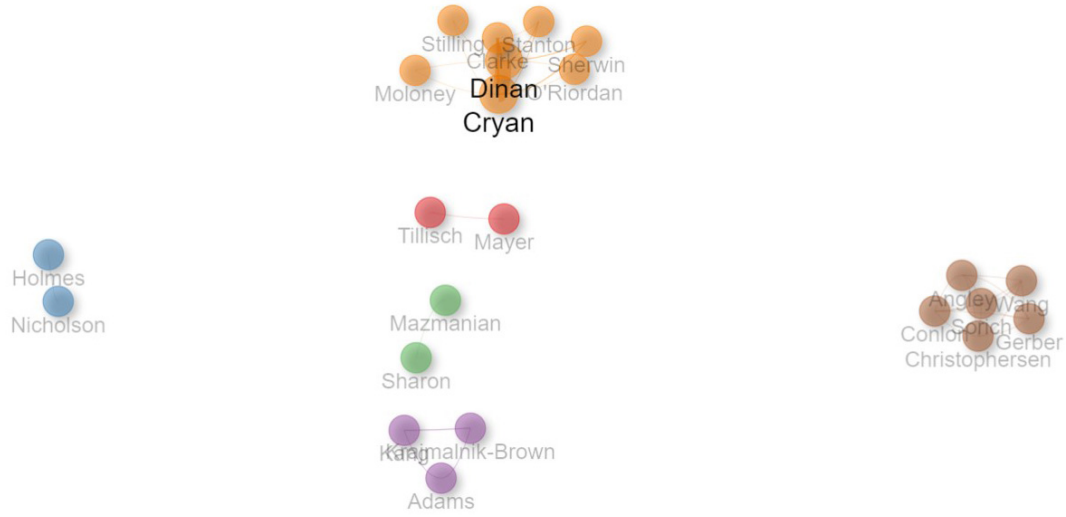

FIGURE 6
Collaboration network between authors. The color of the node represents different collaboration cluster, the width of the curved line indicates the link strength, and the distance between the nodes denotes approximate relatedness among the nodes.

Each journal contributed one to five of the 100 most-cited articles. The number of the articles that the journal contributed was not related to the impact factor of the journal. These findings are consistent with other review papers on impactful studies, such as the landmark studies in burns (Ring et al., 2020). The impact factor was first introduced by Garfield (2006) and was commonly used as a measure to indicate the significance of a journal within its respective field. The impact factor pertains exclusively to journals and does not extend to individual articles. Thus, it is possible for a highly impactful study to be published in a journal with low impact factor. In the current review, one of the highly cited papers "short-term benefit from oral vancomycin treatment of regressive-onset autism" was published in 2000 in *Journal of Child Neurology*, a journal with an impact factor of 1.134 at the time of publication. At that time this article was submitted to a low impact journal was likely due to several reasons. This article focused on short-term effects in a small sample with preliminary nature of findings (Sandler et al., 2000) and this could make high-impact journal hesitant to publish it. Besides, at that time the concept of a link between autism and gut microbiota was not widely accepted, especially in high-impact journals. In addition, the authors at that time might not be aware of the significance of their work or chose the journal based on the audience specialization and journal readership. Nonetheless, this article is one of the early works to suggest a potential link between gut microbiota and autism and is one of the foundational references for researchers exploring this area. It has subsequently cited by numerous papers published in high-impact journals, such as *Nature Reviews Gastroenterology & Hepatology* (Hung and Margolis, 2024), *Clinical Microbiology Reviews* (Yadegar et al., 2024), and *Microbiome* (LaPelusa et al., 2021).

Our findings share certain similarities with other bibliometric analyses on gut microbiota in various conditions. For example, a bibliometric analysis on gut microbiota and Parkinson's disease identified similar main research topics, including "short-chain fatty acids," "probiotics," and 'inflammation" (Li et al., 2024). Another similar analysis on gut microbiota and obesity found similar top journals in this area, such as *Nutrients*, *Scientific Reports*, and *Frontiers in Microbiology* (Wang M. et al., 2024). Various bibliometric analyses related to gut microbiota revealed an overall upward trend in the number of publications and the United States being one of the leading countries in those research fields (Li et al., 2024; Ouyang et al., 2024; Wang M. et al., 2024). Together with other studies on similar fields of investigation, this current study

TABLE 4 Journals with at least two publications.

Name of journal	Number of articles	Journal impact factor in 2023
Microbiome	5	13.8
Cell	4	45.6
Brain Behavior and Immunity	3	8.8
Frontiers in Microbiology	3	4.0
Journal of Proteome Research	3	3.8
Nutrients	3	4.8
PLoS One	3	2.9
Anaerobe	2	2.5
Applied and Environmental Microbiology	2	3.9
International Journal of Molecular Sciences	2	4.9
Molecular Autism	2	6.2
Scientific Reports	2	3.8
Translational Psychiatry	2	5.8
World Journal of Gastroenterology	2	4.3

FIGURE 7
The most-used KeyWords Plus. The font size is proportional to the frequency of the word.

FIGURE 8
The most-used authors' keywords. The font size is proportional to the frequency of the word.

can offer a clear insight into the current research landscape and emerging trends, serving as a valuable reference for researchers entering this field of gut microbiota.

Influential studies

The most-cited article among all the selected papers was "Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders" by Hsiao et al. (2013) published in *Cell*. This is a landmark study, as it demonstrated gastrointestinal barrier defects and microbiota changes in the maternal immune activation mouse model with autistic symptoms. This study found that treatment with the human commensal *Bacteroides fragilis* corrected gut permeability defects, altered the composition of the microbiota, regulated the serum levels of the metabolite of 4-ethylphenylsulfate, and alleviated abnormal communicative, anxiety-like, stereotyped, and

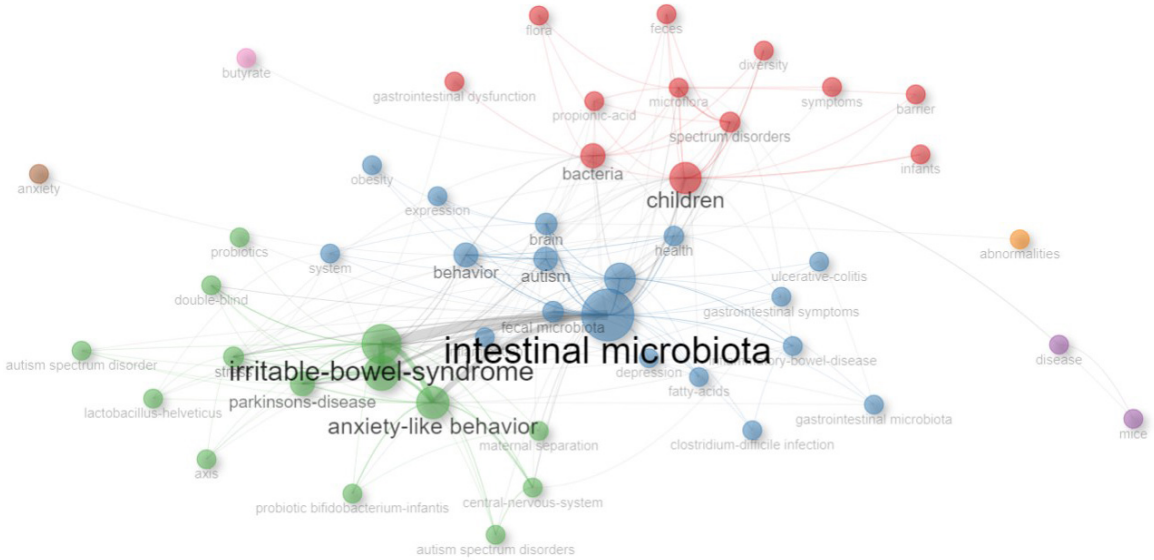

FIGURE 9
The co-occurrence network of KeyWords Plus. The size of the node is proportional to the frequency of the word, the color of the node represents different cluster, and the width of the curved line indicates the link strength.

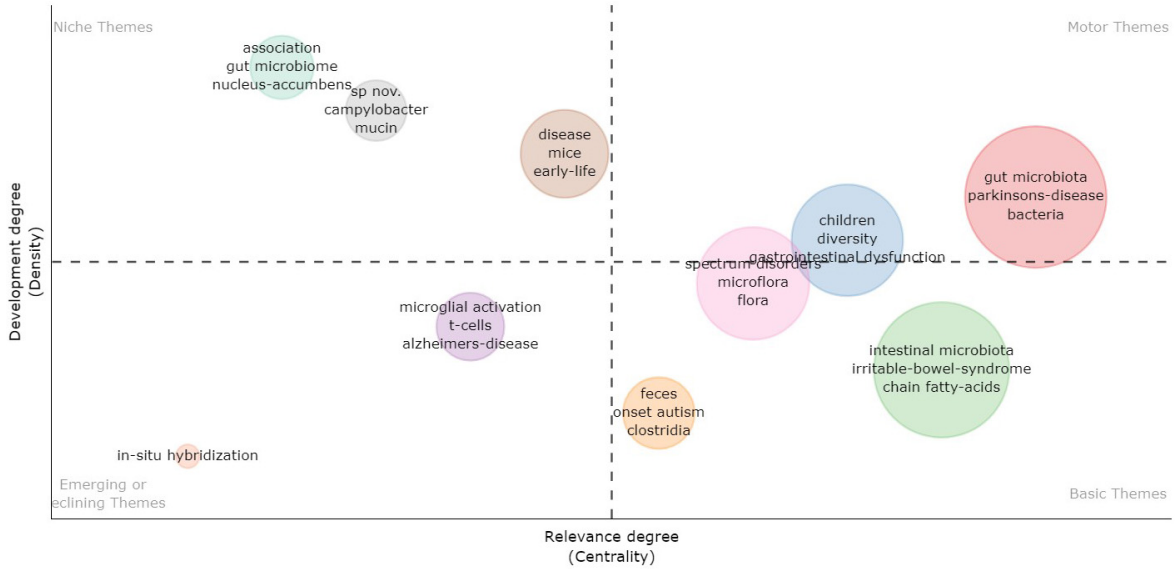

FIGURE 10
Thematic map analysis based on KeyWords Plus. Upper-right quadrant: motor themes – high density (developed) and high centrality (relevant); upper-left quadrant: niche themes – high density (developed) and low centrality (less relevant); lower-left quadrant: emerging or declining themes – low density (less developed) and low centrality (less relevant); lower-right quadrant: basic themes – low density (less developed) and high centrality (relevant).

sensorimotor behaviors. It proposed a groundbreaking idea that ASD could be potentially a disorder related to the gut, and that therapies involving the microbiome might offer a safe and effective approach to treating the disorder.

The human study with most citations was "Microbiota transfer therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study" authored by Kang et al. (2017) and published in *Microbiome*. In this open-label clinical trial, the efficacy of Microbiota Transfer Therapy was evaluated in terms of its impact on gut microbiota, gastrointestinal and autistic symptoms in children diagnosed with ASD. This study found that the abundance of *Bifidobacterium*, *Prevotella*, and *Desulfovibrio* increased after the intervention and the improvement persisted till the end of 8 weeks follow-up. These findings are promising and represent a pivotal advancement in understanding the relationship between gut microbiota and ASD.

The randomized clinical trial among the 100 most-cited articles was performed by Pärtty et al. (2015) who wrote the article "A

possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial" published in *Pediatric Research*. In this clinical trial, 75 infants were randomized to receive *Lactobacillus rhamnosus* GG or placebo during their first 6 months of life and were followed up for 13 years. At 13 years old, Asperger syndrome or attention deficit hyperactivity disorder was diagnosed in 17.1% of children in the placebo group, while none in the probiotic group. This influential study demonstrated, for the first time, that certain probiotics could potentially mitigate the risk of developing specific neurodevelopmental disorders.

The impactful paper published in the journal with the lowest impact factor among all the included articles was "Short-term benefit from oral vancomycin treatment of regressive-onset autism" by Sandler et al. (2000) published in *Journal of Child Neurology*. This open-label clinical trial demonstrated the short-term improvement in autistic symptoms after oral vancomycin treatment among 11 children with regressive-onset ASD. This early study, published in July 2000, indicated the potential existence of a gut-brain connection in a subgroup of children with both ASD and diarrhea.

Future outlook

The co-occurrence network of KeyWords Plus and thematic analysis in this study identified several important hotspots and future directions in this research area, such as microbial metabolites of short-chain fatty acids (SCFAs), role of bacteria, and overlaps of IBS.

Short-chain fatty acids are monocarboxylic acids containing fewer than six carbon atoms (Schonfeld and Wojtczak, 2016). The majority of SCFAs in the human intestine are acetic acid, butyric acid and propionic acid (Iniguez-Gutierrez et al., 2020). These organic acids result from the fermentation of dietary fiber and resistant starch in the intestine (Portincasa et al., 2022). Several well-designed animal studies have been performed to explore the relationship between SCFAs and ASD. For example, one study in Canada found that rats treated with propionic acid displayed more stereotypic behavior, nose pokes and locomotive activity (Meeking et al., 2020). Studies in human participants have also reported changes in SCFAs in the stool of ASD subjects. One recent study revealed that children with ASD and constipation had excessive propionic acid in feces (He et al., 2023). This study provided new clues to understand the etiology and biomarkers for ASD. However, the results in human studies are inconsistent (Lagod and Naser, 2023). The variability in human study outcomes highlights the need for further research on SCFA levels in individuals with ASD.

The composition of bacteria in the human gastrointestinal tract is complex. There are still inconsistences regarding the association between different bacteria and ASD in different studies. Some studies have reported higher abundance of *Lactobacillus* in ASD (Pulikkan et al., 2018; Strati et al., 2017), while it is also reported that *Lactobacillus* has decreased levels (Iovene et al., 2017). The diversity of gut microbiota has been reported to be either increased (Coretti et al., 2017) or decreased (Dan et al., 2020) in persons with ASD. Besides, the ratio between *Firmicutes* and *Bacteroidetes* in persons with ASD has been reported higher in some studies (Strati et al., 2017), and lower in other studies (Zhang et al., 2018). It has been pointed out that the inconsistent conclusions between different studies are likely due to various reasons, including underpowered research design and variation in use of multiple testing corrections (Li et al., 2022). In addition, gut microbiota composition may also be affected by other factors, such as age, body mass index, and dietary habits (Rinninella et al., 2019). Future investigations with more comprehensive and standardized methods may shed light on the intricate connections linking gut bacteria and ASD.

Irritable bowel syndrome is a chronic gastrointestinal disease with the core clinical symptoms of recurrent abdominal discomfort or pain, and altered bowel habits (Huang et al., 2023). IBS is commonly observed as a comorbid condition in individuals with ASD (Penzol et al., 2019). In conditions such as IBS and ASD, where dysbiosis is potentially present, the utilization of prebiotics and probiotics may serve as a low-risk therapeutic approach to improve symptoms (Abdellatif et al., 2020). One recent pilot randomized clinical trial published in *Cell Host & Microbe* tested the effect of bacterial species *Limosilactobacillus reuteri* in children with ASD and found that the bacteria significantly improved the social functioning (Mazzone et al., 2024). Results in recent studies are compelling to encourage additional future research on utilizing probiotics or specific microbes as treatment options for persons with ASD.

Limitations

Although this study provides valuable and comprehensive insights to help researchers and policymakers to understand the research trends and guide feature decision-making, it has several limitations. First, the literature search was conducted only in the Web of Science database. While the Web of Science is the leading database in scientometrics and many studies rely solely on it for bibliometric analysis, our findings could be more comprehensive if additional databases were included. Second, although bibliometric analysis provides a broad overview of research trends and networks, it may lack in-depth analysis as it does not evaluate the quality of the numerous studies. Third, this research field is advancing rapidly. It is possible that some recently published high-quality studies may be overlooked, due to the low accumulated citation numbers.

Conclusion

The present study, to our knowledge, is the first bibliometric analysis to comprehensively explore the 100 most-cited articles in the field of ASD and gut microbiota. By identifying and analyzing these pivotal studies, we provide a detailed overview of the most influential research in this domain. The results highlight key trends, emerging topics, and potential future directions for investigation. This analysis not only illuminates the current landscape of research but also offers valuable insights for researchers, clinicians, and policymakers. It serves as a critical reference for guiding the development and focus of future scientific inquiries and clinical practices related to ASD and gut microbiota.

Data availability statement

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

Author contributions

JY: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft, Writing – review & editing. MZ: Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. K-CW: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. SW: Conceptualization, Methodology, Project administration, Supervision, Validation, Writing – review & editing. MS: Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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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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Investigating the causal impact of gut microbiota on trigeminal neuralgia: a bidirectional Mendelian randomization study

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Background: The etiology and pathogenesis of trigeminal neuralgia remain unclear. This study examines the connection between gut microbiota and trigeminal neuralgia using Mendelian randomization analysis to provide insights into the disorder's origin and propose potential therapies based on our findings.

Methods: We used data from the MiBioGen consortium (13,266 participants) for gut microbiota and the IEU OpenGWAS project (800 cases, 195,047 controls) for trigeminal neuralgia. We checked for heterogeneity and horizontal pleiotropy and used the inverse variance weighting method as our main approach to study the causal link between gut bacteria and trigeminal neuralgia, MR-Egger, simple mode, weighted median, and weighted mode as supplementary methods, with a sensitivity test using leave-one-out analysis. If a bacteria-trigeminal neuralgia link was found, we conducted a reverse analysis for confirmation.

Results: According to the final results, these groups include *Butyricimonas* (Genus, id = 945, *p*-value = 0.007, OR = 1.742, 95% CI: 1.165–2.604), *unknowngenus* (Genus, id = 1000005479, *p*-value = 0.005, OR = 1.774, 95% CI: 1.187–2.651) and *Bacteroidales* (Family, *p*-value = 0.005, OR = 1.774, 95% CI: 1.187–2.651) were causally associated with trigeminal neuralgia. No significant results according to reverse Mendelian randomization analysis.

Conclusion: In our study, we identified specific gut bacteria linked to trigeminal neuralgia. To comprehensively understand their impact and mechanisms, additional randomized trials are necessary.

KEYWORDS

trigeminal neuralgia, gut microbiota, Mendelian randomization, causality, gut-brain axis

Introduction

Trigeminal neuralgia (TN) presents as recurring, paroxysmal bursts of intense pain within the trigeminal distribution area, devoid of any discernible impairment of trigeminal nerve functionality (Cruccu et al., 2016). This affliction predominantly affects females and tends to emerge more frequently in individuals around the age of 40 (Maarbjerg et al., 2017). Diagnosis hinges upon three pivotal criteria: the manifestation of pain localized within one or more branches of the trigeminal nerve, abrupt and excruciating paroxysms of pain

often likened to "shocks" or "electric jolts," lasting a mere fraction of a minute—typically seconds (Bendtsen et al., 2020; Cruccu et al., 2020). The etiology and pathogenesis of TN remain shrouded in ambiguity. Current elucidation posits that the compression of the trigeminal nerve by ectopic, contorted blood vessels within the pontine bridge along the posterior root may instigate localized demyelination, thereby precipitating the onset of painful episodes (Love and Coakham, 2001). Pathological alterations in TN chiefly unveil through the detection of vacuoles within the cytoplasm of trigeminal ganglion cells, along with irregular proliferation, hypertrophy, distortion, or even vanishing of axons. Furthermore, discernible thickening and disintegration of myelin sheaths, coupled with segmental demyelinating changes in the majority of fibers, constitute primary pathological hallmarks of this condition (Love and Coakham, 2001).

In recent investigations, the discerning gaze of scientific inquiry has illuminated the potential influence wielded by metabolites intricately linked to the gut microbiota upon diverse facets of inflammation and immune response (Foster and McVey Neufeld, 2013). Furthermore, the burgeoning body of research accentuates the emergence of a bidirectional conduit for communication between the encephalon and the gastrointestinal realm, heralded as the gut-brain axis (Lyu et al., 2022). This revelation ignites a tantalizing inquiry: might the gut microbiota exert a discernible sway over the pathogenesis and evolution of trigeminal neuralgia? Delving into this provocative query unfurls novel pathways for comprehending the labyrinthine interplay between gut microbiota and TN, thereby furnishing profound insights into the intricate machinations underpinning the inception and progression of this neurological malady. Nevertheless, the definitive establishment of a correlation between gut flora and trigeminal neuralgia remains ensconced in uncertainty, primarily attributed to the paucity of evidence stemming from randomized controlled trials. While randomized controlled trials stand as the pinnacle for corroborating correlation in epidemiological inquiries, their execution is often hindered by ethical constraints and formidable financial burdens. In order to reveal the potential correlation between gut microbiota and trigeminal neuralgia, we explored the causal relationship between gut flora and trigeminal neuralgia by Mendelian Randomization (MR), on the basis of which we hypothesized the correlation between the two (Porcu et al., 2019; Saunders et al., 2022). Mendelian Randomization ingeniously employs genetic variability as an instrumental tool to simulate interventions, thereby augmenting our ability to make more robust inferences concerning the impact of a factor on the incidence of the disease. In the forthcoming investigation, we will wield MR methodologies to scrutinize the putative causal interrelation between gut microbiota and TN.

The paramount aim of this investigation is to delve into the conceivable correlation existing between the intricate makeup of the gut microbiota and the susceptibility to TN, with a deliberate emphasis on unveiling the cryptic biological underpinnings. We envisage that the findings gleaned from this scholarly pursuit will furnish a trove of innovative perspectives and methodologies for the prospective mitigation and management of TN. Beyond the mere elucidation of TN's etiological landscape, this ambitious endeavor harbors the potential to furnish substantial scaffolding for the formulation of tailored therapeutic regimens, thereby heralding the advent of enhanced well-being and ameliorated quality of life for individuals grappling with this neurological affliction.

Abbreviations: MR, Mendelian randomization; TN, trigeminal neuralgia; IVW, inverse variance weighting; GWAS, genome-wide association study; IEU, Integrative Epidemiology Unit; SNPs, single nucleotide polymorphisms; IVs, instrumental variables; OR, Odds Ratio; 95%CI, 95% confidence interval.

Material and methods

Study design

The intricate framework of our comprehensive study design is elegantly depicted in Figure 1. Our methodological rigor was exemplified through the application of Mendelian Randomization (MR), meticulously employed to scrutinize the causal interplay between the gut microbiota and TN. Adherence to the triad of foundational tenets governing MR analysis was paramount: (1) Establishing a robust nexus between genetic variability and exposure factors; (2) Ensuring the independence of genetic variability from confounding variables (Jia et al., 2023); (3) Validating that genetic variability exerts its sole influence on outcomes via exposure factors, while eschewing involvement in alternative pathways (Jin et al., 2023). Concurrently, we undertook a reverse MR analysis, leveraging the statistically significant revelations from the initial MR inquiry to fortify and refine our conclusions.

We procured the succinct summary statistics delineating the composition of the gut microbiota from the preeminent genome-wide meta-analysis conducted to date, meticulously orchestrated by the esteemed MiBioGen consortium (van der Velde et al., 2019).[1] This exhaustive analysis encompassed a cohort of 18,340 individuals of European descent hailing from 11 nations, encapsulating an expansive dataset comprising 122,110 loci of genetic variability. Concomitantly, the pertinent summary statistics germane to the genome-wide association study (GWAS) for TN were acquired from the esteemed bastion of the Medical Research Council Integrative Epidemiology Unit (IEU) Open GWAS project [Trait: Trigeminal neuralgia - IEU OpenGWAS project (mrcieu.ac.uk)] (IEU Open GWAS project, n.d.), updated to the illustrious epoch of 2021.04.06. The TN cohort comprised 800 cases from European descent juxtaposed against 195,047 controls, with a comprehensive amalgam of 16,380,408 single nucleotide polymorphisms (SNPs) delineating the genetic landscape.

The meticulous curation of instrumental variables (IVs) was governed by a systematic protocol, meticulously delineated through the following methodological steps: (1) Initial Identification: Single nucleotide polymorphisms (SNPs) demonstrating association with each genus at the locus-wide significance threshold ($P < 1.0 \times 10-5$) were earmarked as prospective IV candidates (Liu et al., 2022; Zeng et al., 2023). (2) Linkage Disequilibrium Analysis: Following the preliminary selection, a rigorous examination of linkage disequilibrium (LD) was undertaken for all IVs. A stringent threshold of r2 < 0.001 was imposed, with a clumping window size of 10,000 kb, ensuring the independence of

1 https://mibiogen.gcc.rug.nl

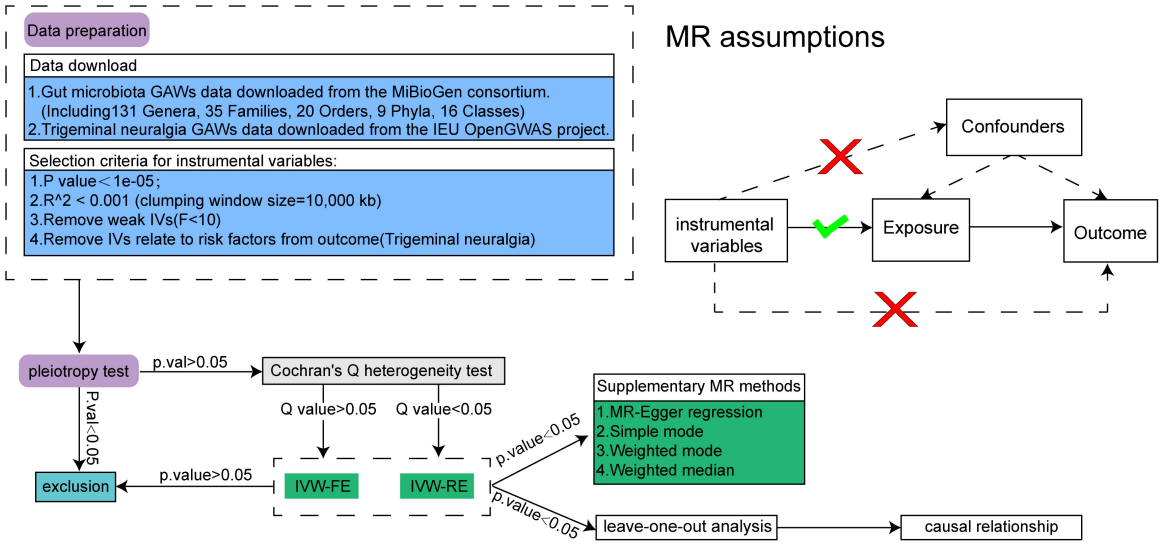

FIGURE 1
The whole study design.

selected IVs. If the number of instrumental variables was less than 10, the bacterial flora was excluded. (3) Relevance Assessment: SNPs linked to the exposure but lacking corresponding matches within the genome-wide association study outcome statistics were judiciously pruned. This elimination process, guided by the formula F = beta^2_exposure / SE^2_exposure, was instrumental in guaranteeing the pertinence of the selected instrumental variables to both the exposure and outcome in the subsequent analysis (Jin et al., 2023). (4) Allele Frequency Threshold: SNPs characterized by a minor allele frequency (MAF) $\leq$ 0.01 were discerningly excluded from further consideration, ensuring the robustness of subsequent analyses. (5) Resolution of Palindromic SNPs: In instances involving palindromic SNPs, meticulous determination of forward strand alleles was meticulously executed based on allele frequency information. This meticulous step aimed to foster consistency and lucidity in the portrayal of genetic information throughout the analytical process (Li et al., 2022).

Statistical analysis

In the quest to unravel potential causal links between bacterial taxa and trigeminal neuralgia, the venerable tool of Mendelian Randomization (MR) was deftly wielded. Prior to commencing our analytical voyage, a meticulous scrutiny for horizontal pleiotropy was undertaken, aimed at identifying and preemptively excluding statistics susceptible to the confounding influence of horizontal pleiotropic effects. This preemptive maneuver, undertaken with sagacious forethought, served as a bulwark fortifying the integrity of our analysis, thereby endowing the inverse variance weighting (IVW) method with the mantle of primacy in adjudicating causality within the realm of MR analysis (Burgess et al., 2016). Moreover, the discerning eye of scrutiny was cast upon the landscape of heterogeneity among instrumental variables, courtesy of Cochrane's Q test. Detection of heterogeneity (P < 0.05) prompted the adoption of a judicious recourse to the random-effects IVW (IVW-RE) model, renowned for its penchant in furnishing conservative estimates. Conversely, in instances where heterogeneity lay dormant, a steadfast embrace of the fixed-effects IVW (IVW-FE) model ensued, deemed apt for scenarios characterized by the steadfast consistency of instrumental variable effects. This adaptive approach, attuned to the ebbs and flows of heterogeneity among the selected instrumental variables, epitomized a hallmark of methodological finesse (Zeng et al., 2023). Subsequent to the unveiling of statistically significant results from the IVW analysis (p < 0.05), a veritable arsenal of additional MR methodologies was marshaled forth, including MR-Egger regression, simple mode, weighted median, and weighted mode. Noteworthy is the selection of weighted median and MR-Egger regression, undertaken to supplement the IVW method, thereby imbuing our analyses with a wider expanse of confidence intervals (CIs). These supplementary forays into the analytical landscape were conceived with the noble intent of buttressing the resilience of our findings and delving into the nuanced tapestry of the causal nexus between the gut microbiota and trigeminal neuralgia (Slob and Burgess, 2020).

In a final stride towards bolstering the veracity of our findings, we subjected the p-values corresponding to statistically significant causality to a meticulous leave-one-out analysis, culminating in resolute outcomes. Subsequent to this, in an earnest bid to fortify the edifice of our conclusions, we embarked upon an inverse Mendelian Randomization (MR) analysis. In this undertaking, we leveraged Genome-Wide Association Study summary statistics stemming from flora causally intertwined with trigeminal neuralgia as the outcome, juxtaposed against those emanating from TN as the exposure. Notably, the arsenal of analytical tools was deftly wielded within the realm of the R programming language, complemented by the versatile "TwoSampleMR" package, thereby furnishing a robust platform for methodical exploration and inference. This methodological rigor, underpinned by the synergistic interplay between analytical precision and computational prowess, lent an aura of credibility

and gravitas to our investigative odyssey (Hemani et al., 2017; Hemani et al., 2018).

Results

We harnessed the comprehensive gut microbiota Genome-Wide Association Study data procured from the esteemed MiBioGen consortium, encompassing a rich tapestry of taxonomic resolution. Within this expansive dataset, a total of 131 genus-level taxa, 35 family-level taxa, 20 order-level taxa, 9 phylum-level taxa, and 16 class-level taxa were meticulously curated, affording a granular insight into the multifaceted landscape of microbial composition. This breadth of taxonomic resolution facilitated a nuanced exploration of potential associations between microbial taxa and trigeminal neuralgia, enriching our analytical endeavors with depth and granularity. Following the outlined methodology, we meticulously executed a horizontal pleiotropy test to discern and exclude statistics potentially influenced by horizontal pleiotropy. Subsequently, we engaged diverse Inverse Variance Weighting (IVW) analysis methods contingent upon the Q-value derived from the heterogeneity test. Remarkably, the Q-value for the heterogeneity test surpassed the threshold of 0.05 across nearly all cohorts, indicative of a notable absence of statistical heterogeneity. As delineated in Figure 2, Supplementary Figure 1, and Supplementary Table 1, the IVW analysis method unveiled associations between trigeminal neuralgia and 7 genus-level flora [*Butyricimonas, FamilyXIIIAD3011group, FamilyXIIIUC, Lactococcus, RuminococcaceaeNK4A214group, Ruminococcus2, unknowngenus*(id.1000005479)] alongside 3 family-level flora (*BacteroidalesS24.7group, Christensenellaceae, FamilyXIII*). However, discerning from the results of the leave-one-out analysis (Figure 3), we prudently excluded seven flora exhibiting sensitivity. The conclusive outcomes of the forward Mendelian Randomization analysis were meticulously tabulated in Table 1 and illustrated in Figure 4. Notably, *Butyricimonas* (Genus, IVW, P = 0.007, OR = 1.742, 95%CI:1.165–2.604; Weighted median, P = 0.030, OR = 1.847, 95% CI: 1.063–3.211), *unknowngenus(id.1000005479)* (Genus, IVW, P = 0.005, OR = 1.774, 95%CI:1.187–2.651; Weighted median, P = 0.011, OR = 2.014, 95% CI: 1.172–3.462), and *BacteroidalesS24.7group* (Family, IVW, P = 0.005, OR = 1.774, 95%CI:1.187–2.651; Weighted median, P = 0.014, OR = 2.014, 95% CI: 1.152–3.524) were substantiated in two MR methods to harbor causality with TN (IVW and weighted median). Furthermore, *BacteroidalesS24.7group* (Family, IVW, P = 0.005, OR = 1.774, 95%CI: 1.187–2.651; Weighted median, P = 0.014, OR = 2.014, 95% CI: 1.152–3.524; Simple mode, P = 0.049, OR = 2.587, 95% CI: 1.142–5.861) evinced causality with TN across three distinct methods (IVW, Simple mode, and weighted median). Detailed information regarding Single Nucleotide Polymorphisms (SNPs) can be found in Supplementary Table 2.

Employing the taxa delineated above, we embarked upon a reverse MR analysis, with the Genome-Wide Association Study data of bacteria enlisted as the exposure. Elaborate details pertaining to the single nucleotide polymorphisms (SNPs) deployed as instrumental variables are meticulously cataloged in Table 2. Regrettably, the outcomes of the reverse MR analysis failed to unveil any significant results, thereby corroborating the notion that trigeminal neuralgia may not exert a discernible impact on the abundance of intestinal flora. This alignment with the concept of a functional disorder of trigeminal neuralgia serves to further bolster the credibility and robustness of our findings, reinforcing the reliability and validity of our investigative endeavors.

Discussion

In the pursuit of unraveling the intricate relationship between gut microbiota and Trigeminal Neuralgia, our paramount objective was to employ a Mendelian Randomization analysis imbued with methodological rigor. To this end, we judiciously harnessed the aggregated gut microbiota statistics gleaned from the extensive Genome-Wide Association Study meta-analysis meticulously orchestrated by the MiBioGen consortium. Concurrently, we complemented this robust dataset with aggregated TN statistics sourced from the IEU OpenGWAS project release data, thus ensuring the foundation of our study rested upon a bedrock of empirical validity.

Within this analytical crucible, our discerning gaze alighted upon three specific microbial taxa: *Butyricimonas*, an *unknown genus (id.1000005479)*, and the *Bacteroidales S24.7 group*, each exhibiting notable causality with Trigeminal Neuralgia (TN). Intriguingly, the results of our exhaustive analysis appear to suggest that *Butyricimonas, the unknown genus (id.1000005479),* and *the Bacteroidales S24.7 group* may act as harbingers of increased risk for the development of trigeminal neuralgia. However, given the enigmatic nature of the unknown genus, our subsequent discussion will pivot upon the implications stemming from the findings related to *Butyricimonas* and *the Bacteroidales S24.7 group*.

The genus *Butyricimonas*, renowned for its production of butyrate, a pivotal energy source for colonic cells, occupies a prominent position in the pantheon of gut microbiota. Butyrate, primarily metabolized into energy by colonic cells, serves as a linchpin in promoting gut health, fostering the release of host-derived antimicrobial peptides (AMPs), and stimulating the secretion of immunoglobulin A (IgA). These activities collectively orchestrate a potent anti-inflammatory immune response, thereby conferring protection against allergen-induced airway issues, ameliorating airway hyperresponsiveness, and thwarting the development of intestinal tumors, as evidenced in murine models.

Recent investigations have underscored the indispensable role of *Butyricimonas* in preserving central nervous system (CNS) homeostasis, orchestrating inflammation modulation, and assuaging neuropathic pain (Linnerbauer et al., 2020; Stoeva et al., 2021). Despite being lauded as a probiotic owing to its multifaceted anti-inflammatory, anti-tumor, and immune-boosting properties, the intriguing findings stemming from our Mendelian Randomization analysis suggest a potential paradox. Contrary to prevailing notions, our analysis posits *Butyricimonas* as a putative risk factor for trigeminal neuralgia, thereby challenging conventional wisdom and underscoring the complexity inherent in the gut-brain axis and its implications for neurological disorders.

Building upon Lin et al. (2021)'s seminal work, which delineated a trigeminal neuralgia mouse model and elucidated the role of infiltrating macrophages and lymphocytes in chronic compression nerve injury, we extrapolate further insights into

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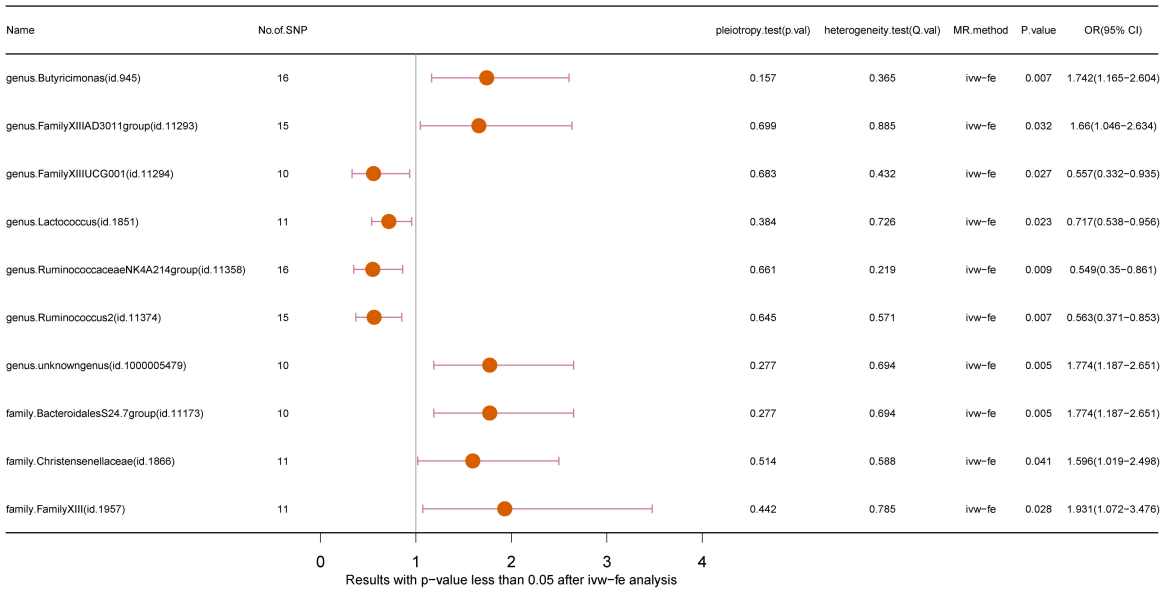

FIGURE 2
Forest plot of GM taxa associated with GBM ($P < 0.05$) identified by IVW-FE method.

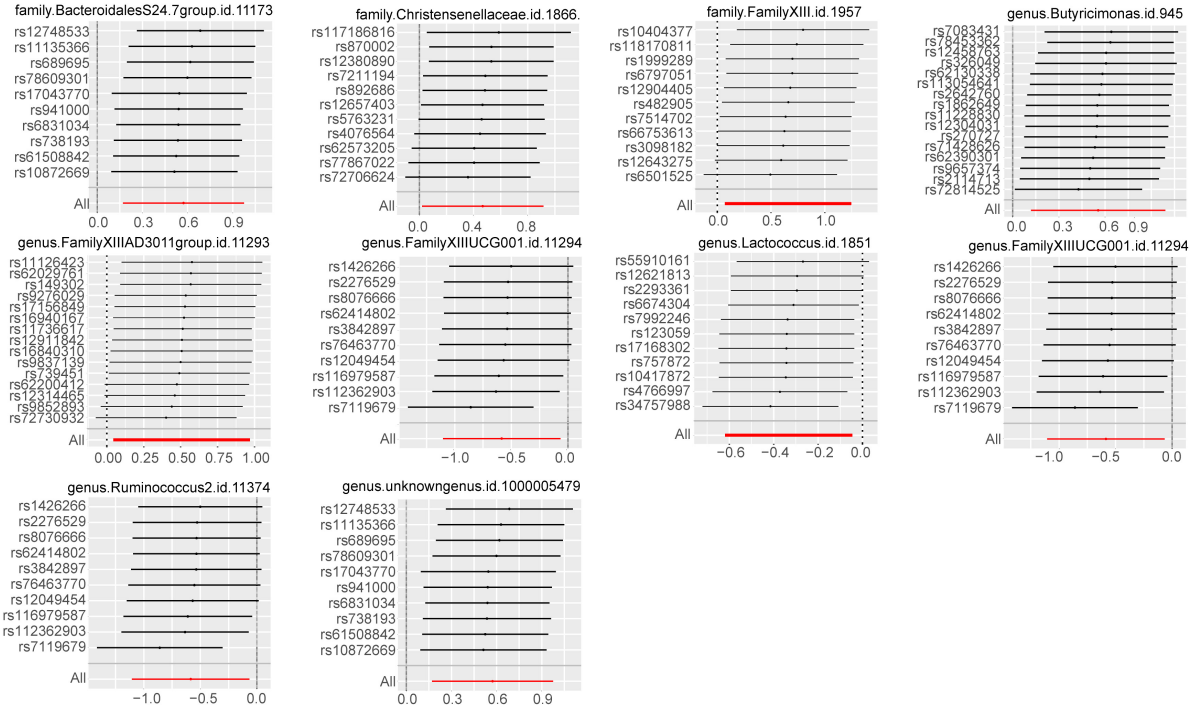

FIGURE 3
Leave-one-out plots for the causal association between gut microbiota and GBM identified by IVW-FE method.

the pathogenesis of this debilitating condition. It is postulated that these immune cells traverse the compromised blood-nerve barrier under chronic compression, guided by specific chemokines, thereby orchestrating the onset and progression of trigeminal neuralgia. In light of these observations, we propose a novel hypothesis implicating *Butyricimonas* and its metabolites in the etiology of trigeminal neuralgia. It is conjectured that *Butyricimonas*-induced alterations in trigeminal ganglion cells manifest as vacuoles in the plasma, irregular proliferation, hypertrophy, distortion, or even disappearance of axons, alongside marked thickening and disintegration of myelin sheaths, and segmental demyelinating changes in the majority of fibers. These structural aberrations may precipitate the onset and progression of trigeminal neuralgia by fostering the release of host-derived

TABLE 1 The results of MR analysis.

Name (id)	No. of SNP	Pleiotropy test (*p*-value)	Cochrane's Q heterogeneity test (Q_pval)	MR method	*p*-val	OR	or_lci95	or_uci95
genus.Butyricimonas (id.945)	16	0.157	0.365	ivw-fe	0.007	1.742	1.165	2.604
				MR Egger	0.447	0.520	0.101	2.674
				Simple mode	0.167	1.919	0.796	4.629
				Weighted mode	0.156	1.826	0.828	4.026
				Weighted median	0.030	1.847	1.063	3.211
genus.unknowngenus (id.1000005479)	10	0.277	0.694	ivw-fe	0.005	1.774	1.187	2.651
				MR Egger	0.107	4.546	0.888	23.277
				Simple mode	0.064	2.587	1.071	6.248
				Weighted mode	0.080	2.194	1.006	4.782
				Weighted median	0.011	2.014	1.172	3.462
family.BacteroidalesS24.7 group(id.11173)	10	0.277	0.694	ivw-fe	0.005	1.774	1.187	2.651
				MR Egger	0.107	4.546	0.888	23.277
				Simple mode	0.049	2.587	1.142	5.861
				Weighted mode	0.081	2.194	1.001	4.806
				Weighted median	0.014	2.014	1.152	3.524

No. of SNP, number of SNPs being used as IVs.; ivw-fe, fixed-effects inverse variance weighting; OR, Odds Ratio; or_lci95-or_uci95, 95% confidence interval; Significant *P*-value was marked in red.

TABLE 2 The detail information of SNPs in trigeminal neuralgia.

SNP	Effect_allele	Other_allele	beta	se	eaf	pval
rs72640584	G	A	0.6285	0.1363	0.03858	3.97E−06
rs116501213	G	A	1.0847	0.2398	0.01381	6.10E−06
rs141103938	A	G	1.9571	0.4319	0.005103	5.85E−06
rs6531785	G	A	−0.7635	0.1614	0.9713	2.24E−06
rs79054949	G	A	0.5838	0.1231	0.04856	2.09E−06
rs10214062	C	T	0.3263	0.0726	0.1448	7.07E−06
rs75568875	G	A	2.2983	0.5177	0.003709	9.03E−06
rs9767181	T	C	0.4203	0.0938	0.08363	7.39E−06
rs62501575	A	T	0.2555	0.0566	0.2958	6.41E−06
rs79459523	G	A	5.0421	1.0424	0.0011	1.32E−06
rs9550562	A	G	0.2491	0.0548	0.3122	5.57E−06
rs111365054	T	C	0.678	0.1443	0.03512	2.61E−06
rs148953513	C	A	1.0163	0.2285	0.01533	8.71E−06
rs3810268	T	C	0.2572	0.0518	0.3826	6.88E−07
rs182954926	C	A	3.3932	0.7661	0.00196	9.46E−06
rs13052134	A	G	0.7143	0.1554	0.02998	4.29E−06

SNP, single nucleotide polymorphism; SE, standard error.

antimicrobial peptides (AMPs) or secretory immunoglobulin A (IgA), thus perpetuating a cascade of inflammatory events culminating in neuronal dysfunction and pain. This hypothesis presents a tantalizing avenue for further investigation, shedding light on the intricate interplay between gut microbiota, immune response, and neurological pathology in trigeminal neuralgia.

Bacteroidales, an order of bacteria nestled within the Bacteroidetes phylum, represents a cadre of Gram-negative

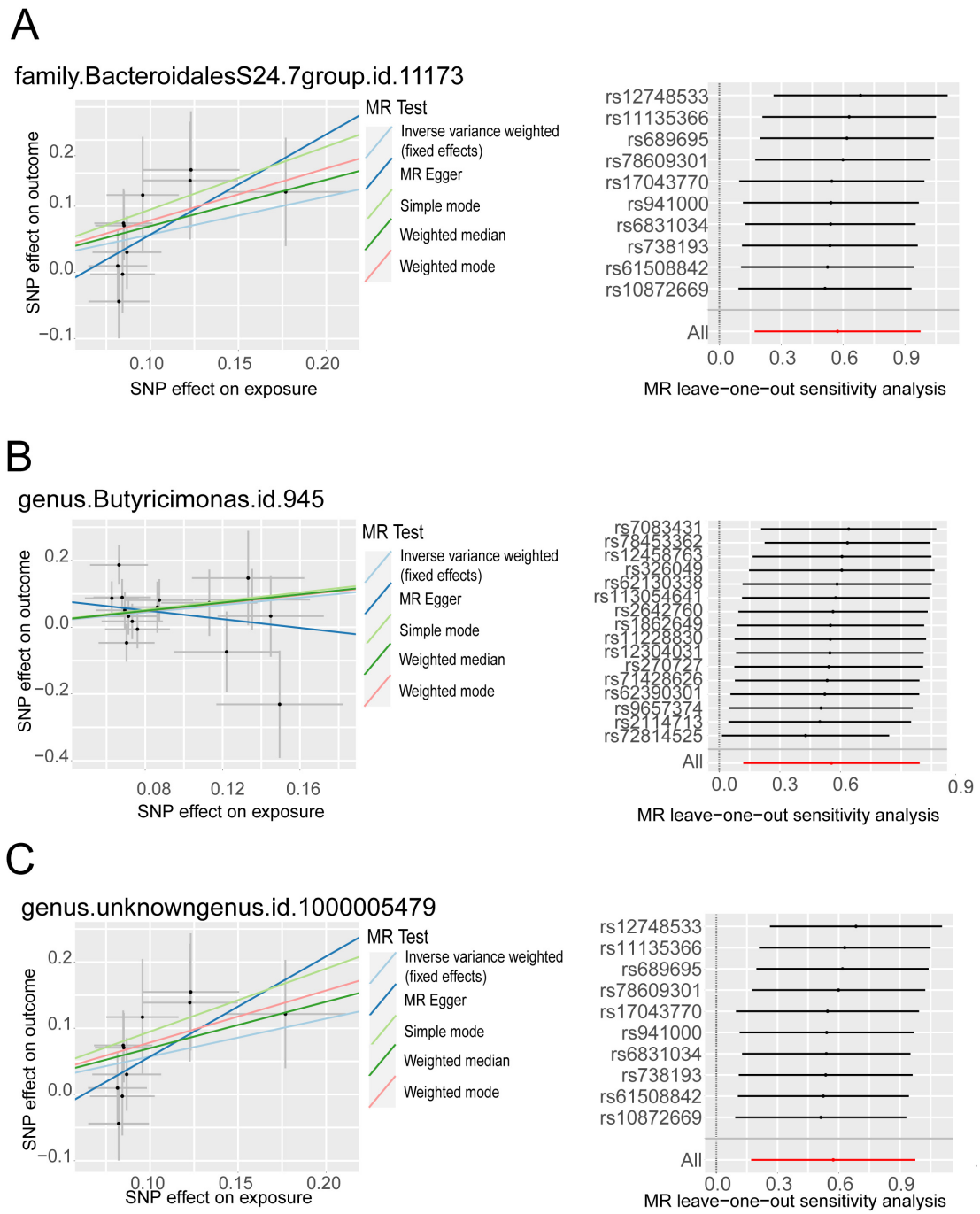

FIGURE 4
The MR positive results screened after the leave-one-out analysis. **(A)** Scatter plot of MR results for *BacteroidalesS24.7group* on the left, Leave-one-out analysis results on the right; **(B)** Scatter plot of MR results for Butyricimonas on the left, Leave-one-out analysis results on the right; **(C)** Scatter plot of MR results for unknown genus (id.1000005479) on the left, Leave-one-out analysis results on the right.

organisms that find ubiquity across diverse ecosystems, including the intricate milieu of the human gut. This taxonomic order harbors a mosaic of bacterial species, each endowed with a repertoire of functions, ranging from beneficial to pivotal in the digestion of complex carbohydrates within the gut milieu. Eckburg et al. (2005)'s observational study stands as a testament to the multifaceted role played by Bacteroidales metabolites, unveiling their profound anti-inflammatory effects. Furthermore, Hu et al. (2020)'s seminal work sheds light on the potential therapeutic implications of Bacteroidales S24.7 in mitigating inflammation within the context of inflammatory bowel disease (IBD). These seminal findings underscore the nuanced interplay between gut microbiota, inflammation, and disease pathogenesis, painting a picture of Bacteroidales as a linchpin in maintaining gut homeostasis and orchestrating immunomodulatory responses (Hu et al., 2020).

The landscape surrounding the Bacteroidales S24.7 group within the family Bacteroidales unfolds with complexity and contradiction, as elucidated by Ormerod et al. (2016)'s report. Their findings shed light on the dual nature of this microbial cohort, capable of both triggering infections by inciting immune responses and synthesizing virulence factors. Furthermore, another Mendelian Randomization analysis, delving into the causal nexus between periodontitis and gut microbiota, lends credence to the notion that the family Bacteroidales S24.7 group might indeed exacerbate the risk of periodontitis (Luo et al., 2023). The discordance observed across these studies underscores the intricacies inherent in the gut microbiome and its multifaceted interactions with inflammatory diseases. Individual variations in gut microbiological composition, coupled with the intricate interplay of multifactorial properties characterizing inflammatory conditions, contribute to this tapestry of inconsistency. In light of these nuances, the MR analysis, grounded upon the assumptions delineated in Figure 1, endeavors to navigate through this labyrinth of complexity. Within this analytical framework, we proffer a conjecture positing that the *Bacteroidales S24.7 group* and its metabolites may indeed play a contributory role in the onset and progression of trigeminal neuralgia. By stimulating the production of inflammatory responses, this microbial cohort potentially orchestrates a cascade of events culminating in neuronal dysfunction and pain. However, further elucidation through rigorous investigation is imperative to unravel the true mechanistic underpinnings of this phenomenon.

Rooted in the conceptual framework of the gut-brain axis elucidated in prior research, this study stands at the vanguard of scientific inquiry, poised to unravel the intricate interplay between the human gut microbiota and the central nervous system (CNS). Through the conduit of the gut-brain axis, the human gut microbiota exerts a profound influence on the development and function of the CNS, underscoring its pivotal role in shaping neurological processes (Dehhaghi et al., 2020). This study boasts several methodological advantages, chief among them being the utilization of Mendelian Randomization (MR) analysis—a powerful tool for establishing causal relationships by harnessing existing genetic variations in nature. Through the mechanism of randomization simulation, MR analysis affords the unique ability to mimic assignment to a control group, thereby furnishing researchers with heightened confidence in formulating causal hypotheses. By employing genetic variation as instrumental variables, MR analysis effectively circumvents issues stemming from confounding and reverse causation, thereby facilitating a clearer elucidation of relationships between variables (Hong et al., 2023). In contrast to observational studies fraught with myriad limitations such as confounding, selection bias, and memory bias, Mendelian Randomization analysis offers a robust alternative. By leveraging genetic variation gleaned from the most comprehensive meta-analysis of global genomic studies, this study ensures the availability of robust instrumental variables for MR analysis. Through this meticulously crafted approach, causal relationships between gut microbiota and Trigeminal Neuralgia are unearthed, thereby attenuating confounding factors and ameliorating concerns pertaining to the reversal of causality in causal inference (Birney, 2022). Furthermore, the employment of a two-sample MR design, leveraging non-overlapping exposure and outcome pooled data, serves to further bolster the integrity of the study design, mitigating bias and enhancing the robustness of the findings. In summation, this study represents a paradigm shift in our understanding of the intricate relationship between gut microbiota and Trigeminal Neuralgia, offering invaluable insights into the pathophysiological mechanisms underpinning this neurological disorder (Long et al., 2023).

While this study employs a rigorous methodology, there are several important caveats to consider. First, the limited number of Single Nucleotide Polymorphisms (SNPs) analyzed under the conventional Genome-Wide Association Study (GWAS) significance threshold ($P < 5 \times 10\hat{}-8$) required adjustments to enhance sensitivity analysis and reduce potential effects of horizontal pleiotropy. This adjustment underscores the complex relationship between statistical thresholds and the reliability of findings in genetic research.

Additionally, the inherent limitations of Mendelian Randomization (MR) analysis, such as vulnerability to demographic and genetic sequencing errors, should be acknowledged. Given that our study population is of European ancestry, the generalizability of our findings to other demographic groups may be limited, emphasizing the need for careful interpretation and contextualization of results within specific population frameworks.

And it is worth mentioning that in our quest for trigeminal neuralgia GWAS data, we were unable to find data typed according to the Eller/Burchiel protocol (Eller et al., 2005). Moreover, the data utilized in this study originated from Finnish databases of trigeminal neuralgia GWAS data, where rigorous typing was lacking (The description of the data can be found here), thus emphasizing a limitation of our study.

Lastly, the classification of *Butyricimonas* and the *Bacteroidales S24.7 group* as probiotics underscores the multifaceted nature of their roles in gut health. Altering the abundance of these flora, albeit potentially beneficial in alleviating TN symptoms, may engender unintended consequences. Therefore, in the development of TN treatment programs, careful consideration of the broader implications of modulating gut microbiota is imperative to ensure holistic and personalized therapeutic interventions.

Conclusion

In this two-sample Mendelian Randomization study, we identified several gut microbiota species with causal associations to trigeminal neuralgia. Particularly significant was the causal relationship observed with *Butyricimonas* and *Bacteroidales S24.7 group*, suggesting potential implications for trigeminal neuralgia treatment. To comprehensively understand the negative role of *Butyricimonas* and *Bacteroidales S24.7 group* in trigeminal neuralgia and unveil its precise mechanisms, additional randomized controlled trials are necessary.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: the esteemed MiBioGen consortium

(https://mibiogen.gcc.rug.nl); GWAS for Trigeminal neuralgia (https://gwas.mrcieu.ac.uk/datasets/finn-b-G6_TRINEU/).

Author contributions

CZ: Conceptualization, Data curation, Software, Writing – original draft, Writing – review and editing. ClZ: Data curation, Writing – original draft. YJ: Data curation, Writing – original draft. HZ: Data curation, Writing – original draft. HS: Funding acquisition, Supervision, Writing – review and editing. CH: Data curation, Resources, Supervision, Writing – review and editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1420978/full#supplementary-material

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Microbiota-gut-brain-axis: a new target of acupuncture therapy for post-stroke cognitive impairment

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Stroke-induced cognitive impairment is a common complication and an important risk factor for disability. Prevention and treatment of secondary stroke injuries are crucial. Modern research has found that the gut microenvironment can directly or indirectly affect neurological function and cerebral ischemic outcomes, and their crosstalk is achieved through the microbiota-gut-brain-axis (MGBA). Acupuncture, as a promising non-drug treatment, has been recommended for improving post-stroke cognitive impairment (PSCI). However, in recent years, few studies have systematically analyzed the potential mechanisms in this field, and whether acupuncture can improve PSCI through the MGBA remains to be explored. This review comprehensively summarizes literature and shows that, acupuncture, as an adjuvant therapy can play a potential important role in the treatment of PSCI by regulating the microbiota-gut-brain-axis. Acupuncture can repair intestinal epithelial barrier, regulate gut microbiota and serum metabolites, alleviate gut inflammation and neuroinflammation, and regulating HPA axis function, etc. From the studies we have included, the evidence for its effectiveness remains limited, these results should be interpreted with caution due to the low quality of evidence. Future high-quality clinical and experimental studies are needed. This review also discussed the development prospects of acupuncture in improving PSCI via the MGBA, such as genomics, personalized therapy, establishment of standards, and combination therapy, etc. providing new research ideas and scientific and reliable evidence for the application of acupuncture in PSCI.

KEYWORDS

acupuncture, post-stroke cognitive impairment, traditional Chinese medicine, gut microbiota, microbiota-gut-brain-axis

1 Introduction

Intra cerebral stroke, also known as cerebrovascular accident (CVA), is divided into ischemic stroke (IS), intracerebral hemorrhage (ICH), subarachnoid hemorrhage (SAH), and stroke of undetermined type. Stroke is the second largest cause of death and disability in the world after ischemic heart disease (GBD 2019 Stroke Collaborators, 2021). Post-stroke cognitive impairment (PSCI) refers to a series of syndromes that meet the diagnostic criteria for cognitive impairment within 6 months after stroke. Although different subtypes

of stroke have different pathogenesis and clinical manifestations, they all have a certain probability of leading to the occurrence of PSCI. The severity of PSCI varies from mild to severe, with up to 60% of stroke survivors experiencing this condition within the first year after stroke (Donnellan and Werring, 2020; Rost et al., 2022). Although there is currently no gold standard for cognitive screening after stroke, several brief cognitive screening tests (≤30 min) have been used to identify PSCI, such as Mini Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA). However, since most screenings are not developed for the heterogeneity of PSCI, stroke defects and individual factors of patients may also limit the practicality of screening tools (Kwon et al., 2020). PSCI has the characteristics of low cure rate, slow recovery effect and rapid changes in the condition. The impact of cognitive impairment on daily living activities sometimes far exceeds that of physical impairment. If not actively intervened, it will seriously affect the daily life of patients and cause a heavy burden on families. Cognitive impairment ranks as one of the leading causes of disability worldwide and contributes significantly to the global burden of disease that requires urgent attention in national health policy (Rost et al., 2022). Due to the limitation of treatment time window, the treatment for PSCI patients is currently very limited. At present, the main treatment method is recommended to give priority to rehabilitation training, with drug assisted treatment (such as antihypertensive drugs, lipid regulating drugs, drugs for treating diabetes, antiplatelet drugs and anticoagulants according to the specific conditions of patients), but the economic cost is high, and most wage earners are difficult to support treatment (Huang et al., 2022). At the same time, drugs also have a certain degree of side effects, such as the development of drug resistance, as well as adverse reactions to gastrointestinal symptoms, neurological and psychiatric symptoms, and other systemic symptoms. It is significant to exploring safe and effective treatment methods to alleviate symptoms of PSCI.

Research has found that in addition to symptoms of neurological and cognitive impairment, gastrointestinal dysfunction is also common in PSCI patients. With the gradual improvement of the brain-gut-microbiome theory, more scholars are paying attention to the connection between the brain and the intestine. The gut microbiota and its metabolites have been proven to be closely related to the occurrence and development of central nervous system (CNS) diseases, especially in stroke patients (Han et al., 2023). Researchers have found significant differences in gut microbiota and metabolites between patients with and without PSCI (Feng et al., 2021). Therefore, it is possible that a better intestinal microenvironment may play an important role in maintaining health, and researchers are gradually realizing that treating PSCI can be achieved through benign regulation of the gut microenvironment. In terms of comprehensive treatment of PSCI, acupuncture, one of the oldest and most widely used traditional Chinese medicine technology in the world, has significant advantages in better biological safety and socio-economic benefits. Acupuncture involves the insertion of metal needles into specific points of the body to modulate the meridians, enhance the circulation of *qi* and blood, thus restoring the balance of *yin* and *yang*, and ultimately achieving the goal of disease prevention and treatment (Kaptchuk, 2002). In recent years, more and more clinical trials show that acupuncture can not only promote the recovery of neurological function after stroke, but also have significant benefits for patients with secondary cognitive impairment, which can improve the quality of life of patients with PSCI (Han et al., 2024; Hung et al., 2019). Acupuncture has a multi-target effect, which can simultaneously regulate multiple neurobiological targets and improve brain function. It can also be personalized based on the patient's specific condition for selecting acupoints and developing treatment plans. Meanwhile, compared to medication treatment, acupuncture has relatively fewer side effects. Acupuncture can regulate the changes of intestinal microbiota, which may be one of the important mechanisms of acupuncture treatment for PSCI, and is also a potential research direction (Zhang Q. et al., 2023). However, in recent years, there have been few studies that systematically analyze the potential mechanisms in this field. This review systematically summarized the relationship between intestinal microenvironment and PSCI, and the role and potential mechanism of acupuncture in preventing and treating PSCI by regulating the intestinal microenvironment, trying to evaluate the role of intestinal microenvironment in the prevention and treatment of PSCI by acupuncture and its possible mechanism, so as to provide new ideas and targets for the research of traditional Chinese medicine in treating PSCI.

2 The relationship between intestinal microenvironment and PSCI

The intestinal microenvironment is the most complex part of the human microbiota, composed of gut microbiota, intestinal mucosa, and gut immune system, which are strongly associated with various types of diseases in the human body (Cryan et al., 2020). The microbiota-gut-brain-axis (MGBA) is a bidirectional communication network between gut microbes and brain. The dysfunction of MGBA induced by metabolic disorder is related to stroke and the development of common stroke risk factors such as hypertension, obesity and atherosclerosis. Similarly, changes in gut microbiota are also reflected in the response to stroke and may affect recovery after injury (Honarpisheh et al., 2022).

In addition to digestion and absorption, normal intestinal function also has a strong barrier function against harmful microbial organisms and various toxins produced by the intestine which is achieved through the intestinal epithelial barrier (IEB). IEB, one of the largest interfaces between outside and the body's internal environment, is composed of epithelial cells, goblet cells, Paneth cells, and enterochromaffin cells (Rodríguez-Colman et al., 2017). Epithelial cells are connected through tight junctions, gap junctions, adhesive junctions and desmosomes, which prevent toxic macromolecules and microbial organisms from passing through and play an innate defense role (Hansson, 2019). Stroke can disrupt the integrity of IEB, leading to damage to the intestinal villous epithelium, increased permeability, damage to intestinal tight junctions, decreased mucus, and decreased defense ability, ultimately leading to an imbalance of microorganisms. Harmful substances cross the intestinal epithelial barrier and enter the extraintestinal organs, increasing the risk of infection after stroke and affecting prognosis (Lee et al., 2023).

Microbial organisms are distributed on the surface and mucous membranes of the human body, with the most abundant part

being the gastrointestinal (GI) tract. The gut microbiota consists of approximately 10^{14} cells, including prokaryotes, eukaryotes, viruses and bacteriophages, which are crucial for host metabolism, immune, and nervous system development (Cryan et al., 2019). PSCI can alter the composition of gut microbiota. A comprehensive RNA sequencing analysis was conducted on differentially expressed genes in the ischemic cerebral cortex of mouse brains at pre-stroke and post-stroke day 1 and day 3. It is found that neurodegenerative pathway is the key enriched pathway. And through research on gut microbiota transplantation, it has been demonstrated that the gut microbe-dependent trimethylamine-N-oxide (TMAO) pathway can impact stroke severity (including infarct size and long-term cognitive outcomes) and induce different mRNA expression profiles (Kaur et al., 2023). Similarly, gut microbiota can also influence stroke outcomes through a bidirectional brain-gut axis. A study has found that dysphagia is associated with poor stroke prognosis, including increased risk of breathing difficulties, malnutrition, and even death, possibly due to oral food intake affecting the oral and gut microbiota of patients undergoing enteral nutrition rehabilitation (Arnold et al., 2016; Katagiri et al., 2019). The gut microbiota can not only affect the brain and behavior of stroke patients, but also affect multiple specific brain activities, such as learning, memory, anxiety, etc. Research has found that compared to non-PSCI patients, PSCI patients have significantly higher levels of gut *Enterobacteriaceae, Proteobacteria*. Furthermore, the similar alterations were also observed at the class, order, family, and genus levels (Ling et al., 2020). It was further found that PSCI mice receiving PSCI patient microbiota presented a higher level of *Enterobacteriaceae*, resulting in more severe intestinal damage and cognitive impairment than mice receiving non-PSCI patient microbiota (Wang et al., 2022b). The oral administration of *Escherichia coli* can also cause memory impairment in mice (Jang et al., 2018). In addition, gut microbiota can also affect the main clinical risk factors of stroke. A comprehensive Mendelian randomization study revealed that the abundance of specific bacterial can influence the risk of stroke subtypes, such as cardioembolic stroke, small vessel stroke and large artery stroke (Meng et al., 2023). Age is also a contributing factor to stroke. It is found that the cognitive impairment caused by transplanting feces from elderly people and aged mice into young mice is significantly more severe than feces from young adults and mice (Lee et al., 2020).

The intestinal immune system plays a crucial role in the progression of PSCI. Intestinal immune cells and their secreted inflammatory factors can migrate to the brain through peripheral circulation after the IEB is damaged during stroke (Jiang et al., 2023). It is reported that a significant migration of $CD45^{hi}$ immune cells from the gut to the brain and meninges can be observed at 3 days after stroke. And it was found that $CD11c^{+}$ cells in the intestine significantly tend to migrate from the small intestine to the brain and meninges, leading to inflammation after stroke (Brea et al., 2021), and inflammatory dysfunction of stroke can lead to long-term learning and memory impairments (Gulen et al., 2023). Excessive dietary salt can upregulate the expression of TH17 cells in the small intestine of stroke mice, leading to a significant increase in plasma IL-17. It further promotes cognitive impairment by inhibiting Rho kinase dependent phosphorylation of endothelial nitric oxide synthase and reducing the production of nitric oxide in brain endothelial cells (Faraco et al., 2018).

The enteric nervous system (ENS) can control intestinal behavior and interact with the immune and endocrine systems. ENS can not only interact with microbiota, metabolites, and nutrients, but also interact with immune cells and inflammatory factors to jointly maintain intestinal homeostasis (Rao and Gershon, 2016). ENS structure and neurochemistry resemble that of the CNS, the external connections between CNS and ENS include sympathetic and parasympathetic nerve fibers that directly connect from the hindbrain to the gastrointestinal tract. The vagus nerve and spinal sensory neurons terminate at different locations on the intestinal wall, including the muscular layer and mucosal epithelium. The CNS communicates with the intestine through the gut brain axis (Elenkov et al., 2000; Yoo and Mazmanian, 2017). In ENS, neurotransmitters nitric oxide (NO) and vasoactive intestinal peptide (VIP) are considered to play important roles in the maintenance and protection of neurons (Sandgren et al., 2003). Administration of VIP after stroke in rats can enhance neurogenesis and improve neurological function (Yang et al., 2015). More and more evidence suggested that stroke can affect the gut nervous system. It is found that stroke can trigger the release of central and peripheral galactin-3 causing enteric neuronal loss through by regulating the transforming growth factor β-activated kinase 1 (TAK1) or AMP activated kinase (AMPK) (Cheng et al., 2016). *Paenalcaligenes* and *Escherichia coli* can penetrate the brain through the blood and vagus nerve, leading to cognitive impairment (Lee et al., 2020). In addition, in the study of PSCI, the regulation mechanism of microbiota-gut-brain-axis mediated by gut microbiota and its metabolites (such as gut probiotic metabolites) through vagus nerve and hypothalamus has gradually been reported (Lal et al., 2001). After stroke, it can lead to ecological imbalance in the intestines, producing harmful substances, causing changes in inflammatory reactions and secretion of signaling molecules such as short chain fatty acids (SCFAs) and 3-indoleacetic acid, et al. The inflammatory response after stroke is also considered the core pathological process of secondary brain injury. Inflammation may lead to abnormal immune activation, tissue damage, and ultimately functional loss. By increasing the permeability of the gastrointestinal epithelial layer, inflammation can lead to local dysregulation of the epithelial layer and cause metabolites to escape from the intestine and enter the blood (Vinolo et al., 2011). SCFAs can induce gluconeogenesis, reduce inflammation, and directly interact with vagus nerve afferent nerves (De Vadder et al., 2014; Guo et al., 2022). As a key link between the gut and the brain, the vagus nerve can transmit signals related to appetite, inflammation, and various processes. SCFAs can affect the blood-brain barrier, neurotransmitter levels, and neurotrophic factors, and directly stimulate the vagus nerve to transmit to the nerves, affecting gut-brain communication (Powley et al., 2019). Various intestinal hormones interact with these incoming fibers and ultimately converge in the nucleus tractus solitaries (NTS) located in the medulla oblongata. NTS acts as a relay station for incoming information to the central automatic network (CAN), which includes key areas such as the hypothalamic paraventricular nucleus, locus coeruleus, brachiocephalic nucleus, and limbic system. It can further regulate the HPA axis that responsible for stress response and the autonomic nervous system (Han et al., 2022). But in patients with cognitive impairment after stroke, there is often a decrease in SCFAs. For example, it is reported that the increase of Fusobacteria in the gut and the deficiency of

SCFA metabolites in the gut microbiota are significantly correlated with PSCI (Ling et al., 2020).

The microbiota-gut-brain-axis is an interactive system jointly constructed by the brain, gut and gut microbiota, which suggests a certain correlation between the central nervous system and the gut nervous system in the human body. The above studies confirm that the interaction between the intestinal epithelial barrier (mechanical barrier), mucus layer (chemical barrier), intestinal immune cells and their secretions (immune barrier), and intestinal microbiota (biological barrier) is involved in the occurrence and development of stroke and PSCI. A deep understanding of the intestinal microenvironment and its cross-talk with stroke is of great significance for the treatment and prognosis improvement of PSCI.

3 Clinical effects of acupuncture treatment for PSCI

We screened the PubMed, Embase, Web of science and Cochrane Library databases for published studies, from January 1998 to December 2023. The search keywords employed were as follows: ["acupuncture" or "electroacupuncture (EA)" or "transcutaneous acupoint electrical stimulation (TAES)" or "transcutaneous electrical acupoint stimulation (TEAS)"] and ["post stroke cognitive impairment (PSCI)" or "vascular cognitive impairment (VCI)" "vascular dementia (VD)"] and ["gastrointestinal microbiome" or "gut flora" or "gut microbiota" or "intestinal microflora" or "intestinal flora"]. A total of 213 articles were identified. The following inclusion criteria were used for screening of the identified articles: stimulation methods included manual acupuncture (MA), EA, TAES and TEAS, and the main diseases studied included cognitive impairment caused by stroke which are related to microbiota-gut-brain-axis. We employed Excel software to manually select references that met the theme. Among them, lacked abstract full text, unrelated to the theme, reviews or meta-analyses were excluded. Finally, 18 full texts of basic research articles and 13 clinical research articles, meeting the theme, were included.

Up to now, many clinical studies have observed the effects of MA or EA on PSCI (Table 1). MoCA, MMSE, the Modified Barthel Index (MBI), the Self-rating Depression Scale (SDS), Activities of Daily Living Scale (ADL), Hamilton Depression Rating Scale (HAMD), Hamilton Anxiety Rating Scale (HAMA), Pittsburgh Sleep Quality Index (PSQI), Loewenstein Occupational Therapy Cognition Assessment (LOTCA) and dementia quality of life questionnaire (DEMQOL) are the main tools for evaluating the efficacy of acupuncture. In clinical practice, the determination of acupuncture treatment plan includes pre-treatment evaluation, personalized selection of acupoints, patient response during treatment, and education for patients and their families after treatment. The principle of selecting acupoints is determined by clinical doctors based on syndrome differentiation and treatment, local acupoint selection, and meridian selection. The scalp is one of the commonly chosen areas for treatment. There is a corresponding spatial relationship between the distribution of acupoints in the head and the functional areas of the cerebral cortex. Acupuncture at acupoints on the head can regulate blood circulation of brain. For example, after 12 weeks of scalp acupuncture and cognitive training, the MMSE total score, orientation, spatial executive function, LOTCA total score, and language orientation control score were improved in PSCI patients. At the same time, brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the serum were also increased, indicating that acupuncture has neuroprotective effects (Xiong et al., 2020). Some researchers have improved the method of scalp acupuncture. a study recruited 660 patients with PSCI and found that after an 8-week course of scalp acupuncture treatment, interactive dynamic scalp acupuncture (IDSA) not only significantly improved cognitive function, but also reduced depression and anxiety, improving patients' self-care ability, that is better than simple combination therapy (SCT) and traditional scalp acupuncture (TSA) (Zhang S. H. et al., 2022). In addition, besides to the acupoints used in the scalp acupuncture group, specific acupoints can also be added based on the syndrome differentiation. Compared with drug therapy, such as nimodipine or citicoline, acupuncture therapy can significantly improve the MoCA score of PSCI patients (Wang et al., 2016; Yang et al., 2019; Zhang et al., 2013). Acupoints have specific characteristics. There are studies comparing the effects of acupuncture at different acupoints on VD using clinical scales and cerebral functional imaging. It was found that acupuncture at conventional acupoints plus GV20 can affect the inner temporal system, thalamencephalon system and prefrontal cortical system; plus GV26 has a greater impact on the prefrontal cortex system, and plus HT7 has a greater impact on memory. The simultaneous action of these three acupoints can comprehensively affect multiple aspects of the nervous system related to intellectual activities (Huang et al., 2007). Acupuncture also has a good effect on the hemorheology, blood lipid content, and nail fold microcirculation of PSCI patients (Liu, 2004; Lun et al., 2006). Vascular dementia (VD) is also caused by cerebrovascular disease and is a group of diseases with dementia as the main clinical manifestation. It is similar to PSCI in terms of etiology, clinical manifestations, diagnosis, and evaluation, etc. Acupuncture also has a good improvement effect on VD patients. EA can improve the MoCA score of VD patients. And over time, the MoCA score was improved better without any adverse events (Huang et al., 2021; Shi et al., 2015; Zhao et al., 2009). Some studies have also observed the effect of acupuncture on traditional Chinese medicine syndromes of VD patients, and it was found that acupuncture at *Baihui* (GV20), *Sishencong* (EX-HN1), *Shenting* (GV24), *Danzhong* (CV17), *Zhongwan* (CV12), *Qihai* (CV6), *Xuehai* (SP10), *Zusanli* (ST36) and *Neiguan* (PC6) is more effective in treating excess syndromes, such as Liver-yang hyperactivity or phlegm obstruction of the orifices than deficiency syndromes such as kidney essence deficiency (Shi et al., 2014). Acupuncture can also improve superoxide dismutase (SOD) activity in erythrocytes, and lipid peroxide (LPO) level in plasma of VD patients (Gao et al., 2001). It is found that the functional connectivity between the posterior cingulate gyrus-left middle frontal gyrus and the posterior cingulate gyrus-right superior temporal gyrus is positively correlated with cognitive function. Functional connectivity analysis of fMRI showed that after EA, the default mode network function in brain regions such as the posterior cingulate gyrus, left middle frontal gyrus, left anterior cingulate gyrus, left and right superior temporal gyrus, right insula, left precentral gyrus and other brain regions were significantly increased. EA can increase the functional connectivity between the posterior cingulate gyrus and other gyrus

in VD patients (Lin et al., 2024). Sum up, acupuncture may be used as a supplementary therapy, further improving the clinical efficacy of PSCI patients.

4 Acupuncture alleviate PSCI via microbiota-gut-brain-axis

Some clinic and basic studies have shown that acupuncture can alleviate symptoms of stroke and PSCI. It has been proved that acupuncture can effectively regulate intestinal microbiota to treat diseases and keep body in balance. The treatment strategy based on gut microbiota has great clinical potential in acupuncture treatment of PSCI, but its regulatory mechanism is still unclear. Exploring the mechanism of action is crucial for the clinical application of acupuncture in the treatment of PSCI by regulating the gut microbiota. We reviewed and found that acupuncture can improve the symptoms of patients with PSCI by protecting the IEB, remodeling the structure of gut microbiota, inhibiting inflammation, regulating metabolic pathways and regulating the hypothalamic-pituitary-adrenal (HPA) axis (Tables 1, 2).

4.1 Protecting the intestinal epithelial barrier

Acupuncture can protect the structure and function of the IEB and prevent pathogenic substances from crossing the physical barrier, thus maintaining human health. The function of IEB depends on the apical junction complexes (AJC), composed of tight junctions (TJ), adherent junctions (AJ) and desmosomes, can control the entry of gut microbiota into intestinal connective tissue, prevent antigens from passing through IEB (Alonso et al., 2014). TJ is the apical junction of intestinal epithelial cells (IECs), composed of cytoplasmic attachment proteins, transmembrane proteins, and cytoskeletal proteins. They can play a crucial role in maintaining barrier integrity and participate in intestinal epithelial repair (Krug and Fromm, 2020). Electroacupuncture (EA) at Zusanli (ST36) can upregulation TJ proteins (ZO-1, Occludin, and Claudin-1) and mucin protein (E-Cadherin, MUC2) to reduce the apoptosis and proliferation of IECs and intestinal permeability, further repair the mucus and epithelial barriers (Liu et al., 2023; Wang et al., 2020a). Current evidence suggests that stroke destroys the integrity of the IEB and can lead to enterogenic sepsis (Stanley et al., 2016). EA can improve the mitochondrial dynamic balance mediated by the heme oxygenase 1 (HO-1)/PTEN-induced putative kinase 1 (PINK1) pathway and protect the patient's intestinal barrier (Zhang Y. et al., 2023). Manual acupuncture (MA) at Baihui (GV20), Yintang (GV29) and ST36 can also effectively alleviate the destruction of the intestinal mucosal barrier, manifested as orderly arrangement of intestinal gaps and narrow connecting gaps (Hao et al., 2022).

4.2 Regulating the structure of gut microbiota and metabolic pathways

Acupuncture treatment modulates the species abundance of the gut microbiota. A clinical study shows that EA treatment at GV20 and GV24 can effectively alleviate cognitive impairment and balances gut microbiota ecology in patients with subjective cognitive declines. EA can increase the abundance of beneficial bacteria (such as *Roseburia*, *Ruminococcus UCG-014*, *Ruminococcus UCG-013* and *Bacteroides*), and downregulate harmful bacteria such as *escherichiacoli, e.coli* and *Klebsiella* (Tan et al., 2023). Preclinical studies have shown that MA at ST36, *Sanyinjiao* (SP6), *Guanyuan* (CV4), *Qihai* (CV6) and GV20 can enhance the abundance of *Candidatus Arthromitus*, *Lactobacillus* and *Clostridia_UCG-014_unclassified* and decrease the abundances of *Escherichia-Shigella*, *Burkholderia-Caballeronia-Paraburkholderia* and *Streptococcus* (Lv et al., 2022). MA at GV20 and GV29 can enhance the abundance of *Bacteroidota*, *Firmicutes*, *Proteobacteria*, *Campilobacterota*, and *Actinobacteriota* at the phylum level, decrease *Proteobacteria* and *Escherichia-Shigella*, while the bacterial community was dominated by *Proteobacteria* in the MA group. The study also found that acupuncture combined with oral probiotics has a synergistic effect on enhancing efficacy (Zhang Y. et al., 2022). EA at GV20, GV14, BL23 and ST36 can reduce the relative abundance of harmful bacteria (such as the *Catabacter*, *Robinsoniella* and *Desulfovibrio*), while the relative abundance of *Clostridiales-unclassified* is upregulated in the EA + probiotics group, they both can improve cognitive impairment in vascular dementia mice (Chen et al., 2022).

Acupuncture treatment can also modulate the metabolic pathways. Some studies have shown that gut microbiota and its metabolite TMAO have been proved to be related to atherosclerosis. The increased level of TMAO may reflect the severity of atherosclerosis and carotid stenosis, and induce stroke related cognitive dysfunction. Naochang Tongtiao acupuncture applied at anterior oblique line of vertex-temporal, Zhongwan (CV12), CV4, Tianshu (ST25), ST36, Shangjuxu (ST37) and Xiajuxu (ST39) can decrease plasma level of TMAO and improve the nerve function in stroke patients (Wang et al., 2022a). EA can regulate gut microbiota to activate more indole-3-propionic acid, thereby affecting melatonin receptors and improving symptoms in middle cerebral artery occlusion (MCAO) mice (Li S. et al., 2022). Serum metabolomics analysis showed that acupuncture can regulate the N-methylnicotinamide, β-glycerophosphoric acid, geranyl acetoacetate, serotonin and phenylalanine, tyrosine and tryptophan biosynthesis, taurine and hypotaurine, and beta-alanine metabolic pathways (Lv et al., 2022). Acupoint is one of the key factors of clinical effectiveness of acupuncture. Hypertension is a clinical risk factor for stroke, a clinical study used high-performance liquid chromatography tandem mass spectrometry to compare the targeted metabolic phenotype changes induced by two different acupoint treatments. It was found that only in the active acupoint treatment group, Taichong (LR3), Renying (ST9), Taixi (KI3), and Neiguan (PC6) were selected and the metabolites sucrose, fibrodisaccharides, and hypoxanthine were proven to be the most important targets for active treatment (Yang et al., 2018).

4.3 Regulating the gut microbiota and immune system

When the IEB is damaged, the permeability of the IEB increases, lipopolysaccharides and other endotoxins produced by

TABLE 1 Clinical effects of acupuncture treatment for PSCI.

References	Acupuncture method	Acupoints	Acupuncture parameters	Outcomes
Xiong et al., 2020	MA	GV20, GV24, GB20, EX-HN1	3~4h, 3 times a week, 8-week course	MMSE↑, LOTCA↑, FMA↑, mADL↓, BNDF↑, NGF↑
Zhang S. H. et al., 2022	MA	MS6, MS7	30-min, 6 times a week, 8-week course	MMSE↑, MoCA↑, HAMD↓, HAMA, PSQI↓, MBI↑
Zhang et al., 2013	MA/EA	Main acupoints: GV20, GV24, GB20, EX-HN1 TCM syndrome: the kidney essence deficiency syndrome + KI3; phlegm blocking meridian syndrome + ST40; liver-Yang hyperactivity syndrome + LR3; Qi and blood deficiency syndrome + ST36	30-min, 3 times a week, 8-week course	MMSE↑, clock drawing test↓, picture recognition↑
Wang et al., 2016	MA	GV20, EX-HN1, ST2, GB20, GB12, BL10, GV26, HT7, PC6, ST40, SP36, LR3	30-min, 6 times a week, 12-week course	MoCA↑
Yang et al., 2019	MA	ST36, SP10, CV17, CV12, CV6, GV20, GV16, BL15, BL45, HT5, KI6, KI3, GB39, ST40, PC6, BL17	30-min, twice a week (at an interval of 2–4 days), 12-week course	ADAS-cog↓
Huang et al., 2007	MA	LI15, LI11, TE5, LI4, SP10, ST36, SP6, LR3, GV20, GV26, HT7	20-min, 5 times a week, 4-week course	MMSE↑, FAQ↓, Blood Flow↑, glucose metabolism↑, inner temporal system↑, thalamencephalon system activation↑, prefrontal cortical system activation↑, prefrontal cortical system activation↑
Lun et al., 2006	MA + EA	Excess syndrome: LI11, ST40, LR3; Deficiency syndrome: BL18, BL23, ST36	MA: 20-min; EA:30-min, 5 times a week, 9-week course	NO↓, NOS↓
Huang et al., 2021	EA	GV20, GV29, GV24, GV26, GV17, EX-HN1, GB20, HT7, SP6	30-min, 3 times a week, 8-week course	MoCA↑, MMSE↓
Shi et al., 2015	MA	GV20, EX-HN1, GV24, CV17, PC6, CV12, CV6, SP10, ST36, GB20, ST40, LR3, SP6, ST25	30-min, once every other day, 6-week course	MMSE↑, ADL↓
Zhao et al., 2009	EA	EX-HN1, GV20, GV24, GB20	30-min, 5 times a week, 6-week course	MMSE↑, P_{300} (P_3 latency↓, P_3 wave amplitude↑)
Shi et al., 2014	MA	Main acupoints: GV20, EX-HN1, GV24, CV17, CV12, CV6, SP10, ST36, PC6 TCM syndrome: the kidney(Shen)-essence deficiency: GB39; phlegm obstruction of the orifices: ST40; blood-letting puncture, for collateral obstruction due to blood stasis: EX-HN11; liver(Gan)-Yang hyperactivity syndrome: LR3; fire hyperactivity: ST44; organ-turbidity retention: ST25; qi and blood deficiency syndrome: CV4	30-min, once every other day, 6-week course	SDSVD↓
Gao et al., 2001	MA	EX-HN1, GV26, PC6, SP6, ST40	15-min, once daily with an interval of 2 days between 2 weeks, 6-week course	HDS↑, SOD↓, LPO↓, P_{300} (P_3 latency↓, P_3 wave amplitude↑), Rheoencephalogram↓
Lin et al., 2024	EA	GV20, GV24	30-min, 5 times a week, 8-week course	MoCA↑, STROOP↑

↑, upregulated by acupuncture; ↓, downregulated by acupuncture.

bacteria can activate the peripheral immune system, activate immune cells and related signaling pathways, promote the release of inflammatory factors and cause systemic inflammation, and also promote the migration and infiltration of peripheral immune cells to the brain, leading to central nervous system inflammation through the blood-brain barrier (BBB) (Caspani and Swann, 2019). Stroke can trigger inflammatory response and studies have shown that inflammation may affect synaptic function and exacerbate cognitive impairment. A systematic review and meta-analysis evaluated the levels of inflammatory markers in PSCI patients and found that their inflammation (such as CRP, hs-CRP, MMP9, IL-1β, IL-6, IL-10, TNF-α) in blood increased and was negatively correlated with cognitive function. Interventions targeting inflammation can also improve cognitive function in animals, which also seems to explain the clinical use of complement inhibition and engomod in the treatment of PSCI (Tack et al., 2023). Acupuncture can not only repair the intestinal epithelial barrier, but also repair the blood-brain barrier and inhibit the

TABLE 2 Effects and mechanisms of acupuncture on microbiota-gut-brain-axis.

References	Acupuncture	Acupoints	Intestinal epithelial barrier	Gut microbiota	Metabolites and metabolic pathways	Immune system	Nervous system	HPA axis
Liu et al., 2023	EA	ST36	ZO-1↓, Occludin↓, Claudin-1↓, MUC2↓	–	–	IL-6↓, IL-1β↓, IFN-γ↓, TNF-α↓	–	–
Wang et al., 2020a	EA	ST36	ZO-1↑, Occludin↑, E-Cadherin↑, MUC2↑, IECs↓	Muribaculaceae↓, Roseburia↓, Faecalibacterim↓, Bifidobacterium↓, Muribaculaceae↓, Roseburia↓, Faecalibacterium↓, Bifidobacterium↓, EscherichiaShigella↑, Erysipelatoclostridium↑	–	MAPK↑(p-ERK1/2↑, p-JNK↑, P-p38↑), TNF-α↓, IL-1β↓, IL-6↓, iNOS↓	–	–
Zhang Q. et al., 2023	EA	ST36, LI4	DAO↑	–	ROS↓, ATP↑, HO-1/PINK1↑, Drp1↓, Mfn1↓, Mfn2↓	IL-1β↓	–	–
Hao et al., 2022	MA	GV20, GV29, ST36	ZO-1↑, Occludin↑, GFAP↓	Bacteroidetes↑, Proteobacteria↓, EscherichiaShigella↓	–	TNF-α↓, LPS↓	–	–
Lv et al., 2022	MA	ST36, SP6, CV4, CV6, GV20	ZO-1↑, Claudin-5↑, Occludin↑	Candidatus↑, Arthromitus↑, Lactobacillus↑, Clostridia_UCG014_unclassified↑, EscherichiaShigella↓, Burkholderia Caballeronia-Paraburkholderia↓, Streptococcus↓	–	IL-1β↓, IL-6↓, TNF-α↓	–	CRH↑, ACTH↓, CORT↑
Zhang Y. et al., 2022	MA	GV20, GV29, ST36	–	Bacteroidetes↑, Proteobacteria↓, Firmicutes↓, EscherichiaShigella↓	–	LPS↓, IL-1β↓, TNF-α↓	–	–
Chen et al., 2022	EA	GV20, GV14, BL23, ST36	–	Elusimicrobia↓	–	IL-1β↓, IL-18↓	–	–
Li S. et al., 2022	EA	GV20, GV14, ST36	–	Akkermansia↑, Lactobacillus↑	IPA↑, UCP2↓, MTR1/PGC-1α↑	–	SOD↑, GSH/GSSG↑, MDA↓, Bcl-2/Bax/Caspase3↓	–
Wang H. et al., 2023	EA	GV26	–	Bacteroidetes↑, norank_f_Bacteroidales_S24-7_group↑, Bacteroides↑	TC↓, TG↓, HDL↑, LDL↓, ALT↓, AST↓, ALP↓	IL-10↑, IL-6↓, TNF-α↓	–	–
Qiuping et al., 2023	MA	CV17, CV12, CV6, ST36, SP10, SJ5, CV24, CV23, ST7, ST4	–	–	–	Treg↑, Th-17↓, CD4 + RORγt↑, CD4 + Foxp3↓	–	–
Liu et al., 2020	EA	ST36	ZO-1↑, Claudin-1↑	Firmicutes↓	–	CD11b↓, F4/80↓, TLR4↓, MyD88↓, CRP↓, IFN-γ↓	–	–

(Continued)

10.3389/fmicb.2025.1425054

TABLE 2 (Continued)

References	Acupuncture	Acupoints	Intestinal epithelial barrier	Gut microbiota	Metabolites and metabolic pathways	Immune system	Nervous system	HPA axis
Wang et al., 2020b	MA	GV20, ST36	–	–	–	miR-93↓, TLR4↓, MyD88↓, NF-κB↓, IL-6↓, TNF-α↓	IL-6↓, TNF-α↓	–
Du et al., 2018	MA	ST36, GV20	–	–	–	TXNIP ↓, NLRP3↓, caspase-1↓, IL-1β↓	–	–
Cao et al., 2021	MA	GV20, ST36	–	–	–	IL-1β↓, IL-6↓, TNF-α↓, p-JAK2↑, p-STAT3↑	α7nAChR↑	–
Dou et al., 2020	EA	ST25, CV4, ST36, SP6	–	Proteobacteria↑, Firmicutes↓, cyanobacteria↓, Bacteroidetes↓, Lachnospiraceae-NK413↓, staphylococci↓, Enterobacter↑, Stenotrophomonas↑, Helicobacter↑	–	–	PGP9.5↓, ChAT↓, nNOS↓	–
Mengzhu et al., 2022	EA	ST25, ST36	–	Ileibacterium↑, Dorea↑, Candidatus Saccharimonas Dubosiella↑, Lactobacillus↑, Lactobacillales↑, Erysipelotrichales↑	–	–	–	CRF↓
Le et al., 2016	EA	ST36, CV4	–	–	–	–	CRH↓, 5-HT↑, 5-HT1AR↑	ACTH↓, CORT↓,
Sun et al., 2015	EA	ST25, ST36, LR3	–	–	–	–	5-HT↓, CGRP↓, NPY↑	–

↑, upregulated by acupuncture; ↓, downregulated by acupuncture. PSCI, post-stroke cognitive impairment; CVA, cerebrovascular accident; IS, ischemic stroke; ICH, intracerebral hemorrhage; SAH, subarachnoid hemorrhage; MMSE, Mini Mental State Examination; MoCA, Montreal Cognitive Assessment; MGBA, microbiota-gut-brain-axis; IEB, intestinal epithelial barrier; GI, gastrointestinal; TMAO, trimethylamine-N-oxide; ENS, enteric nervous system; NO, nitric oxide; VIP, vasoactive intestinal peptide; TAK1, transforming growth factor β-activated kinase 1; AMPK, AMP activated kinase; HPA, hypothalamic-pituitary-adrenal; AJC, apical junction complexes; TJ, tight junctions; AJ, adherent junctions; ZO-1, Zonula Occludens-1; MUC2, mucin2; IECs, intestinal epithelial cells; EA, electroacupuncture; ROS, reactive oxygen species; ATP, adenosine triphosphate; DAO, diamine oxidase; HO-1, heme oxygenase 1; PINK1, PTEN-induced putative kinase 1; Mfn 1/2, Mitofusin 1/2; Drp1, Dynamin-related protein 1; MA, manual acupuncture; MCAO, middle cerebral artery occlusion; BBB, blood-brain barrier; TLRs, toll like receptors; MyD88, myeloid differentiation factor 88; NF-κ B, Nucler factor kappa B; CRP, C-reactive protein; hsCRP, hypersensitive C reactive protein; DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; RORγt, orphan nuclear receptor; Foxp3, forkhead box p3; JAK, intracellular Janus Kinase 2; STAT, Activator of Transcription 3; TXNIP, Thioredoxin-interacting protein; NLRP3, NOD-like receptor protein 3; α7nAChR, Alpha-7 nicotinic acetylcholine receptor; CORT, cortisol; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; GR, glucocorticoid receptors; CRF, corticotropin-releasing factor; 5-HT, 5-hydroxytryptamine; CGRP, calcitonin gene-related peptide; NPY, Neuro-peptide Y; IL-6, Interleukin 6; IL-1β, Interleukin 1β; IL-10, Interleukin 10; IFN-γ, Interferon γ; TNF-α, tumor necrosis factor; MAPK, Mitogen-activated protein kinase; p-ERK1/2 phosphorylation extracellular signal-regulated kinases 1/2, p-JNK, nNOS, neurogenic Nitric Oxide Synthase; ChAT, acetylcholine transferase; PGP9.5, protein gene product 9.5; GFAP, glial fibrillary acidic protein; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ALT, alanine transferase; AST, aspartate transferase; ALP, alkaline phosphatase; LOTCA, Loewenstein Occupational Therapy Cognition Assessment; FMA, Fugl-Meyer assessment; mADL, modified Daily Living Scale; BNDF, neurotrophic factor; NGF, nerve growth factor; HAMD, Hamilton Depression Rating Scale; HAMA, Hamilton Anxiety Rating Scale; PSQI, Pittsburgh Sleep Quality Index; MBI, Modified Barthel Index; ADAS-cog, Alzheimer's disease assessment scale-cognitive subscale; ADL, Daily Living Scale; FAQ, Family Attitude Questionnaire; NOS, Nitric Oxide Synthase; SDSVD, Scale of differentiation of syndromes of vascular dementia; HDS, SOD, superoxide dismutase; LPO, lipid peroxide; STROOP, Stroop color-naming condition. Acupoints: ST36, *Zusanli*; SJ5, *Waiguan*; LI4, *Hegu*; LI11, *Quchi*; GV14, *Juque*; GV16, *Fengfu*; GV20, *Baihui*; GV24, *Shenting*; GV26, *Shuigou*; GV29, *Yintang*; SP5, *Shangqiu*; SP6, *Sanyinjiao*; SP9, Yinlingquan; SP10, *Xuehai*; CV4, *Guanyuan*; CV6, *Qihai*; CV12, *Zhongwan*; CV17, *Danzhong*; ST2, *Sibai*; ST25, *Tianshu*; ST37, *Shangjuxu*; ST39, *Xiajuxu*; ST40, *Fenglong*; ST44, *Neiting* LR3, *Taichong*; ST9, *Renying*; KI6, *Zhaohai*; KI3, *Taixi*; PC6, *Neiguan*; HT5, *Tongli*; HT7, *Shenmen*; GB20, *Fengchi*; GB39, *Xuanzhong*;BL10, *Tianzhu*; BL15, *Xinshu*; BL17, *Geshu*; BL18, *Ganshu*; BL23, *Shenshu*; BL45, *Yixi*; EX-HN1, *Sishencong*; MS6, *Anterior oblique line of vertex-temporal*; MS7, *posterior oblique line of vertex-temporal*; EX-HN11, *Shexia*.

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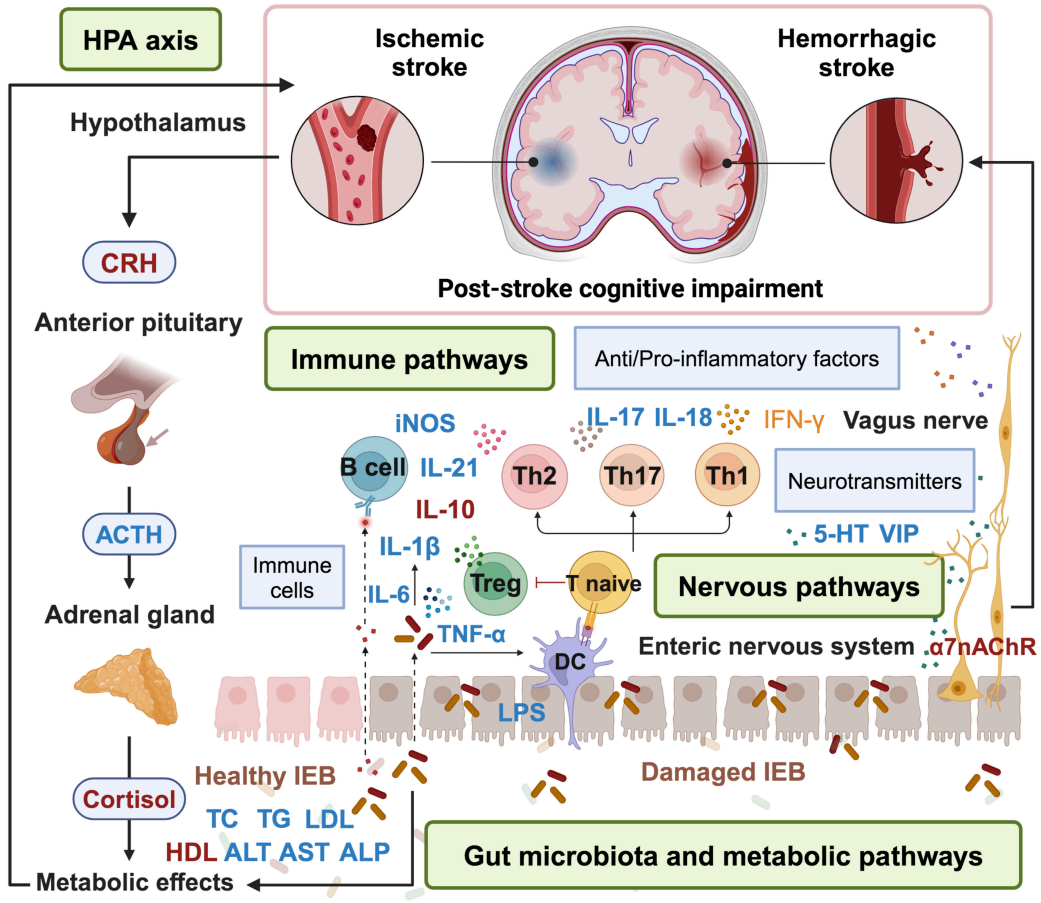

FIGURE 1
Acupuncture treatment for PSCI via microbiota-gut-brain-axis. Acupuncture can alleviate symptoms of PSCI by repairing the damaged IEB, remodeling the structure of gut microbiota, inhibiting inflammation, regulating the release of neurotransmitters, metabolic pathways and HPA axis. Factors in blue are down-regulated by acupuncture, while factors in red are up-regulated by acupuncture. HPA, hypothalamic-pituitary-adrenal; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; DC, dendritic cell; IEB, intestinal epithelial barrier; VIP, vasoactive intestinal peptide; 5-HT, 5-hydroxytryptamine. The figure is created with BioRender.com.

entry of inflammatory factors into the brain. Acupuncture may also improve cognitive impairment after stroke by inhibiting the activation of inflammation related pathways and the expression of inflammatory factors. The content of some types of gut microbiota (such as Bacteroides) is often positively correlated with MMSE and MOCA scores, and has anti-inflammatory properties (Zhuang et al., 2018). Acupuncture can upregulate the expression of Bacteroides and decrease the systemic inflammatory response (Wang H. et al., 2023). EA at GV20 can regulate Treg/γδ T cell polarization (increased the percentage of Treg in the small intestine while decreasing the rate of γδ T cells) and Th17/Treg balance to improve the intestinal barrier integrity of stroke rats (Qiuping et al., 2023; Wang Y. L. et al., 2023). EA at GV20 can not only regulate Tregs and γδ T cells in the small intestine, but also regulate them in the ischemic brain, inhibit the mobilization of intestinal T cells to brain, which indicated its effect on inhibiting inflammation (Wang Y. et al., 2023). Intestinal epithelial cells contain various pattern recognition receptors, such as toll like receptors (TLRs), which can recognize invading damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), and regulate subsequent inflammatory responses (Wicherska-Pawłowska et al., 2021). EA can alleviate both macroscopic and microscopic colonic inflammation, upregulate the expression of claudin-1 and ZO-1 to repair the intestinal barrier, remold the overall structure of the gut microbiota, and inhibit pro-inflammatory factors IFN-γ, TNF-α, and IL-6 through the MyD88-dependent TLR4 signaling pathway, preventing excessive immune response throughout the body (Liu et al., 2020). MA at ST36 can inhibit TLR4/MyD88/NF-κB signaling pathway mediated by miR-93 in experimental vascular dementia rats to alleviate cognitive impairment associated with inflammation. The expression of inflammatory cytokines (IL-6 and TNF-α) in the hippocampus and plasma were significantly decreased after treatment (Wang et al., 2020b). Thioredoxin-interacting protein (TXNIP) plays an important role in oxidative stress and NOD-like receptor protein 3 (NLRP3) inflammasome activation. MA at ST36 and GV20 can decrease TXNIP, NLRP3, caspase-1, and IL-1β in the hippocampus of vascular dementia rats (Du et al., 2018). The above studies have found that acupuncture can downregulate the expression of inflammatory factors in the gut, serum and brain, regulating the gut microbiota and immune system, improving stroke and cognitive impairment. Alpha-7

nicotinic acetylcholine receptor (α7nAChR) is a ligand gated ion channel expressed in the central and peripheral nervous systems and has been proven a key component to regulate inflammation. MA at these two acupoints can also promote cognitive function by activating α7nAChR and its downstream JAK2-STAT3 pathway, and found that this combination of acupoints is better than GV20 + GV24 and ST36 + SP10 acupoints combination (Cao et al., 2021). A systematic analysis of 42 studies with a total of 1,486 animals also showed that among all acupuncture regimens, GV20 + ST36 combined with 14-day MA had the best improvement effect on cognitive function (Li G. et al., 2022).

4.4 Regulating the gut microbiota and nervous system

ENS can act as a local neural mechanism to independently control intestinal behavior and interact with the immune and endocrine systems. Gut microbiota can activate stress circuits in the CNS through the vagus and sensory neurons of ENS (Cryan et al., 2020). NO and VIP are considered to play important roles in maintaining and protecting neurons in ENS (Joly et al., 2021). Stroke can affect the ENS, and acupuncture may improve stroke and subsequent cognitive impairment by regulating gut microbiota and ENS. Studies have found that EA at ST25, ST36, CV4 and SP6 promotes the diversity of intestinal microbiota, significantly improving the pathological ENS score, serum neurotransmitter levels, and intestinal transport rate in mice (Dou et al., 2020). The functional activity of the brain depends on signal transmission between neurons and glial cells, which mainly relies on neurotransmitters (Alkasir et al., 2017). Neurotransmitters are chemical substances that can transmit information between neurons through synapses, thereby controlling behaviors such as movement, emotion, and memory. The neurotransmitter substances produced by gut microbiota are also closely related to the host's behavior (O'Donnell et al., 2020). In additional, certain bacterial taxa in the intestine can also produce enzymes that catalyze the synthesis of neurotransmitters in the brain. A study has found that neurotransmitters from the intestine act on the brain through blood circulation, local stimulation of the intestinal nervous system, and rapid signal transduction of the vagus nerve (Chen et al., 2021). Another study found that focal cerebral ischemia and global cerebral ischemia have different effects on the survival of intestinal neurons, which may reflect the difference in the immune response of peripheral nerves, also indicating the mechanism of acupuncture application also needs further exploration (Cheng et al., 2018).

4.5 Regulating HPA axis

The HPA axis is one of the main neuroendocrine systems that respond to stress, and can produce glucocorticoids, participate in cognitive processes such as neural development, learning and memory. The HPA axis includes cortisol (CORT), the corticotropin-releasing hormone (CRH), and the adrenocorticotropic hormone (ACTH). Research shows that stress can significantly affect microbiota-gut-brain-axis through HPA axis (Rusch et al., 2023). For example, CORT receptors are expressed on epithelial cells, immune cells, and intestinal endocrine cells of the intestine. They can not only directly affect intestinal function, but also affect the gut microbiota by altering intestinal transport time, intestinal permeability, and nutrient availability. Cortisol can affect brain function by binding to glucocorticoid receptors (GR) in the hippocampus, amygdala, and prefrontal cortex of the brain (Carabotti et al., 2015). The imbalance of the HPA axis can affect gut microbiota and further affect post-stroke cognitive function. Acupuncture may improve post-stroke cognitive impairment by regulating the HPA axis. MA at ST36, SP6, CV4, CV6 and GV20 can increase the expression of CRH and CORT and inhibit the expression of ACTH (Lv et al., 2022). EA at ST25 and ST36 can modulate the dysbiosis of gut microbiota and suppress the overexpression of corticotropin-releasing factor (CRF) in colon tissues (Mengzhu et al., 2022). EA at CV4 and ST36 can reduce the expression of CRH mRNA in the hypothalamus, downregulate ACTH and CORT levels in plasma, and significantly increase hippocampal serotonin concentration and 5-hydroxytryptamine receptor 1A (5-HT1AR) mRNA and proteins expression, improving adverse emotions in rats (Le et al., 2016). It is found that neurotransmitter 5-HT can mediate changes in the hypothalamus, spinal cord, and colon, and spinal cord 5-HT and calcitonin gene-related peptide (CGRP) are closely related to behavioral assessment. EA at ST25, ST36 and LR3 can regulate the expression of 5-HT, CGRP, and Neuro-peptide Y (NPY) in the distal colon, spinal cord, and hypothalamus (Sun et al., 2015). The role of acupuncture in regulating the HPA axis has been widely reported. However, limited studies have shown whether acupuncture can improve PSCI by regulating the HPA axis. Currently, most of the evidence is related rather than direct, and further exploration is needed in the future.

5 Discussion and conclusion

Prevention and treatment of secondary stroke injuries are crucial. As an important participant in the pathological and physiological events of cognitive impairment in stroke, the structure and function of the gut microenvironment can directly or indirectly affect neurological function and cerebral ischemic outcomes, and their crosstalk is achieved through the microbiota axis. The microbiota-gut-brain axis includes the nervous system (central and enteric nervous systems), endocrine system (HPA axis), and immune system (intestinal and central immune systems), and is an information exchange network connecting the gut and brain. It can transmit information "bottom–up" from the gut to the brain and "top–down" from the brain to the gut bidirectionally. Here are certain correlations between intestinal epithelial barrier, gut microbiota and serum metabolites, gut inflammation, neuroinflammation and HPA axis function. This review demonstrates that, acupuncture, as an adjuvant therapy can play a potential important role in the treatment of PSCI by regulating the microbiota-gut-brain-axis (Figure 1). Acupuncture is a promising non-drug treatment for reducing cognitive symptoms. In the analysis of acupoints included in the literature, it was found that the commonly used local acupoints on the head are *Baihui* (GV20), *Shenting* (GV24), *Shuigou* (GV26), *Yintang*

(GV29), and *Fengchi* (GB20) and *Fengfu* (GV16). The commonly used acupoints on the limbs include *Hegu* (LI4), *Waiguan* (SJ5), *Neiguan* (PC6), *Zusanli* (ST36), *Sanyinjiao* (SP6), *Yinlingquan* (SP9), *Xuehai* (Xuehai), *Taixi* (KI3), *Zhaohai* (KI6), and *Taichong* (LR3). The abdominal acupoints include *Guanyuan* (CV4), *Qihai* (CV6), *Zhongwan* (CV12), *Danzhong* (CV17), *Tianshu* (ST25), etc. The acupoints on the head have the functions of clearing the brain, awakening the brain, and enhancing intelligence. In the theoretical system of acupuncture and meridians, the Du meridian plays an important role in the cognitive function of the brain. Acupuncture of head points can protect damaged neurons by affecting glucose metabolism, regulating neurotransmitters, releasing and improving patients' intelligence, and promoting the improvement of cognitive function. Acupuncture at acupoints in the abdomen and lower limbs can improve cognitive impairment by regulating the balance of gut microbiota, improving the gut microenvironment, promoting the release of beneficial gut microbiota and neuroactive substances, regulating the immune system. We also found that the acupuncture protocols used in clinical trials lack consistency, which may be related to the diversity of acupoint selection and compatibility, heterogeneity of operating parameters and stimulation techniques, diversity of acupuncture methods, and lag in mechanism research and standardization. Traditional Chinese medicine emphasizes "acupoint selection based on syndrome differentiation", and different studies select acupoint combinations based on syndrome types. However, these acupuncture protocols reflects the advantages of individualized treatment, but weakens the comparability between different studies. The operation of acupuncture also depends on the doctor's experience, and it is difficult to blind the performer. Only a few trials can blind subjects, evaluators, and statistical analysts. There are various cognitive function assessment tools, including MMSE, MoCA, ADL scales, but the threshold definition and sensitivity are different. Some studies combine neuroimaging (such as fMRI) or biomarkers, but there is a lack of unified standards for detection methods and time points. There is still no international consensus on specific acupoint combinations and stimulation parameters for acupuncture treatment of PSCI. In the future, it is necessary to develop core acupoint combinations and develop layered treatment plans based on syndrome classification. Doctors need to unify operating parameters, standardize electroacupuncture frequency, needle retention time, and treatment course. It is also necessary to optimize the design of sham acupuncture to reduce the interference of comfort effect and promote multi center large sample research. It is expected to improve the quality of clinical evidence for acupuncture treatment of PSCI and promote its international application.

Although studies have shown that acupuncture may affect PSCI through the MGBA, the specific mechanism of action is still not very clear. The MGBA involves complex interactions between multiple systems such as the nervous system, endocrine system, and immune system. Further research is needed on how acupuncture can precisely regulate these systems to improve cognitive impairment. At present, research on acupuncture for treating PSCI through the MGBA is mostly small sample clinical studies or animal experiments, lacking large-scale, multicenter clinical studies to further confirm its effectiveness and safety. In the future, more basic studies and multicenter randomized controlled studies should be conducted to explore whether acupuncture can improve PSCI through microbiota-gut-brain-axis, and determine the causal relationship among them. By utilizing modern biotechnology such as genomics, proteomics, metabolomics, etc., explore the effects of acupuncture on gut microbiota, neurotransmitters, inflammatory factors, and the relationship between these factors and cognitive function improvement. There is no unified standard for the optimal time point, treatment frequency, and duration of acupuncture treatment, which poses certain difficulties for clinical application. Different treatment plans may produce different therapeutic effects, and further exploration is needed to determine the optimal treatment plan. Further clinical treatment standards for acupuncture treatment of PSCI through the MGBA can be developed, including diagnostic criteria, treatment plans, efficacy evaluation, etc., in order to improve the reproducibility and reliability of treatment. Exploring the combined application of acupuncture and other treatment methods is also a research direction that we can consider, such as drug therapy, rehabilitation training, psychological therapy, etc. By integrating multiple treatment methods and exerting synergistic effects, the therapeutic effect can be improved.

In addition, in the literature we have compiled, we found that two different stimuli, MA and EA, were used for PSCI. There may be some differences in the mechanisms of using MA and EA to treat diseases. MA can stimulate acupoints and regulates the circulation of meridians, qi, and blood. EA, on the basis of traditional acupuncture, can enhance the intensity and duration of acupuncture by stimulating acupoints with electric current. Different frequencies and waveforms of current can also produce different therapeutic effects. These two are interrelated, both regulating physiological functions of the human body through stimulation of acupoints to achieve the goal of treating diseases. In the study of acupuncture treatment for PSCI, there is currently no comparison between MA and EA treatment. In our study, we found that the reason why researchers used MA for stimulation may be to eliminate interference from electrical currents. We also searched some other literature and found that in some diseases, researchers compared the therapeutic effects of MA and EA. For example, it was found that EA treatment is not superior to MA treatment for lower back pain, and the two therapies have similar efficacy in reducing pain and disability in chronic non-specific back pain (Comachio et al., 2020). However, due to the relatively limited research in this area, experts have indeed provided us with new research directions that we can further explore in the future. There is also no unified standard for the optimal time point, treatment frequency, and duration of acupuncture treatment, which poses certain difficulties for clinical application. Different treatment plans may produce different therapeutic effects, and further exploration is needed to determine the optimal treatment plan. Further clinical treatment standards for acupuncture treatment of PSCI through the MGBA can be developed, including diagnostic criteria, treatment plans, efficacy evaluation, etc., in order to improve the reproducibility and reliability of treatment. Exploring the combined application of acupuncture and other treatment methods is also a research direction that we can consider, such as drug therapy, rehabilitation training, psychological therapy, etc. By integrating multiple treatment methods and exerting synergistic effects, the therapeutic effect can be improved.

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TB: Writing – original draft, Writing – review and editing, Conceptualization. RN: Writing – original draft, Methodology. JG: Writing – original draft, Methodology, Writing – review and editing, Formal Analysis, Investigation. WL: Writing – original draft, Formal Analysis, Investigation, Writing – review and editing. MD: Writing – original draft, Formal Analysis, Investigation, Writing – review and editing. JD: Writing – original draft, Formal Analysis, Investigation, Writing – review and editing. YG: Funding acquisition, Resources, Writing – review and editing. SW: Writing – review and editing. ZL: Writing – original draft, Conceptualization, Supervision. YL: Conceptualization, Funding acquisition, Writing – review and editing. NL: Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft.

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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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