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The role of probiotics, postbiotics, and microbial metabolites in preventing and treating chronic diseases

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The role of probiotics, postbiotics, and microbial metabolites in preventing and treating chronic diseases

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Editorial: The role of probiotics, postbiotics, and microbial metabolites in preventing and treating chronic diseases

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Editorial on the Research Topic
The role of probiotics, postbiotics, and microbial metabolites in preventing and treating chronic diseases

Chronic diseases are defined as non-communicable diseases that are difficult to cure and cause host damage due to long-term accumulation, which mainly include cardiovascular and cerebrovascular diseases (hypertension, coronary heart disease, stroke, etc.), cancer, diabetes, and chronic obstructive pulmonary disease (chronic bronchitis, emphysema, etc.). Currently, there is no effective treatment methods for chronic diseases, and probiotics, postbiotics and microbial metabolites have become promising approaches for the improvement of chronic diseases.

In this Research Topic, Lv et al. conducted a clinical intervention experiment to investigate the therapeutic effects of precision probiotic strains transplantation capsules, fecal microbiota transplantation capsules and live combined *Bacillus subtilis* and *Enterococcus faecium* capsules on patients with severe diarrhea irritable bowel syndrome (IBS). Their results showed that precision probiotic strains transplantation capsules exhibited a more pronounced impact on IBS QoL, stool frequency, stool character, degree of abdominal pain, GAD-7 score and gut microbiota compared with other viable agents. Wu et al. explored the effect of dietary fiber on colitis using a mice model of antibiotic-induced *Clostridioides difficile* infection, their findings showed that different diets exerted varying effects on intestinal epithelial permeability, pectin consistently increased the diversity of the microbiome, and reduced the level of inflammation in serum and colonic tissue. Furthermore, the activator FICZ and the inhibitor CH2223191 of the aromatic hydrocarbon receptor (AhR) were used to confirm that pectin worked through the AhR signaling pathway. Similarly, Dong et al. discovered that sodium butyrate (NaB) and fecal microbiota transplantation (FMT) reversed ulcerative colitis to induce prostate enlargement, and up-regulated the expression of GPER and butyric acid in the prostate by a mouse model of ulcerative colitis (UC), suggesting that butyric acid and FMT may be potential treatments for UC-induced prostate enlargement. In addition, Hu et al. and Guo

et al. made a comprehensive overview of the relationship between *Helicobacter pylori* and the development of gastric cancer (GC). In summary, *H. pylori* plays different roles in different stages of GC, and eradicating *H. pylori* and improving the diagnosis rate of early GC are effective ways to reduce the incidence of GC and improve the survival rate of GC.

For the brain diseases, Chen et al. predicted the poor outcome of acute ischemic stroke (AIS) with hyperlipidemia (POAH) patients and good outcome of AIS with hyperlipidemia (GOAH) patients by analyzing gut microbial characteristics. Their results indicated that there was a difference in the characteristics of the gut microbiota in POAH and GOAH, and that the relative abundance of *Enterococcaceae* and *Enterococcus* were enhanced, and *Lachnospiraceae*, *Faecalibacterium*, *Rothia* and *Butyricicoccus* were reduced. Besides, the receiver operating characteristic (ROC) model constructed based on gut microbiota can distinguish between POAH and GOAH, revealing that the microbial composition of POAH have a close correlation to clinical parameters, and the characteristics of gut microbiota can be used as important indicators for the diagnosis of POAH. Pan et al. studied the therapeutic effect and mechanism of probiotic *Pediococcus pentosaceus* on the mouse model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease (PD) by regulating the gut-brain axis. The results showed that *P. pentosaceus* could improve the movement disorder, dopaminergic neuronal degeneration, α-synuclein accumulation, significantly increase the levels of SOD1, Gpx1 and Nrf2 in the brain, reduce the level of Keap1, improve gut microbial dysbiosis, and increase GABA levels. These findings suggest that *P. pentosaceus* may be a promising candidate in the treatment of PD.

For other diseases, Qiao et al. explored the role of gut microbiota and fecal metabolites in the pathogenesis of disuse-induced osteoporosis (DIO), disclosing that gut microbiota and fecal metabolites may be potential factors leading to bone loss. Zhu et al. investigated the improvement of the extracts of the male zooid of *Antheraea pernyi* (EMZAP) on non-alcoholic fatty liver disease (NAFLD) induced by high-fat diet (HFD), indicating that EMZAP as a dietary supplement and functional food may improve HFD-induced NAFLD in the future. Tong et al. made a comprehensive overview of the effects of gut microbiota on the gout physiology and the effects of gout on gut microbiota, illustrating that prebiotics, probiotics, traditional Chinese medicine, and fecal transplantation may treat gout by changing the composition of the gut microbiota. Han et al. provided an overview of bacterial membrane vesicles in the pathophysiology of pneumonia and its complications. In general, targeting the key components of outer membrane vesicles that interact with human lung cells or macrophages may become a new strategy for the treatment of pneumonia in the future. Xie et al. comprehensively reviewed the role of *Lactobacillus* as a new therapeutic agent of atopic dermatitis (AD), suggesting that *Lactobacillus* may become a food supplement to improve AD. Furthermore, Pan et al. explored the regulation of tryptophan metabolism in lactic acid bacteria (LAB) by multi-omics methods, demonstrating that multi-omics technology will help to explore more LAB with tryptophan metabolic potential.

In conclusion, there are 13 papers in this Research Topic that provide a detailed overview of the potential roles of probiotics, postbiotics and microbial metabolites in the occurrence and development of various chronic diseases, which promote our understanding of the improvement of chronic diseases. In addition, we also hope that this Research Topic can help researchers put forward new strategies in the improvement of chronic diseases.

Author contributions

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

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Conflict of interest

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

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Clinical study on sequential treatment of severe diarrhea irritable bowel syndrome with precision probiotic strains transplantation capsules, fecal microbiota transplantation capsules and live combined bacillus subtilis and enterococcus faecium capsules

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Objective: To study the effect of precision probiotic strains transplantation capsules on diarrhea irritable bowel syndrome compared with fecal microbiota transplantation capsules and live combined bacillus subtilis and enterococcus faecium capsules.

Methods: Two patients with severe irritable bowel syndrome were treated with precision probiotic strains transplantation capsules, fecal microbiota transplantation capsules and live combined bacillus subtilis and enterococcus faecium capsules in sequence. IBS-SSS, IBS-QoL, GSRS, stool frequency, stool character, degree of abdominal pain, GAD-7, and PHQ9 scores of patients at 0, 2, 4, 6, 8, 10, and 12 weeks of treatment were monitored and recorded, and stool samples were collected for metagenomics and metabolomics.

Results: It was found that the IBS-SSS score of patient case 1 decreased by 175 points and that of patient case 2 decreased by 100 points after treatment of precision probiotic strains transplantation capsules. There was no significant decrease after fecal microbiota transplantation capsules and live combined bacillus subtilis and enterococcus faecium capsules were used. At the same time, compared with fecal microbiota transplantation and live combined bacillus subtilis and enterococcus faecium capsules, the IBS QoL, stool frequency, stool character, degree of abdominal pain and GAD-7 score of patient case 1 improved more significantly by the precision probiotic strains

transplantation capsules. And the stool frequency and stool character score of patient case 2 decreased more significantly. Intestinal microbiota also improved more significantly after the precise capsule transplantation treatment. And we found *Eubacterium_ Eligens* showed the same change trend in the treatment of two patients, which may play a role in the treatment.

Conclusion: precision probiotic strains transplantation capsules is more beneficial to improve the intestinal microbiota of patients than microbiota transplantation capsule and live combined bacillus subtilis and enterococcus faecium capsules, so as to better alleviate clinical symptoms. This study provides a more perfect and convenient therapeutic drugs for the treatment of IBS.

KEYWORDS

Irritable bowel syndrome, precision transplantation, fecal microbiota transplantation capsules, live combined bacillus subtilis and enterococcus faecium capsules, gut microbiota

Introduction

Irritable bowel syndrome (IBS) is a common functional gastrointestinal (GI) disorder characterized by recurrent abdominal pain associated with changes in stool frequency and form (Hellström et al., 2019). According to the predominant bowel habits, IBS is divided into diarrhea (IBS-D), constipation (IBS-C), mixed (IBS-M), and irregular (IBS-U).10%-15% of people worldwide are troubled by IBS (Defrees and Bailey, 2017). According to current research, the gut microbiota, gastrointestinal motility, visceral paresthesia, changes in intestinal permeability and brain-gut axis, and even infection or psychological stress may be related to the occurrence of IBS (Ford et al., 2017). The current management of IBS is include diet and lifestyle changes, probiotics, psychotherapy and medications (opioid receptor agonists, smooth muscle antispasmodics, bile acid chelators, antidepressants, 5-HT3 antagonists, etc.) (Cangemi and Lacy, 2019).

More and more studies suggest the influence of microbiota on IBS. The Rome Foundation Working Team acknowledged that the dysbiosis of microbiota is a possible contributor to IBS (Simrén et al., 2013). The ratio of *Firmicutes* and *Bacteroidetes* in the microbiota of IBS patients increased, and the ratios of *Clostridiales* and *Bifidobacterium* decreased (Rajilić-Stojanović et al., 2011). So treatments based on regulating the microbiota may be effective for IBS. Ruggiero et al. used a probiotic mixture of Bacillus subtilis and Streptococcus faecium to treat patients with IBS with celiac disease. Compared with placebo, the effective rate was significantly higher (15.3% vs. 3.8%; $P<0.04$) (Francavilla et al., 2019).

Although probiotics are effective in treatment, their effective rate is low. At present, an emerging treatment method fecal microbiota transplantation (FMT), is gradually applied in clinical practice. FMT is an established treatment for recurrent Clostridium difficile infection which cure rate reaches 80–90% (Quraishi et al., 2017). FMT was also be tried to use to treat IBS with an effective rate of 65% (Johnsen et al., 2018). By adjusting the FMT dose, the effective rate can be increased to 89.1% (El-Salhy et al., 2020).

Although the above studies have suggested the possible therapeutic effect of FMT on IBS, the current understanding of fecal bacteria used for FMT treatment can hardly provide precise quantitative data or quantitative indicators of colonization for this microbial population, and different donors are quite different. It is difficult for fecal bacteria to meet the standards in terms of preparation, standard type and quality control. Therefore, there is an urgent need to accurately supplement the gastrointestinal microbiota in response to the changes in the stool of IBS patients. Selective microbiota transplantation (SMT), that is, by supplementing IBS-friendly bacteria and quantifying them to patients, precise treatment of IBS can be achieved.

In this article, we describe two cases of IBS patients, and report the comparison of their efficacy after using our self-developed precision probiotic strains transplantation capsules, fecal microbiota transplant capsules, and live combined bacillus subtilis and enterococcus faecium capsules. We also described the changes in intestinal microbes and inflammation indicators before and after treatment.

Material and methods

Precision strains transplantation capsules

Based on most studies, the difference in the microbiota between IBS patients and normal people is mainly manifested in the decrease in the number of *Lactobacillus* and *Bifidobacterium*. We plan to supplement the microbiota of *Lactobacillus* and *Bifidobacterium*. Screen the *Bifidobacterium* and *Lactobacillus* species in the "The list of strains that can be used for food in China". Strains that are mainly used for diarrhea or have immunomodulatory effects were selected, and finally 9 kinds of probiotics were included: *Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus plantarum, Lactobacillus reuteri and Lactobacillus rhamnosus*. Mix 9 kinds of strains powder to make precision strains transplantation capsules (IBS-diarrhea type). The main components of the precision strains transplantation capsules are: each capsule contains 45mg of each strain, each capsule contains 400mg of 9 strains, and the capsule shell is enteric-coated capsules. The study was conducted from November 2020 to March 2021 at Daping Hospital, Chongqing, China, and was approved by the Ethics Committee of Daping Hospital Affiliated Army Medical University (Third Military Medical University. All study participants provided written informed consent.

Fecal microbiota transplantation donor

According to the FMT donor guidelines, a donor was recruited and screened. By exploring his medical history and living habits, any possibility of contact with infectious pathogens or dangerous social or sexual behaviors (such as drug abuse) can be ruled out. He also underwent physical examinations and blood tests to rule out gastrointestinal, metabolic or neurological diseases (complete blood count, blood sugar, electrolytes and inflammation markers). Serological screenings for HIV, syphilis and hepatitis A, B and C were also carried out. The results of all these tests and inspections are negative. The donor was a 22-year-old Chinese soldier. He was non-smoker, healthy, did not take any drugs, and had a body mass index of 21 kg/m^2. He has not relationship with any of the patients in the trial. He trains five times a week for 1 hour each time. Regular diet and work and rest. The donor has donated his stool within 12 months, and his stool samples are tested every 3 months. The sample maintains a normal biomass, with only minor changes in the composition of bacteria.

Live combined bacillus subtilis and enterococcus faecium capsules

Live combined bacillus subtilis and enterococcus faecium capsules are commonly used intestinal probiotics, containing bacillus subtilis R-179 (5.0×10^7 cfu) and enterococcus faecium R-026 ($4.5\times10^{\wedge 8}$ cfu), which can regulate the human intestinal tract The environment promotes the growth and reproduction of normal Gastrointestinal microbiota and inhibits the growth of intestinal pathogenic bacteria, thereby effectively protecting the intestinal tract.

Therapeutic schedule

Two patients were given sequential treatment with three drugs. First, the precision probiotic strains transplantation capsules (diarrhea type) was given orally, once a day, 4 capsules each time, after 2 weeks of use, the drug was stopped for 2 weeks to elute the effect of the drug. Then in the fourth week, he was treated with fecal microbiota transplantation capsules once, and after two weeks of observation, he was given another two-week drug washout period. In the 8th week, the treatment with live combined bacillus subtilis and enterococcus faecium capsules was started, 3 times a day, 2 capsules each time, and after the same use for 2 weeks, the drug was stopped for 2 weeks. Monitor the changes in clinical symptoms of IBS before use, 2 weeks after use, and 2 weeks after drug withdrawal, and record the number of stools and changes in stool characteristics. Used IBS-SSS, GSRS, IBS-QoL, GAD7, PHQ9, SAS, and SDS scales monitor the changes in bowel symptoms before and after treatment, changes in life treatment, and changes in anxiety and depression. In addition, stool specimens were collected for microbiota analysis, and changes in inflammatory factors were monitored at the same time. Safety indicators (including liver function, kidney function, electrolytes, blood routine, etc.) are also monitored during drug use, and adverse reactions are recorded at any time.

Clinical response criteria

The main research index is the severity of the patient's disease, which is evaluated using the IBS-SSS score (Francis et al., 1997). Secondary indicators include the patient's stool frequency, stool characteristics, and abdominal pain score. The Bristol Stool Trait Scale and Facial Expression Scale were used to evaluate fecal traits and abdominal pain. In addition, we used the IBS-QoL scale to evaluate their quality of life, and used the GAD-7 and PHQ-9 scales to evaluate the changes in their anxiety and depression status.

The patient's IBS-SSS score decreased by at least 50 points or the severity decreased by one level, indicating clinical remission. The times of stools more than 4 drops to 4 or less, the abdominal pain score is dropped by 30%, GSRS score is dropped by 30%, and the stool characteristics are changed to types 3, 4, and 5 for patients which with stool types 6 and 7 were consider effective. A 30% drop in IBS-QoL score indicates an improvement in quality of life. The improvement of anxiety and depression is manifested in the degree of anxiety and depression drops at least one level.

Metagenomic and metabolomic analysis

The fecal samples of patients were analyzed by metagenomics and metabolomics. Select small fragment library on SL5 platform for sequencing (Nuohe Zhiyuan biology company, China). Basic quality control, species and function notes: use fastp (version 0.20.0) to control the original data, and the parameter is cut_ tail -W4 -M20 -n5 -c -l50 -w6. Bmtagger (version 1.1.0) is used to remove the host from the data after quality control, and the parameter is default. The human genome (hg19) metaphlan (version 3.0) was used for species annotation. Humann (V3.0.0. Alpha. 4) for function annotation. The metabolic data acquisition instrument system mainly includes ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS). Based on the self-built targeted target database MWDB (metware database), the information and secondary spectrum data are analyzed qualitatively according to the retention time RT (retention time) and parent and daughter ions of the detected substances.

Statistical analysis

R version 4.0.2 was used for correlation analysis and mapping. Alpha diversity calculation: package vegan version 2.5-7, based on the species level abundance table. Bray Curtis distance calculation: package vegan version 2.5-7, based on the species level abundance table. PCoA analysis: package ape version 5.5. Heat map drawing: package pheatmap version 1.0.12. Other drawings: package ggplot2 version 3.3.5. Spearman statistical method was used to calculate the correlation between metabolites and total abundance of different flora. $P < 0.05$, suggesting that the difference was statistically significant.

Results

Case presentation

Case 1: A 31-years-old male patient. The patient had abdominal pain and diarrhea starting from 20 months before admission without obvious triggers. The abdominal pain was a dull pain around the umbilical cord, which could be relieved after defecation. The diarrhea continued to occur, up to 10+ times/day. It was loose or watery stools, occasionally adheres mucus or foam, with no pus or blood, no tenesmus, no fever, nausea, or vomiting, etc. Gastrointestinal endoscopy and abdominal CT showed no obvious abnormalities. He has been treated with probiotics, pinaverium bromide and other drugs, with poor efficacy. Only three sequential drugs were used during this study.

Case 2: A 20-years-old male patient. Patients have recurrent diarrhea for 1+ years, mostly after a cold, spicy diet, with intermittent attacks, about 4-7 times/day, and 9 times/day at most; stools are mostly mushy stools with mucus; with abdominal pain and bloating, and they are slightly relieved after defecation. Gastrointestinal endoscopy and abdominal CT showed no obvious abnormalities. Probiotics, pinaverium bromide and other drugs have also been used. Only three sequential drugs were used during this study.

Main indicators

The severity of the disease of the two patients was significantly improved after using the precision probiotic strains transplantation capsules, which was not obvious after standard fecal microbiota transplantation and live combined bacillus subtilis and enterococcus faecium capsules. As shown in Table S1, the IBS-SSS score of case 1 patient dropped by 175 points, achieving remission after the use of precision probiotic strains transplantation capsules; but there was no remission after standard fecal microbiota transplantation and live combined bacillus subtilis and enterococcus faecium capsules. The case 2 patient also achieved remission (IBS-SSS score dropped by 100 points) after treated with precision probiotic strains transplantation capsules. Even more, after stopping the drug for 2 weeks, there was still a score reduction of 60 points. In comparison, there were only 10 points reduction after standard fecal microbiota transplantation and live combined bacillus subtilis and enterococcus faecium capsules. The IBS-SSS score trend of the two patients before and after the 3 treatments is shown in Figure 1A and Figure 2A.

Secondary indicators

As shown in Table S2, the frequency of stools in the two patients was reduced to 4 times/day after treatment with precision probiotic strains transplantation capsules. Fecal microbiota transplantation is still effective for the case 2 patient, that it can maintain the stool frequency at 4 times/day. But it was increase to 5 times/day after the case 1 patient used fecal microbiota transplantation. Both of them got not

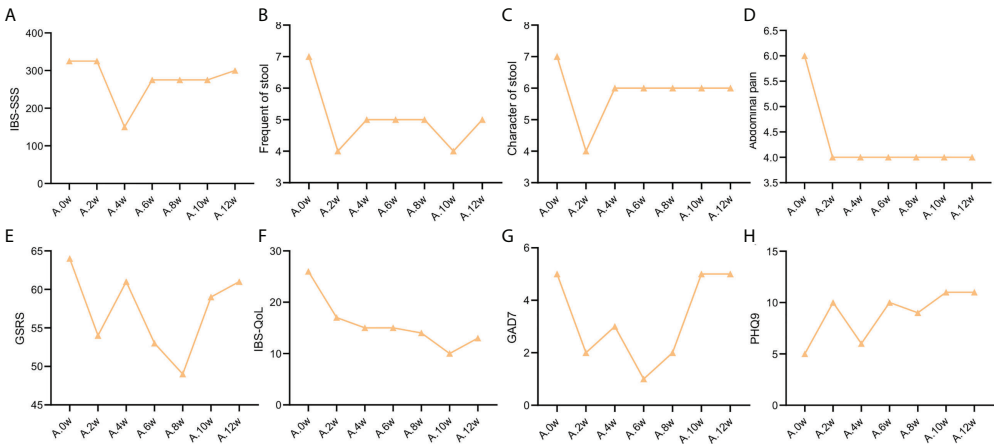

FIGURE 1
Change trend of IBS clinical indicators of patient case 1. **(A)**. IBS-SSS score. **(B)**. stool frequency. **(C)**. Character of stool. **(D)**. Abdominal pain score. **(E)**. GSRS. **(F)**. IBS-QoL. **(G)**. GAD7. **(H)**. PHQ9.

remission after live combined bacillus subtilis and enterococcus faecium capsules treatment. The change trend of stool frequency is shown in Figure 1B and Figure 2B.

For the case 1 patient, although the frequency of defecation decreased significantly, but after the use of the three drugs, the stool properties were not significantly improved, only from watery stool to mushy stool (Table S2, Figure 1C). In contrast, the times of stools of the case 2 patient decreased, and the stool character also improved significantly. After the use of precision probiotic strains transplantation capsules, the patient changed from pasty stool to visible broken edge massive stool (Table S2, Figure 2C).

Both of the two patients had obvious abdominal pain before treatment. Both of them got 6 of the Facial Expression Scale. It dropped to 4 after the use of the precision transplant capsule in the case 1 patient (Table S2, Figure 1D). But there was no significant change after the use of the latter two drugs. The case 2 patient had no relief of abdominal pain after three treatments (Table S2, Figure 2D).

GSRS scores of both patients showed no significant improvement in gastrointestinal symptoms (Figure Figures 1E, 2E)

Irritable bowel syndrome also has a significant impact on the quality of life of patients. Table S3 shows that for the case 1

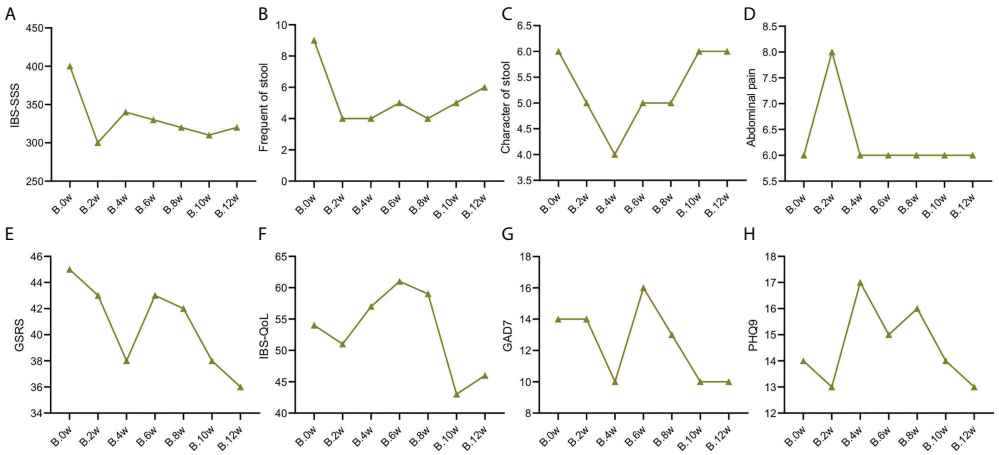

FIGURE 2
Change trend of IBS clinical indicators of patient case 2. **(A)**. IBS-SSS score. **(B)**. stool frequency. **(C)**. Character of stool. **(D)**. Abdominal pain score. **(E)**. GSRS. **(F)**. IBS-QoL. **(G)**. GAD7. **(H)**. PHQ9.

patient, the IBS QoL score decreased after the use of precision probiotic strains transplantation capsules, suggesting that the quality of life improved significantly, 34.6% after 2 weeks and 42.3% after 4 weeks. However, there was no significant improvement after the other two treatments. The case 2 patient had no improvement in quality of life after three treatments. The change trend of stool frequency is shown in Figure 1F and Figure 2F.

The standard for evaluation of anxiety and depression of GAD-7 and PHQ-9 was 0-4 normal, 5-9 mild, 10-14 moderate, 15-27 severe. Case 1 patient suffered a mild anxiety and depression. The anxiety was obviously relieved after the use of precision probiotic strains transplantation capsules, that the other two treatments were ineffective (Table S4, Figures 1G, H). The case 2 patient suffered a moderate anxiety and depression. The precision probiotic strains transplantation capsules and standard fecal microbiota transplantation treatment had no effect on his anxiety and depression, but live combined bacillus subtilis and enterococcus faecium capsules improved the patient's depression (Table S4, Figures 2G, H).

Gut microbiota

In order to evaluate the changes of gut bacterial community after different treatment. We collected stool samples from patients at each follow-up time point for macrogenomic sequencing analysis.

The composition of gastrointestinal microbiota was different before and after treatment. At the phylum level, we can observe that the gastrointestinal microbiota of the two patients was mainly composed of *Firmicutes*, *Bacteroides*, *actinomycetes* and *Proteus*. After treatment, it was found that the abundance of *Bacteroidetes* in patient case 1 decreased after the use of precision probiotic strains transplantation capsules, but there was no such change after FMT and live combined bacillus subtilis and enterococcus faecium capsules treatment (Figure S1A). In patient case 2, it was observed that the abundance of *Firmicutes* decreased after the use of precision probiotic strains transplantation capsules, but increased after the other two treatments (Figure S1C). The picture shows the changes of major microbiota at the genus level of gastrointestinal microbiota in two patients. Before treatment, patient case 1 was mainly composed of *Blautia* and *Bacteroides*. *Blautia* decreased after treatment with live combined bacillus subtilis and enterococcus faecium capsules, while *Bacteroides* decreased after treatment with precision probiotic strains transplantation capsules (Figure S1B). However, in patient case 2, *Blautia* decreased after precision capsule transplantation (Figure S1D). At the level of species, we listed the main strains contained in the intestine of patient a and found that *Blautia_wexlerae* was the most abundant strain. It decreased after treatment with precision probiotic strains transplantation capsules, but this change was not found after the other two treatments (Figure 3A). In patient B, the abundance of *Faecalibaterium prausnitzii* was higher, but decreased at four weeks, eight weeks and twelve weeks, that is, after the withdrawal of each treatment (Figure 4A).

In patient case 1, the diversity (Shannon index) of gastrointestinal microbiota increased most significantly after precision probiotic strains transplantation capsule, and decreased after drug withdrawal (Figure 3B). The diversity of gastrointestinal microbiota also increased after the treatment of patient case 2 precision probiotic strains transplantation capsules (Figure 4B). Principal coordinate analysis (PCoA) was used to investigate the similarity of microbial communities at different time points. No obvious time point aggregation was found, but there was obvious heterogeneity before and after treatment (Figures 3C, 4C).

We analyzed the changes of gastrointestinal microbiota species before and after each treatment to further evaluate the colonies that may play a role in the treatment. The Figure S2 and Figure S3 show the differences of strains at the level of phylum, and the top 20 microbiota with the most obvious differences at the level of genus and species. In patient case 1, 12 strains with the same change trend before and after treatment were screened (Figure 3D). *Fusicatenibacter_ saccharivorans Eubacterium_ eligens Actinomyces_ sp_ Hmsc035g02* were up-regulated after treatment, while *Lostridium_ sp_ CAG_ 58, Clostridium_ symbiosum, Blautia_ hansenii, Coprococcus_ comes, Eubacterium_ ramulus, Erysipelatoclostridium_ ramosum, Blautia_ wexlerae, Streptococcus_ salivarius, Actinomyces_ sp_ Icm47* were down regulated after treatment. The correlation between related strains and bacterial metabolites was analyzed. Four strains *Eubacterium-eligens, Blautia-hanserii, Clostridium-symbiosum* and *Erysipelatoclostridium* were found to be negatively correlated with metabolites (Figure 3E). In patient case 2, 20 strains with the same change trend before and after treatment were selected (Figure 4D). *Eubacterium_ hallii, Agathobaculum_ butyriciproducens, Arabacteroides_ merdae, Bacteroides_ stercoris, Roseburia_ inulinivorans, Dorea_ longicatena, Romboutsia_ ilealis, Oscillibacter_ sp_ 57_ 20, Adlercreutzia_ equolifaciens, Lactobacillus_ rogosae, Eubacterium_ eligens, Roseburia* were up-regulated after treatment, while *Megamonas_ hypermegale, Megamonas_ funiformis, Streptococcus_ mitis, Streptococcus_ parasanguinis, Gemella_ sanguinis, Megamonas_ rupellensis, Collinsella_ aerofaciens, Dialister_ Pneumosintes* were down regulated after treatment. The picture shows their correlation with metabolites. Among them, 6 strains are related to metabolites in pos mode (Figure 4E). *Streptoccus_ Parasanguinis* and *dialist_ Pneumosintes* decreased after treatment and were negatively correlated with 13 (S)-HOTrE. *Parabacteroides _ merdae, Bacteroides_ stercoris, Eubacterium_ hallii, Adlercreutzia_ Equolifaciens* were positively correlated with the corresponding

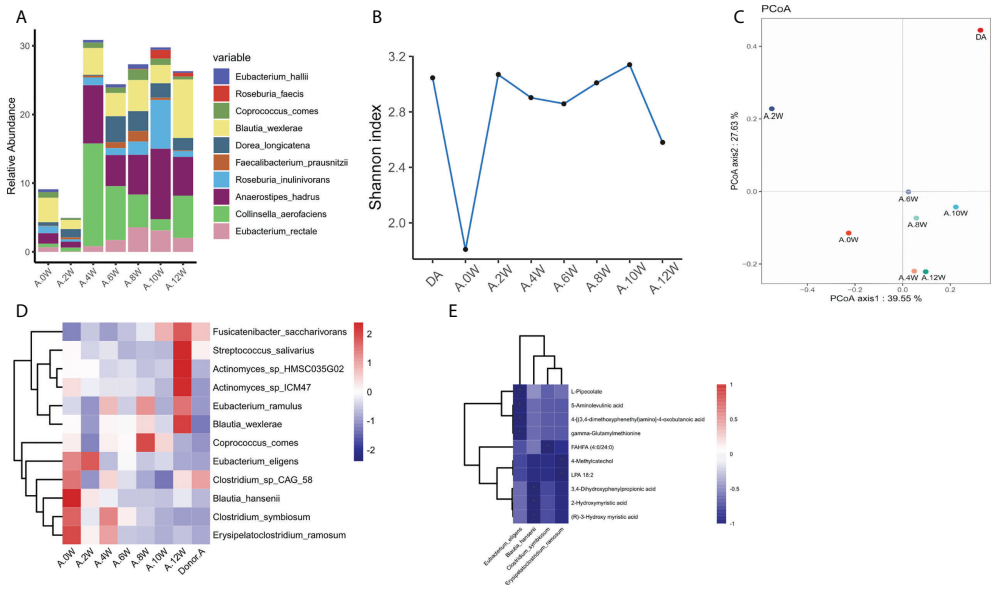

FIGURE 3
Characteristics of gastrointestinal microbiota in patient case 1. **(A)**. Composition of the top 10 species at the species level. **(B)**. The diversity (Shannon index) of gastrointestinal microbiota. **(C)**. Principal coordinate analysis (PCoA) was used to investigate the similarity of microbial communities at different time points. **(D)**. Strains with the same change trend before and after treatment with three treatments. **(E)**. Correlation between strains with the same change trend and metabolites in neg mode. *p < 0.05.

metabolites. 12 strains are related to metabolites in neg mode (Figure 4F).

Interestingly, it is found that the same change trend of *Eubacterium_eligens* before and after different drug treatments in the two patients. Figure 5 shows the change at each time point.

Side reaction

The two patients had no obvious side effects during the study.

Discussion

IBS is a chronic intestinal functional disease (Mearin et al., 2016; Holtmann et al., 2016). The incidence rate of the world is high, and its impact on quality of life is comparable to other chronic diseases, such as diabetes and liver diseases. The economic burden is also very heavy (Everhart and Ruhl, 2009). IBS is characterized by abdominal pain, abdominal distension, abdominal discomfort and irregular stool. The two patients we observed were mainly IBS-D patients with abdominal pain and diarrhea. According to their IBS-SSS score, they were severe IBS-D patients. According to the brief GAD-7 and PHQ-9 scores, patient case 1 was complicated with mild anxiety and depression, while patient case 2 was complicated with moderate anxiety and depression. Although the pathophysiology of irritable bowel syndrome has not been fully clarified, researchers increasingly believe that the imbalance of gastrointestinal microbiota is related to it.

Gastrointestinal microbiota is a diverse and numerous ecosystem, which is distributed in the whole gastrointestinal tract and has an impact on all systems of our body. However, due to its huge complexity and high variability among individuals, the role of gastrointestinal microbiota in human physiology has not been fully understood. In this experiment, the use of three drugs was designed to regulate the gastrointestinal microbiota, observe their curative effects, and detect the changes of microbiota before and after use to further clarify the role of microbiota.

Gut microbiota refers to bacteria, viruses, parasites (Sender et al., 2016). The adult gut microbiota is composed of more than 2000 kinds of bacteria, with increasing density and diversity from the stomach to the colon. Healthy gastrointestinal microbiota is a microbial ecosystem with diversity, stability, resistance and adaptability (Lozupone et al., 2012). Gastrointestinal microbiota in abiotic state is increasingly involved in the pathogenesis and development of many diseases. Animal model experiments show that transplanting the gastrointestinal microbiota of IBS patients into sterile animals can cause visceral hypersensitivity, damage intestinal permeability and change gastrointestinal transport time, indicating the importance and possible etiological role of

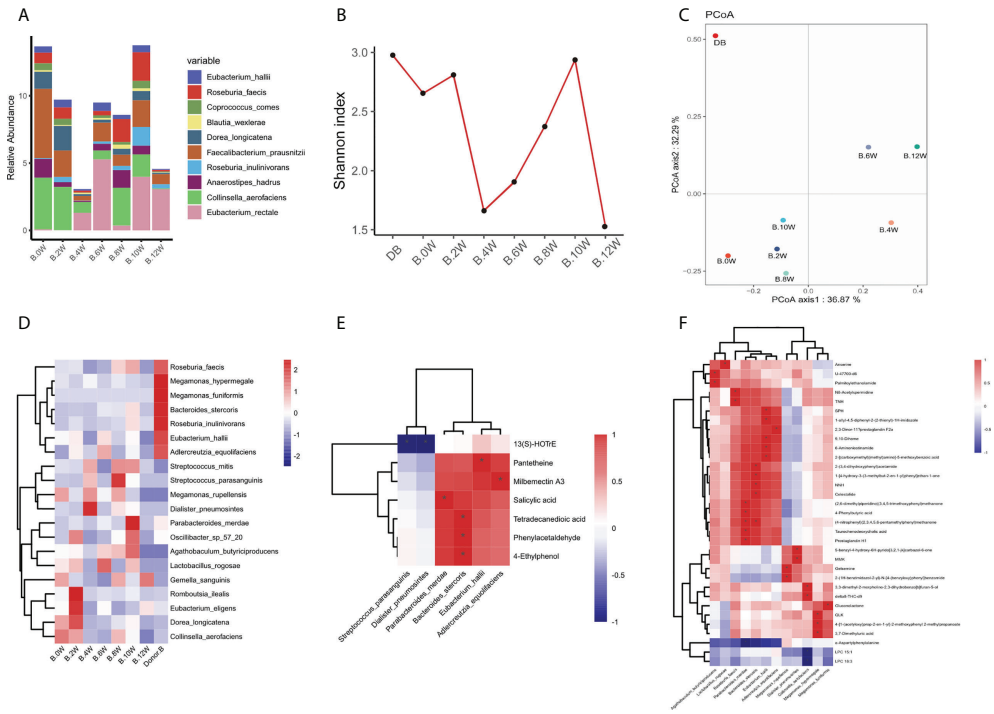

FIGURE 4
Characteristics of gastrointestinal microbiota in patient case 2. **(A)**. Composition of the top 10 species at the species level. **(B)**. The diversity (Shannon index) of gastrointestinal microbiota. **(C)**. Principal coordinate analysis (PCoA) was used to investigate the similarity of microbial communities at different time points. **(D)**. Strains with the same change trend before and after treatment with three treatments. **(E)**. Correlation between strains with the same change trend and metabolites in pos mode. **(F)**. Correlation between strains with the same change trend and metabolites in neg mode. *p < 0.05.

gastrointestinal microbiota in IBS (Crouzet et al., 2013). The microbiota characteristics of IBS patients are as follows: *Clostridium, Bacteroides, Bifidobacterium* and *Faecalibacterium* are significantly reduced in IBS (Rajilić-Stojanović et al., 2011; Jalanka-Tuovinen et al., 2014), while the bacteria associated with *Ruminococcus torques* (a species of *Lachnospiraceae*) are abundant in IBS patients (Scully et al., 2010; Rajilić-Stojanović et al., 2011; Saulnier et al., 2011), and the level is positively correlated with intestinal symptoms (Malinen et al., 2010; Rajilić-Stojanović et al., 2011; Saulnier et al., 2011). In

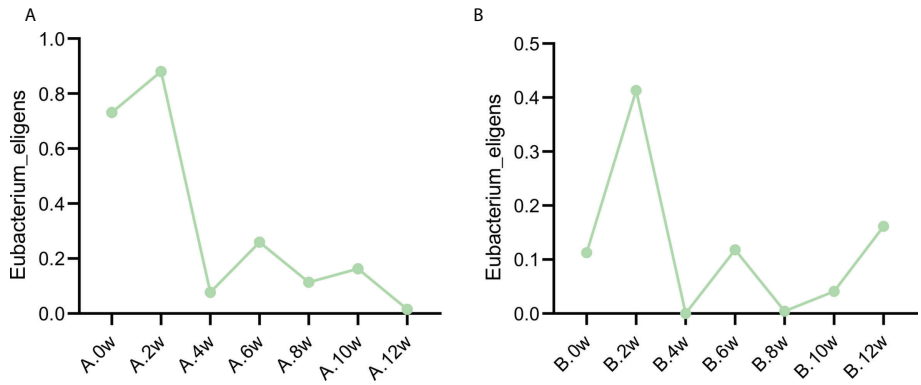

FIGURE 5
Variation trend of Eubacterium eligens before and after different treatment. **(A)** Case 1, **(B)** Case 2.

addition, the study found that an increase in the ratio of *Firmicutes* to *Bacteroidetes* has been observed at the phylum level (Rajilić-Stojanović et al., 2011). Therefore, the Rome Foundation Working Team (Simrén et al., 2013) also believes that microbiota imbalance is a reasonable pathogenic factor of IBS.

The use of probiotics can improve the symptoms of IBS patients. Probiotic products containing lactic acid bacteria can significantly reduce specific symptoms (i.e. abdominal pain and flatulence) and improve the quality of life of patients (Asha and Khalil, 2020). *Lactobacillus* is reported to be beneficial to abdominal pain in functional gastrointestinal diseases (Horvath et al., 2011; Jadrešin et al., 2017). Probiotics containing *Lactobacillus* or *Bifidobacterium* were found to increase stool frequency and reduce intestinal transit time (Miller et al., 2017).

FMT is the transfer of fecal microbial content from healthy donors to individuals with gastrointestinal diseases. The mechanism of action is not completely clear, but the recovery of disordered microbiota seems to be the basis of the observed effect (Quraishi et al., 2017). FMT is mainly used to treat patients with recurrent *Clostridium difficile* infection (rCDI) (Debast et al., 2014; McDonald et al., 2018), and the cure rate is 80 - 90% (Quraishi et al., 2017). FMT can be taken orally through duodenal tube or capsule through the upper digestive tract (Kao et al., 2017) (van Nood et al., 2013). A study has shown that patients treated with oral capsule FMT have a high cure rate, and this method may reduce patients' discomfort (Kao et al., 2017).Our study also used oral fecal bacteria capsule to treat patients with FMT. Johnsen et al. Published the first RCT to study the effect of FMT in patients with IBS (Johnsen et al., 2018). The authors found an overall improvement in symptoms in 58% of patients treated with FMT. A meta-analysis of five clinical studies found that fresh or frozen donor feces delivered through colonoscopy or naso jejunal bowel may be beneficial to IBS (Ianiro et al., 2019). Although FMT seems safe and easy to implement, it should be used with caution because its long-term effects are unclear or unidentifiable. FMT is not a standardized treatment, and the treatment methods are different in different places. The fecal bacteria used for transplantation are difficult to meet the standards in preparation, standard type and quality control. Using all the fecal bacteria of donors cannot provide accurate quantitative data or quantitative indicators of colonization, nor can it avoid harmful microorganisms, and the samples of different donors are quite different. That makes the application of FMT have some limitations.

We hope to design a selective microbiota transplantation capsule for IBS-D, so we designed accurate transplantation capsules according to the microbiota characteristics of IBS-D. We mainly screened *Bifidobacteria* and *Lactobacillus* which decreased in patients with IBS. Nine probiotics were screened, including: *Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus*. Fuqiang yuan et al. Included five randomized controlled trials of treating IBS with probiotics containing *Bifidobacterium infantis* for meta-analysis. The results showed that compound probiotics containing *Bifidobacterium infantis* could effectively treat IBS without obvious adverse reactions (Yuan et al., 2017). *Lactobacillus helveticus* and *Lactobacillus rhamnosus* supplements can significantly reduce the duration of antibiotic related diarrhea like defecation (Ren et al., 2018). *Bifidobacterium longum* can significantly improve the depression and quality of life of patients with IBS-D (Pinto-Sanchez et al., 2017). *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus (*Preston et al., 2018) and *Lactobacillus plantarum (*Ducrotté et al., 2012) can improve intestinal symptoms and quality of life in patients with IBS. There is clear evidence that *Bifidobacterium breve* has strong immunomodulatory properties. The research of Jianjun Ren et al. Shows that *Bifidobacterium breve* mainly restores the Th1/Th2 balance by inhibiting Th2 response (Ren et al., 2018). *Bifidobacterium infantis* can reduce the ability of systemic pro-inflammatory biomarkers and make INF-α decreased in chronic fatigue syndrome and psoriasis, and IL-6 decreased in ulcerative colitis and chronic fatigue syndrome (Groeger et al., 2013). It is reported that *Lactobacillus reuteri* can prevent oxidative stress and inhibit the increase of intestinal oxidation products (Amaretti et al., 2013). *Lactobacillus helveticus* can control the intestinal microbiota and enhance the infant's immune system (Xiao et al., 2017), and inhibit immune cell proliferation and proinflammatory cytokines (IL-6 and IL-1β) (Yamashita et al., 2014). *Bifidobacterium longum* can regulate the proliferation of mouse immune B cells and inhibit Th1 (IL-2, IFN- γ)/Th2 (IL-4, IL-10) cytokine imbalance and immune cytokine production (Choi et al., 2019). In conclusion, the probiotics we screened mainly have the role of treating diarrhea and regulating immunity. Through practice, it is found that the therapeutic effect is gratifying.

Based on the severity of IBS-SSS, it can be seen that both the two patients belong to patients with severe IBS. The IBS-SSS score decreased by 50 points was defined as clinical response (El-Salhy et al., 2020). Both the two patients had clinical response after the use of precision probiotic strains transplantation capsules, and the severity of their condition decreased. However, no obvious clinical reactions were found after the use of fecal microbiota transplantation capsule and live combined bacillus subtilis and enterococcus faecium capsules. At the same time, the patient's stool characteristics, stool frequency and abdominal pain were also significantly improved after the use of precision probiotic strains transplantation capsules. Fecal microbiota transplantation capsule and live combined bacillus subtilis and enterococcus

faecium capsules also improved the stool characteristics of patients. The improvement of quality of life was assessed by IBS-QoL scale. It was found that only patient case one's quality of life improved after using precision probiotic strains transplantation capsules. In terms of the impact on patients' anxiety and depression, we found that precise transplantation of capsule improved the anxiety and depression of the first patient, but for the second patient, live combined bacillus subtilis and enterococcus faecium capsules showed the effect of improving emotional disorders. No obvious effect was found in the use of fecal microbiota transplantation capsule. In general, precision capsule can improve the severity of IBS-D patients, reduce the number of stools, improve the characteristics of stools, relieve abdominal pain, improve the quality of life of patients, and improve the anxiety and depression of patients. Fecal microbiota transplantation capsule can improve the characteristics of stool, and live combined bacillus subtilis and enterococcus faecium capsules can play a certain role in improving anxiety and depression. This suggests that the effectiveness of precision probiotic strains transplantation capsules for IBS-D patients is better than fecal microbiota transplantation capsule and live combined bacillus subtilis and enterococcus faecium capsules. It has the advantages of more convenient production, controllable safety and more advantages in future application.

Based on the mechanism of regulating intestinal microbiota, we chose these three drugs for treatment, so we monitored the changes of intestinal microbiota and metabolites during treatment through macrogenomic analysis. Through the analysis of microbiota, it was found that there were great differences in intestinal microbiota after the three treatments. It has been found that the proportion of *Firmicutes* and *Bacteroidetes* increased significantly in the intestinal microbiota of IBS patients (Rajilić-Stojanović et al., 2011). In our trial, it was found that the *Bacteroidetes* level of patient case 1 decreased after the use of precision probiotic strains transplantation capsules, and the *Firmicutes* also decreased in the 4th week after treatment. This change was not found after treatment with the other two drugs. This suggests that the treatment of precision probiotic strains transplantation capsules seems to be changing the disordered microbiota structure of patients. In patient case 2, it was also found that the *Firmicutes* decreased significantly after the use of precision probiotic strains transplantation capsules. *Blautia* was found to be more abundant in the fecal microbiota of patients with irritable bowel syndrome and ulcerative colitis than in healthy individuals (Rajilić-Stojanović et al., 2011; Nishino et al., 2018). The intestinal microbiota of our two patients was dominated by *Blautia* at the genus level. *Blautia* decreased in patient case 1 after treatment with live combined bacillus subtilis and enterococcus faecium capsules, and decreased in patient case 2 after treatment with precision probiotic strains transplantation capsules. This suggests that precise transplantation of capsules promotes the reduction of adverse microbiota in patients. This change is also reflected in the level of species. In patient case 1, *Blautia_wexlerae* decreased after the use of precision probiotic strains transplantation capsules. After precise capsule transplantation, the microbiota structure of patients changed significantly, but the richness and diversity of microbiota increased.

Different treatments have different effects. In order to explore the functional microbiota, we analyzed the characteristics of different microbiota before and after treatment. Some strains have the same change trend before and after the use of the three treatment methods. Among patients case 1, 3 strains were up-regulated after treatment, while 9 strains were down regulated after treatment. Among patients case 2, 12 strains were up-regulated after treatment, while 8 strains were down regulated after treatment.

Further evaluating the relationship between intestinal microbiota and its metabolites, we found that some strains had a certain correlation with metabolites. In patient case 1, we found that *Eubacterium_ eligens, Blautia_ hansenii, Clostridium_ symbiosum, Erysipelatoclostridium_ Ramosum* was negatively correlated with metabolites. *Clostridium_ Symbiosum* decreased significantly after treatment, and its negatively related FAHFA (Fatty acid esters of hydroxy fatty acids) has anti-inflammatory effect and can inhibit the production of LPS induced pro-inflammatory cytokines in macrophages and dendritic cells (Yore et al., 2014). *Erysipelatoclostridium_ Ramosum* that reduced after treatment was negatively correlated with LAP (plasma lysophosphatidic acid) and 4_ Methylcatechol, both of which could promote tumor cell apoptosis (Zheng et al., 2004; Karatug Kacar et al., 2018). In patient case 2, *Streptoccus_ Parasanguinis* and *dialist_ Pneumosintes* decreased after treatment and were negatively correlated with 13(S)-HOTrE which can produce anti-inflammatory effect by inactivating NLRP3 inflammatory body complex (Kumar et al., 2016). Several strains increased after treatment, *Parabacteroides _ merdae, Bacteroides_ stercoris, Eubacterium_ hallii, Adlercreutzia_ Equolifaciens* were positively correlated with the corresponding metabolites. Milbemectin A3 has antifungal activity (Silva et al., 2013). Salicylic acid mainly destroys eicosanoic acid metabolism, thus changing the levels of prostaglandins and leukotrienes (Mitchell et al., 1993). Phenylacetaldehyde can enhance the postsynaptic effect of dopamine and act as a neuromodulator of catecholamine neurotransmission in the brain (Paterson et al., 1990). Many different strains showed significant positive correlation with metabolites, such as *parabacteroides_Merdae* increased after treatment, which was positively correlated with 4 −phenylbutyric acid and taurochenodeoxycholic acid. 4 −phenylbutyric acid can improve lipotoxicity and stimulate fatty acids β (He and Moreau, 2019). Taurochenodeoxycholic

acid can inhibit proinflammatory cytokines (IL-1 β、 IL-6 and TNF- α) to inhibit inflammation (Liu et al., 2011). The change of different strains leads to the production of different metabolites, which affects the intestinal function.

In the analysis, we found that the change trend of *Eubacterium_eligens* was the same in the two patients, and increased after the treatment of three drugs. It shows that it may play an important role in the improvement of symptoms and microbiota after treatment. *Eubacterium_Eligens* were negatively correlated with 5 – aminolevulinic acid, which induced the accumulation of protoporphyrin IX in malignant tissues (Fratz et al., 2014). It has been found that *Eubacterium_eligens* is a professional pectin degrading agent, which may provide anti-inflammatory activity by promoting the production of IL-10 by epithelial cells (Chung et al., 2017). Therefore, after treatment, the increase of *Eubacterium_eligens* improves the anti-inflammatory activity of microbiota. Whether Eubacterium plays a certain role in IBS needs further experiments.

Since it is not easy to include patients in sequential therapy, only 2 patients were included in this study for observation. Fen Zhang et al. Also found the changes of intestinal flora after FMT treatment during the treatment of only one patient, providing a new idea for the diagnosis and treatment of severe colitis associated with graft-versus-host disease in the future (Zhang et al., 2021). Our research shows that, compared with fecal microbiota transplantation capsule and live combined bacillus subtilis and enterococcus faecium capsules, precision probiotic strains transplantation capsules can significantly improve the severity of IBS-D patients, reduce the number of stools, change the nature of stools and reduce abdominal pain. Moreover, precision probiotic strains transplantation capsules can improve the quality of life of patients, and may also have a certain effect on patients with anxiety and depression. On the other hand, it was found that precision probiotic strains transplantation capsules had more beneficial improvement on the intestinal micriobiota of patients than microbiota transplantation capsule and live combined bacillus subtilis and enterococcus faecium capsules, resulting in better clinical symptom relief. Compared with live combined bacillus subtilis and enterococcus faecium capsules, precision probiotic strains transplantation capsules has more abundant microbiota. Compared with fecal microbiota transplantation capsules, it is more convenient to prepare and can accurately quantify the dose. This study provides a more perfect and convenient treatment scheme for the treatment of IBS-D. However, there are still deficiencies in this study. The interval between discontinuation of the three drugs is short, and the delayed effect of the drugs has an impact on the treatment. The interval needs to be extended in future experiments. And a larger sample size is needed in the future to further verify the effect of precision probiotic strains transplantation capsules.

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 human participants were reviewed and approved by the Ethics Committee at the Army Medical University (Third Military Medical University) affiliated with Daping Hospital. In addition, the study was registered at the Chinese Clinical Trial Registry (www.chictr.org.cn); the trial registration number was ChiCTR2100043160, through which the trial protocol can be accessed. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: YW, DC, LL, and GR. Formal analysis: LL, GR, and XZ. Data curation: YT, ZX, LL, and GR. Writing—original draft preparation: LL and GR. Writing—review and editing: YW, DC, LL, GR, and YP. Supervision: YW and DC. Validation: YP, YC, YT, and ZX. Funding: YW. All authors contributed to the article and approved the submitted version.

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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/fcimb.2022.1025889/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Composition of the top 10 species at the phylum and genus level. **(A)**. Phylum level in patient case 1. **(B)**. Genus level in patient case 1. **(C)**. Phylum level in patient case 2. **(B)**. Genus level in patient case 2.

SUPPLEMENTARY FIGURE 2
The differences of strains at the level of phylum, and the top 20 microbiota with the most obvious differences at the level of genus and species in patient case 1. A1. The differences of strains at the level of phylum before and after use of precision probiotic strains transplantation capsule. A2. The top 20 microbiota with the most obvious differences at the level of genus before and after use of precision probiotic strains transplantation capsule. A3. The top 20 microbiota with the most obvious differences at the level of species before and after use of precision probiotic strains transplantation capsule. B1. The differences of strains at the level before and after use of fecal microbiota transplantation. B2. The top 20 microbiota with the most obvious differences at the level of genus before and after use of fecal microbiota transplantation. B3. The top 20 microbiota with the most obvious differences at the level of species before and after use of fecal microbiota transplantation. C1. The differences of strains at the level of phylum before and after use of live combined bacillus subtilis and enterococcus faecium capsules. C2. The top 20 microbiota with the most obvious differences at the level of genus before and after use of live combined bacillus subtilis and enterococcus faecium capsules. C3. The top 20 microbiota with the most obvious differences at the level of species before and after use of live combined bacillus subtilis and enterococcus faecium capsules.

SUPPLEMENTARY FIGURE 3
The differences of strains at the level of phylum, and the top 20 microbiota with the most obvious differences at the level of genus and species in patient case 2. A1. The differences of strains at the level of phylum before and after use of precision probiotic strains transplantation capsule. A2. The top 20 microbiota with the most obvious differences at the level of genus before and after use of precision probiotic strains transplantation capsule. A3. The top 20 microbiota with the most obvious differences at the level of species before and after use of prcesion probiotic strains transplantation capsules. B1. The differences of strains at the level before and after use of fecal microbiota transplantation. B2. The top 20 microbiota with the most obvious differences at the level of genus before and after use of fecal microbiota transplantation. B3. The top 20 microbiota with the most obvious differences at the level of species before and after use of fecal microbiota transplantation. C1. The differences of strains at the level of phylum before and after use of live combined bacillus subtilis and enterococcus faecium capsules. C2. The top 20 microbiota with the most obvious differences at the level of genus before and after use of live combined bacillus subtilis and enterococcus faecium capsules. C3. The top 20 microbiota with the most obvious differences at the level of species before and after use of live combined bacillus subtilis and enterococcus faecium capsules.

SUPPLEMENTARY TABLE 1
IBS-SSS score change.

SUPPLEMENTARY TABLE 2
Changes of IBS related symptoms.

SUPPLEMENTARY TABLE 3
Changes in quality of life scale scores IBS-QoL.

SUPPLEMENTARY TABLE 4
Changes in anxiety and depression scale scores.

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Sodium butyrate treatment and fecal microbiota transplantation provide relief from ulcerative colitis-induced prostate enlargement

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The ability to regulate the gut environment has resulted in remarkable great breakthroughs in the treatment of several diseases. Several studies have found that the regulation of the gut environment might provide relief from the symptoms of benign prostatic hyperplasia. However, the correlation between the gut microenvironment and the colon and prostate glands is still unknown. We found that ulcerative colitis (UC) induced an increase in prostate volumes that could be reversed by sodium butyrate (NaB) and fecal microbiota transplantation (FMT). The mechanism by which UC induced changes in the prostate gland was examined *via* RNA-Seq. The results show that the expression level of GPER was significantly lower in the prostate gland of UC mices than in normal mices. The expression of GPER could be increased *via* treatment with NaB or FMT. We found that prostate tissues exhibited higher butryic acid levels after they were treated with NaB or FMT. In experiments conducted *in vitro*, NaB or the fecal filtrate (FF) from healthy mice up-regulated of the expression of GPER, inhibited cell growth, and induced apoptosis in BPH-1 cells. These changes could be alleviated by treatment with the G15 or in GPER-silenced cells.

KEYWORDS

sodium butyrate, fecal microbiota transplantation, ulcerative colitis, prostate enlargement, G protein-coupled estrogen receptor (GPER)

Introduction

Benign prostatic hyperplasia (BPH) is associated with dysuria and urinary retention, and is common in elderly people (Woodard et al., 2016). It results in a decreases in the quality of life of patients because of its persistent symptoms and long treatment cycle (Zhang et al., 2022). The regulation of the gut environment is widely used as a treatment with low economic burden in a variety of diseases (Gupta et al., 2020). The consumption of foods such as nuts, fruits, and vegetables which encourage the production of short-chain fatty acids (SCFAs), could help relieve the symptoms of BPH (Russo et al., 2021).

The prostate gland is located near the colon in the body. An inflammation of the colon, known as ulcerative colitis (UC), is similar to BPH. It is difficult to cure and it is easy for the patient to relapse (Ferretti et al., 2022). Alternations in SCFAs and gut microbiota diversity lead to the development of both ulcerative colitis and BPH (Ratajczak et al., 2021; Xu et al., 2022). However, few studies have reported on the relationship between changes in the prostate gland and UC. According to the results of our previous study, butyric acid production levels are correlated with the effect of fecal microbiota transplantation (FMT) during UC treatment (Xu et al., 2021a). Butyric acid, as an SCFA, is the main source of energy for the colon (Pituch et al., 2013). Sodium butyrate (NaB) is a salt of butyric acid (Pituch et al., 2013). We found that NaB and FMT inhibit the progression of UC (Zhao et al., 2020; Xu et al., 2021a; Xu et al., 2021b). In this study, we aim to examine the changes in the prostate gland and explore whether FMT or NaB affects the prostate gland during the progression of UC.

Materials and methods

Establishment of a mouse model

All animal experiments were performed in accordance with the protocols used in our previous studies (Xu et al., 2021a). The animal study was reviewed and approved by The institutional review board of Guangzhou Medical University. Sixteen eight-weeks-old male BALB/c mice were obtained from the Animal Center at Guangdong Medical Laboratory. The DSS-induced colitis model symptomatically resembles epithelial damage and inflammatory state in UC. Water containing 2.5% DSS (Sigma, United States) was provided every other week for 5 weeks to induce UC. Mice in the control group were provided water alone. The UC-NaB groups included mice with colitis that were fed with 0.2g/kg NaB for 7 days. The UC-FMT group was gavaged with filtrates of fresh feces obtained from healthy mice from the control group one day at a time, for a total of 7 days. The disease activity index (DAI) was used to grade colitis severity and determine the stool consistency (0=normal, 1=pasty and not sticking to the anus,2=pasty and slightly sticking to the anus, 3=pasty and stuck and to the anus, 4=watery), weight loss percentage (0 = 0-1%, 1 = 1-5%, 2 = 5-10%, 3 = 10-15%, 4 = 15-100%), and extent of rectal bleeding (0= hemoccult (−), 1= hemoccult (±), 2=hemoccult (+), 3=hemoccult (++), 4=obvious blood in stool) (Murthy et al., 1993). Fecal samples (500 mg) were collected from healthy mice in the control group solubilized in PBS, and passed through a 0.2 μm membrane filter (Shute et al., 2021). Prostate volume was calculated by height × length × width × π/6.

Western blot

Tissue or cell proteins were separated *via* 10% SDS-PAGE and transferred onto 0.22 μm PVDF membranes (Millipore, #ISEQ00010). The membranes were blocked with 5% skimmed milk (BD Difco, #232100) in TBS-Tween 20 and incubated with GPER (Abcam, ab260033) and Anti-β-Actin Antibody (Boster, #BM0627). The results were visualized with the SImmobilonWestern Chemiluminescent HRP substrate (Millipore, #WBKLS0100). β-Actin was used as a control for internal loading.

Immunohistochemistry

Expression levels and subcellular localization were detected using anti-ZO-1 (Abcam, ab216880), anti-occludin (Abcam, ab168986), and anti-GPER (Abcam, ab260033). Two pathologists who were blinded to the clinical data scored the results of the immunostaining process independently. The scoring scheme was based on the percentage of positive cells (0 = 0%, 1 = 1-10%, 2 = 11-50%, 3 = 50%-80%, 4 = 81%-100%) and the staining intensity (0=no staining, 1=weakly stained, 2=moderately stained, 3=strongly stained). These antibodies were diluted by 1:200 times.

Gene silencing with shRNAs

Plasmid-targeting GPER shRNA was obtained from the HYY Med company (Guangzhou, China). The framework plasmid was used as a lentiviral vector. BPH-1 cells were infected with GPER-shRNA and its control vector. The shRNA targeting sequences in this study: GPER-shRNA-a: GCAACATCCTGATCCTGGTGGTGAA, 1111; GPER-shRNA-b: CCGACTCCCTCATTGAGGTGTTCAA, 1213; GPER-shRNA-c: CAGGTCAACATGTACAGCAGCGTCT, 1296. Stable cell lines were obtained after the medicinal sieving. The efficacy of these three shRNAs was evaluated by western blot. The sequence with the highest inhibition efficacy for GPER and GPER-shRNA-c, was identified by western blot.

Sequencing data analysis

Prostate tissue was selected for RNA-Seq. Total RNA was extracted using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked *via* electrophoresis using RNase-free agarose gel. After total RNA was extracted, eukaryotic mRNA was enriched using Oligo(dT) beads. Then, the enriched mRNA was broken down into short fragments using fragmentation buffer and reverse transcribed into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs). The end repair of purified double-stranded cDNA fragments was performed, a base was added, and the fragments were ligated to Illumina sequencing adapters. The ligated sequences were purified using AMPure XP Beads (1.0X) and subjected to PCR amplification and size selection *via* agarose gel electrophoresis. The resulting cDNA library was sequenced using an Illumina Novaseq6000 instrument, which was developed by Gene Denovo Biotechnology Co. (Guangzhou, China). The results suggested that the sample could be used for further analysis. DEGs were mapped to GO terms in the Gene Ontology (http://www.geneontology.org/) and KEGG Pathway (http://www.genome.jp/kegg/) database. The raw sequencing data have been deposited in the Genome Sequence Archive (http://bigd.big.ac.cn/)

Cell cultures

BPH-1 is a benign prostate epithelial cell line. Cells were purchased from Sigma-Aldrich and authenticated by the HYY Med company (Guangzhou, China). The BPH-1 cell line was maintained in RPMI-1640 medium (HyCloneTM, #SH30027.01). The media were supplemented with 10% fetal bovine serum (Gibco, #10438-026), 2 mM L-glutamine, and 1% antibiotics. Both cell lines were maintained at 37°C and 5% CO2.

Cell viability

BPH-1 cells were seeded in 96-well plates and treated or not treated with a medium containing 1 mM NaB, 10 μM G15, fecal filtrate, both 1 mM NaB and 10 μM G15,and both fecal filtrate and 10 μM G15. After 24 h, a 10% CCK-8 (Beyotime, C0038) solution was added to each well. A spectrophotometer (Multiskan MK3, Thermo Scientific) was used to detected the absorbance values at 450 nm.

Cell cycle analysis

BPH-1 cells that were treated or not treated with a medium containing 1 mM NaB, fecal filtrate, both 1 mM NaB and 10 μM G15,and both fecal filtrate and 10 μM G15 for 24 h were collected, washed, and fixed in 70% ethanol at 4°C overnight. A mixture containing PBS, propidium iodide, RNaseA, and 0.3% Triton X-100 was added to the cells and then be incubated at 4°C for half an hour. A flow cytometer (BD FACSVerse, BD Biosciences, USA) and FlowJo software were used to for analysis. Three independent tests were conducted at least three times.

Apoptosis detection

BPH-1 cells were cultured for 24 h in 5 conditions: medium alone, 1 mM NaB, fecal filtrate, both 1 mM NaB and 10 μM G15, and both fecal filtrate and 10 μM G15 for 24 h. Then, the cells were harvested *via* trypsinization and incubated with Annexin V-APC and 7AAD. After vortexing, the cells were incubated at about 26°C in the dark for a quarter. Then, 485 μL of 1× binding buffer was added to each sample. The results were analyzed with a flow cytometer (BD FACSVerse, BD Biosciences, USA). Three independent tests were conducted at least three times.

Butyric acid ELISA kit

We collected tissue homogenates from the prostate or colon of mice from the control group, UC group, UC-NaB group, and UC-FMT group. The level of butyric acid (BA) was measured using the BA ELISA Kit, according to the manufacturers' instructions (Elk biotechnology, ELK8174).

Statistics

Relevant data are shown in terms of mean ± standard deviation (SD) values. Statistical analysis was performed with independent samples using the t-test, along with SPSS V16 and Graph Pad Prism 8 software. Data results were considered statistically significant if $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p<0.0001$.

Results

NaB or FMT inhibit the progression of UC

We analyzed and compared the colons of healthy mice and mice with colitis treated or not treated with NaB or FMT. All samples were handled by investigators who were blinded to the data, in accordance with legal and ethical standards. The lengths of colons in the UC group were significantly shorter than in the control, UC-NaB, and UC-FMT groups (Figures 1A, B), while the DAI values in the UC group were higher than those in the other groups (Figure 1C). Occludin and ZO-1, which are the

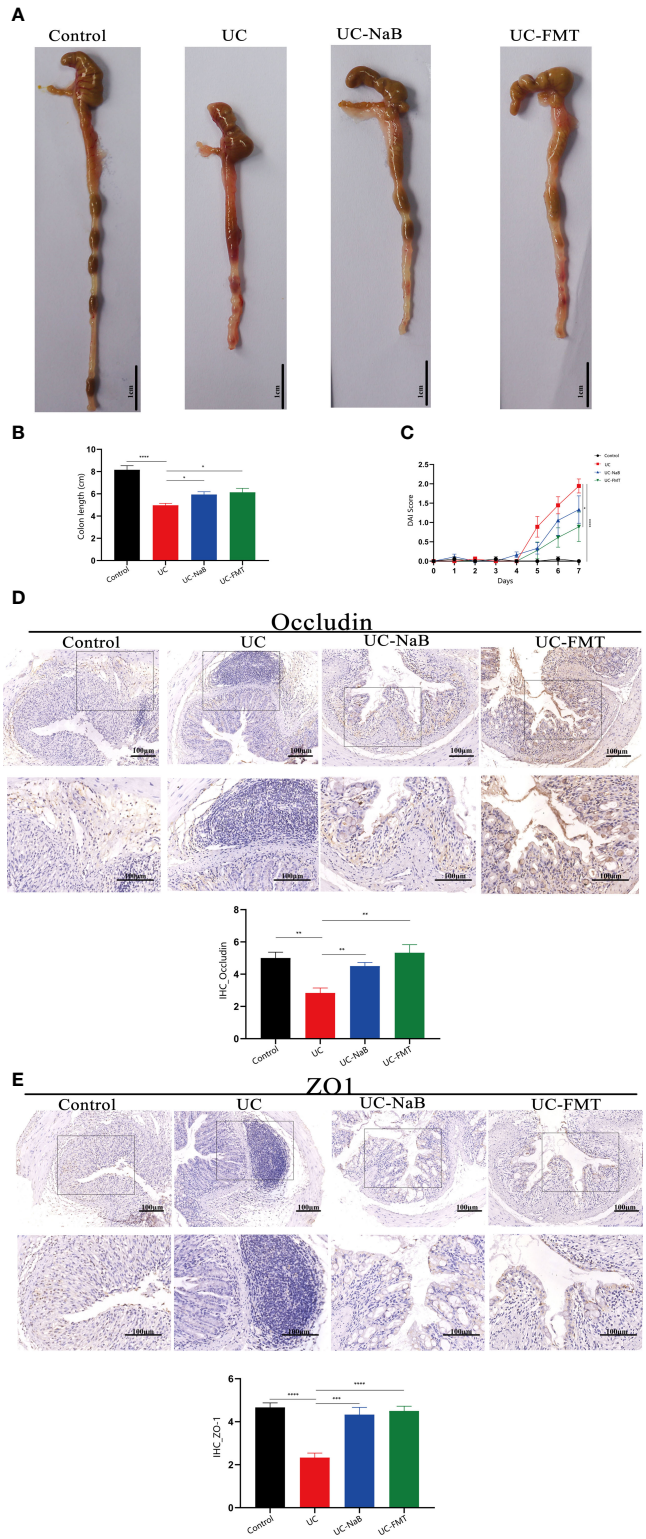

FIGURE 1
Pathological model of the colon in the mouse. **(A)** Anatomical map of the colorectum. Mouse colon lengths **(B)** and disease activity index (DAI) **(C)** in each group. Occludin **(D)** and ZO1 **(E)** expression in colonic tissues. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

biomarkers of gut barrier, were negatively correlated at the intestinal inflammation level (Kato et al., 2007). Immunohistochemistry was used to detect the expression levels of occludin (Figure 1D) and ZO-1 proteins (Figure 1E). As observed for the control group, the expression level of occludin and ZO-1 in the FMT group or NaB group was significantly higher than that in the UC group; this was consistent with our previously reported results.

NaB or FMT inhibit UC-induced prostate enlargement

Prostate tissues were also collected from these animals. The volume of the prostate gland was increased in the UC group when compare to the control group. It was smaller after treatment with NaB or FMT in mice with colitis (Figure 2A, C). The results of histological and morphological analyses reveal that the epithelial cell layer and lumen space were increased in the UC group, compared to the control group (Figure 2B). These changes were reversed after treatment with NaB or FMT. We detected different levels of butyric acid (BA) levels in the prostate tissues of these mices. The results show that the level of BA was higher in the UC-NaB group and UC-FMT groups than in the UC group (Figure 2D).

Sequencing data were analyzed in order to understand the mechanisms underlying the action of NaB and FMT in this model. Principal component analysis (PCA) indicated that there were differences among the control, UC, UC-NaB, and UC-FMT groups (Figure 3A). This result suggested that the sample could be used for further analysis. There were 99, 267, and 758 differentially expressed genes (DEGs) between the control and UC group, UC group and UC - NaB group, UC group and UC-FMT group, respectively (Figure 3B). Among all the enriched KEGG pathways, after excluding the irrelevant information, some

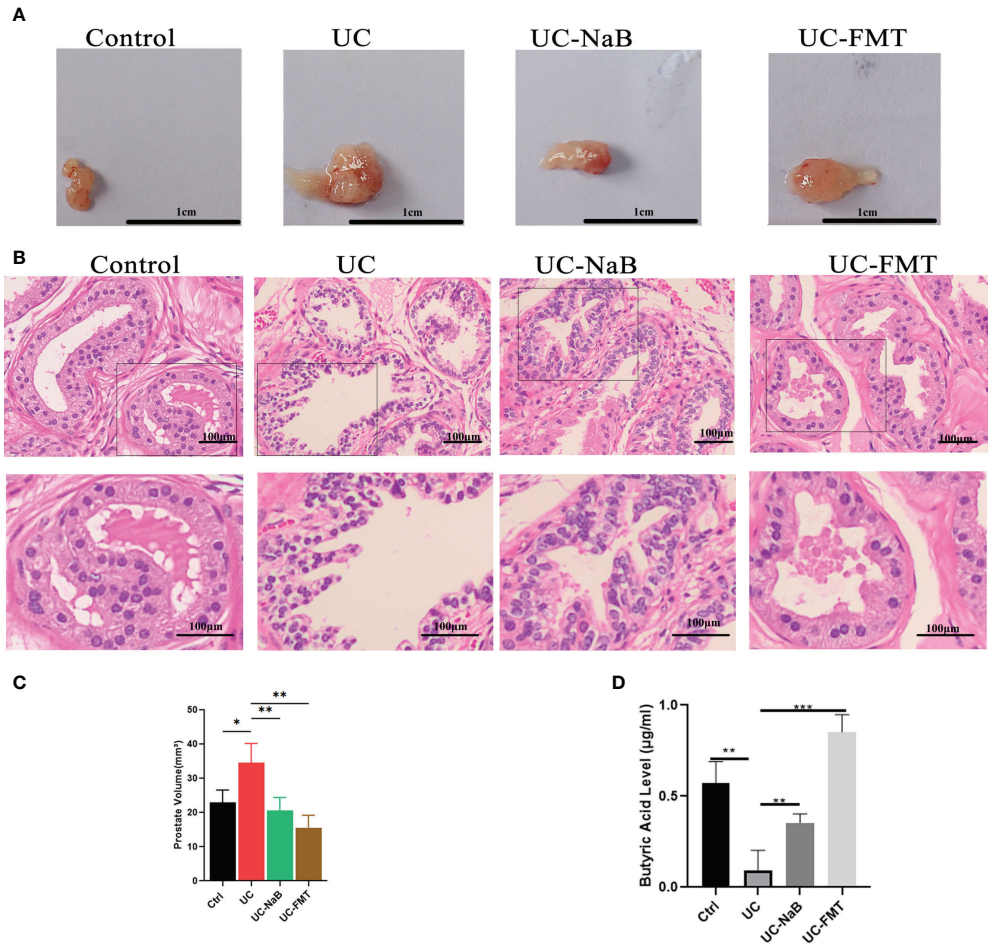

FIGURE 2
Pathological model of the prostate in the mouse. **(A)** Anatomical map of the prostate. **(B)** Histopathological images of HE staining of the prostate glands. Histopathological images of HE staining **(B)** and volume **(C)** of the prostate glands. **(D)** The levels of butyric acid in prostate gland. *p<0.05, **p<0.01, ***p<0.001.

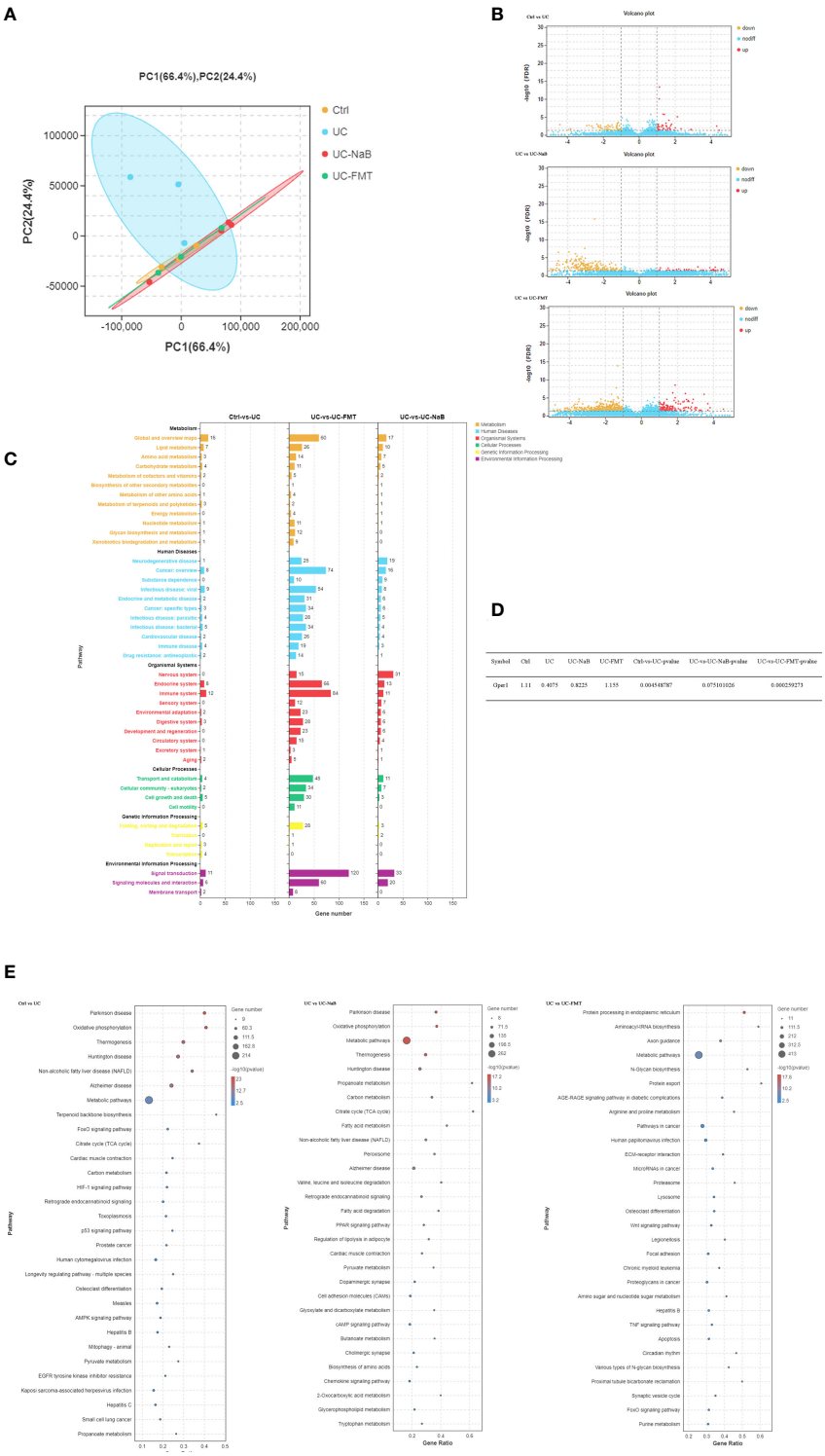

FIGURE 3
PCA was performed with the R package gmodels (http://www.rproject.org/) in this experiment for each of the sample groups, including their biological replicates. **(A)**. The expression profiles of the identified DEGs **(B)**. Yellow and red points represent significant DEGs with an FDR<0.05 and |log2FC|>1, and blue points indicate non-significance DEGs. **(C)**. The vertical axis represents the number of DEGs per pathway in KEGG annotation. The X-axis shows the gene numbers for each different term. **(D)**. The GPER expression levels in each group were compared. **(E)**. Enriched GO terms identified during the analysis of DEGs.

human diseases and metabolism-related pathways are the most significant, including the "cancer pathways", "liquid metabolism pathway", and "cell growth and death pathways" (Figures 3C, E). We found that G protein-coupled estrogen receptor 1 (GPER/GPR30) was down-regulated in the UC group and responded to all these pathways (Figure 3D). GPER is a member of the GPCR family of proteins. We have demonstrated that the activation of GPER inhibits BPH-1 cells proliferation (Dong et al., 2019).

GPER associated with UC-induced prostate enlargement

We examined the levels of GPER in prostate tissues *via* immunohistochemistry (Figure 4A) and western blot (Figure 4B) analyses. The level of GPER in the UC-FMT, UC-NaB, or control groups was significantly higher than that in the UC group, which is consistent with the results of RNA sequencing.

The sh-GPER cell line was established and verified by western blot analyses (Figure 5A). The GPER expression levels observed *in vitro* are higher in BPH-1 cells treated with NaB or filtrates of fresh feces from healthy mice (Figure 5A), compared with the control group. Such upregulation of GPER appears to be weaker in GPER-silencing group or cells treated with GPER antagonist G15. Cell growth is affected by cell proliferation, cell cycle, and apoptosis. The results of the CCK-8 assay (Figure 5B) showed that NaB or FF inhibited the growth of BPH-1 cells. The cell proliferation assay results showed that these stimuli increased the percentage of G1 phase cells (Figure 5C). We also found that NaB and FF induce apoptosis in the BPH-1 cells (Figure 5D). However, the extent of induction of apoptosis, inhibition of cell proliferation, and prolongation of the G1 phase appeared to be reduced in GPER-silenced cells or cells treated with G15 (Figures 5B–D). Therefore, we believe that GPER plays an important role in the enhancement of cell growth that was inhibited by NaB or FMT in BPH-1 cells.

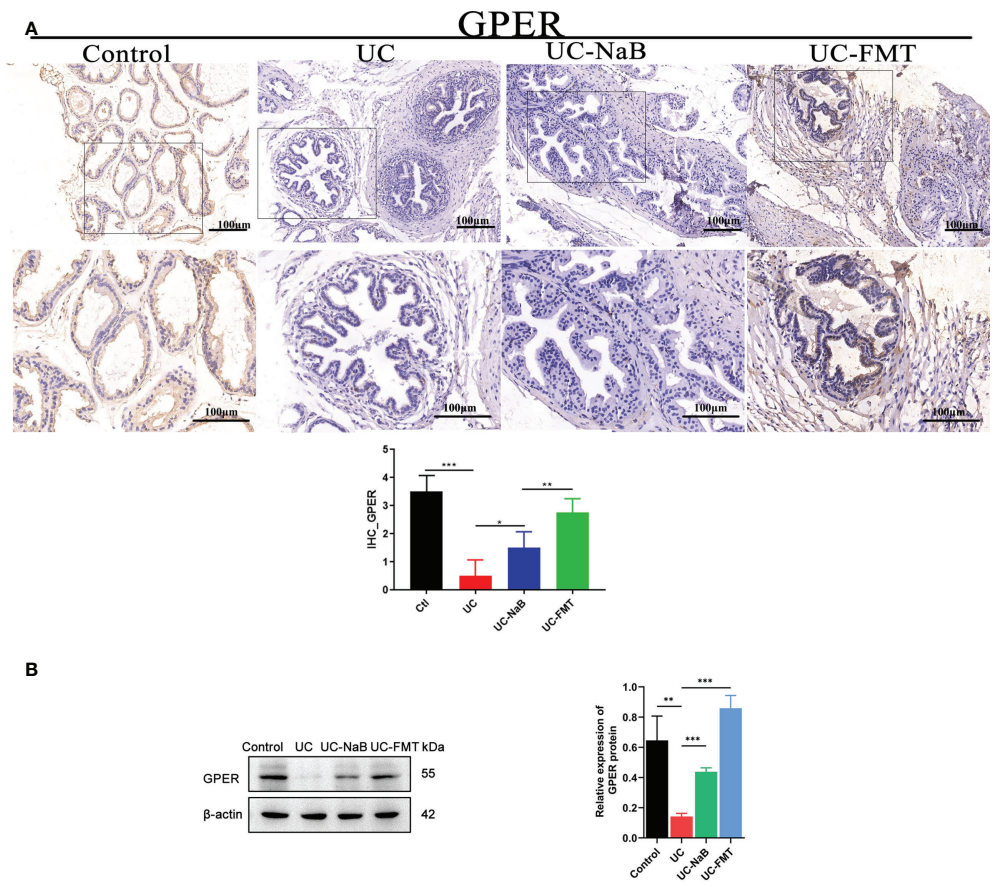

FIGURE 4
GPER expression in the prostate tissue. Immunohistochemistry **(A)** and western blot **(B)** analysis of proteins in the prostate tissue under different conditions. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

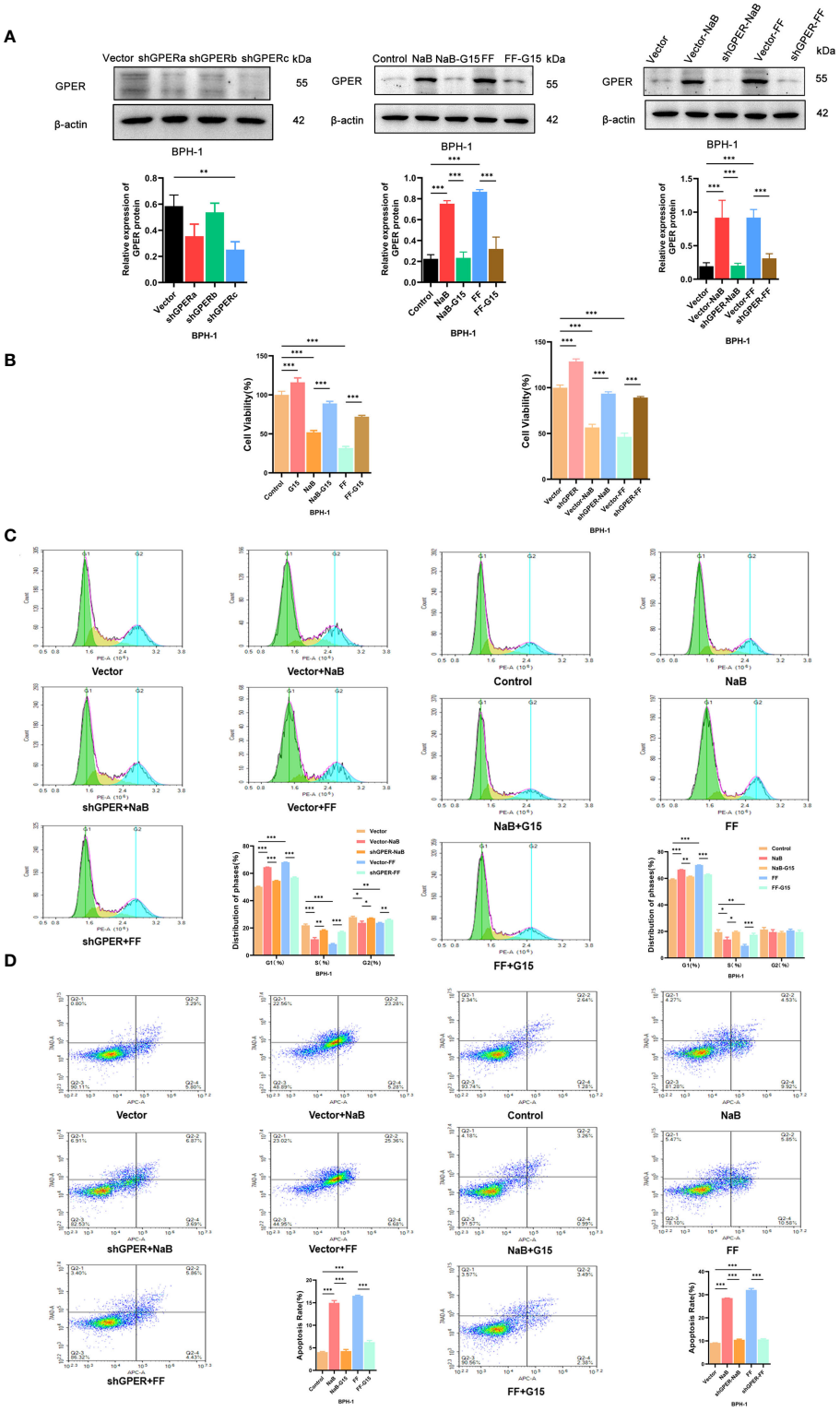

FIGURE 5
(A). Western blot of proteins in BPH-1 cells. **(B)**. Cell viability assays were conducted with BPH-1 cells under different conditions. **(C)**. Cell cycle analysis results for the control, NaB, NaB+G15, FF, FF+G15, Vector, Vector+NaB, shGPER+NaB, Vector+FF, and shGPER+FF groups. **(D)**. Cell apoptosis assay results of the Control, NaB, NaB+G15, FF, FF+G15, Vector, Vector+NaB, shGPER+NaB, Vector+FF, and shGPER+FF groups. *p<0.05, **p<0.01, ***p<0.001.

Conclusion

Our study provides a better understanding of the intestinal microenvironment affects the process of BPH development. UC-induced prostate enlargement which could be inhibited by NaB and FMT, and might be correlate to cancer and metabolic pathway. NaB and FMT might inhibit prostate growth by resulting in the expression of GPER.

Discussion

The change in the diversity of gut bacteria in patients has attracted considerable attention from researchers. Not only do gut bacteria act as novel biomarkers, but they also act as novel disease-modifying targets (Durack and Lynch, 2019; Manor et al., 2020). A recent study reported on the change in gut bacteria in BPH patients (Li et al., 2022). The existence of the gut-genitourinary axis was also proposed in this study (Li et al., 2022). However, the mechanism by how gut affect prostatic diseases is still relatively unknown.

Both BPH and UC are diseases that occur repeatedly and necessitate long-term treatment. FMT and postbiotics are promising potential therapies, especially for relapsing diseases, because of their relatively fewer side effects and low toxicity levels (Yoshimatsu et al., 2015; Salminen et al., 2021; Mendelsohn et al., 2022). Butyrate has been considered as a postbiotic (Panebianco et al., 2022). The colon is the main site at which butyric acid absorption and gut microbiological colonization occur. We analyzed 12 UC patient samples in our previous study and found that the FMT efficacy was associated with the level of butyric acid-producing bacteria (Xu et al., 2021a). Here, we not only verified that supplementary butyrate treatment or FMT had a positive effect on ulcerative colitis again but also found that NaB and FMT suppressed UC-induced prostate enlargement. The period at which BPH and ulcerative colitis can be predicted is different. This might be attributable to the fact that it is difficult to detect a slightly enlarged prostate at an early stage. Subsequently, butyrate enters the circulation and metabolizes substrates (Zhang et al., 2021). Therefore, FMT is more beneficial because it results in a higher level of butyric acid in the systemic circulation; this needs to be verified in future studies. We believe that NaB treatment and FMT could lower the risk of BPH. Therefore, FMT might represent a potential new therapy for the treatment of BPH.

We performed RNA sequencing to further examine how FMT and NaB affected UC-related prostate changes in the prostate. We found that UC-related changes in the prostate were correlated with cancer and the metabolic pathway. Though SCFAs activate multiple GPCRs, the mechanism by which butyrate affects GPER is still unknown (Park et al., 2019). The direct effects of bacteria in the gut and changes in the BA concentration may results in a change in the prostate. We verified that NaB and filtrates of fresh rat dejecta up-regulated the expression of GPER and promoted the process of cellular apoptosis, which could be suppressed in GPER-silenced cells. This indicated that GPER was one of the most significant factors affecting this process. The detailed mechanisms of action need to be further elucidated at the molecular level, to uncover the relationship between NaB and FF. This study provides a better understanding of the mechanism of action of GPER in the gut-genitourinary axis. Butyrate and FMT may be a protential therapy for UC-induced prostate enlargement. The regulation of GPER levels might help to improve the therapeutic effects of FMT and NaB with BPH.

Data availability statement

The data presented in the study are deposited in the Genome Sequence Archive (http://bigd.big.ac.cn/), accession number: CRA008379.

Ethics statement

The animal study was reviewed and approved by the institutional review board of Guangzhou Medical University.

Author contributions

WD, XJ, and HX designed the study. WD, JZ, HX, HT, and SY established the animal model and collected the samples. JZ, YH, ZZ, XL, HL, and GZ carried out the experiments and analyzed the data. WD, XJ, HX, and WZ drafted and revised the article. All authors have read and approved the final manuscript.

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Conflict of interest

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

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Male zooid extracts of *Antheraea pernyi* ameliorates non-alcoholic fatty liver disease and intestinal dysbacteriosis in mice induced by a high-fat diet

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The male zooid of *Antheraea pernyi* (*A. pernyi*) accumulates several nutrients and physiological activity-related substances for reproduction. Some components in the extracts of the male zooid of *A. pernyi* (EMZAP) have several functions, such as protecting the liver, enhancing immunity, antiatherosclerosis, anti-aging, and antitumor effects. In this study, we investigated the ameliorating effects on high-fat diet (HFD)-induced non-alcoholic fatty liver disease (NAFLD). The EMZAP treatment could ameliorate NAFLD and effectively decrease the serum total cholesterol, triglyceride and low-density lipoprotein levels and a significant increase in serum high-density lipoprotein levels was observed. Additionally, the EMZAP treatment reduced the levels of liver-function enzymes and pro-inflammatory cytokines (i.e., IL-6, IL-8, TNF-α, TGF-β_1) and also the oxidative stress indices and regulated the expression of genes associated with fatty acid metabolism (*SREBP-1c, PPARα, ACOX-1, CPT-1*) in the liver to prevent the development of NAFLD. Furthermore, EMZAP enhanced the diversity and richness of the beneficial intestinal microbes, suggesting its potential as a dietary supplement and functional food to combat NAFLD induced by HFD.

KEYWORDS

tussah male moth, NAFLD, hypolipidemic, intestinal microbial diversity, high-fat diet

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the result of diffuse fatty infiltration of the liver caused by factors other than alcohol, such as obesity and diabetes (Tilg et al., 2021). Patients with NAFLD are more likely to develop hepatocellular carcinoma (HCC) due to continuous damage to hepatocytes. NAFLD has become the leading cause of HCC

in some western countries (Huang et al., 2021). Currently, the widely accepted pathogenesis for this is the "multiple strike" theory that involves the parameters of genetic susceptibility, dietary factors, obesity, insulin resistance, gut microbiota disorder, liver detoxification, chronic oxidative stress, lipid metabolism disorder, inflammatory cytokines, adipokines, and changes in the immune system (Nyangale et al., 2012; Ma et al., 2017; Wang et al., 2021).

Microorganisms that live in the gastrointestinal tract are called gut microbiota. By balancing the local and systemic immune responses, maintaining the normal gut-liver circulation, and inhibiting pathogen colonization, a balanced gut microbiota environment plays an important role in the physiological regulation of the host. Dysbacteriosis can lead to various diseases, such as metabolic diseases, immune diseases, respiratory diseases, and even tumors (He et al., 2021). Considering the anatomical position of the liver, the blood from the intestine to the portal vein accounted for 70% of the total liver blood supply. Thus, the liver forms the first line of defense against intestinal antigens such as bacteria and bacterial byproducts, and it is also most vulnerable to intestinal stimulations (Park et al., 2021). According to these results, gut microbiota is closely associated with NAFLD occurrence and development. Gut microbiota participates in energy metabolism through several mechanisms. Gut microbiota and its metabolism may be a decisive factor in the occurrence and development of NAFLD (Cui et al., 2019). Natural drugs exhibit good efficacy and fewer adverse events and can effectively improve the biochemical and histological changes in NAFLD both *in vivo* and *in vitro*, with only minor adverse effects recorded on the gut microbiota (Sun et al., 2022). Several natural drugs have been reported to improve the gut microbiota disorder in NAFLD rats and NAFLD induced by a high-fat diet, including Jiangzhi Ligan Decoction (which is composed of *Rhizoma alismatis*, cassia seed, *Salvia miltiorrhiza*, turmeric, seaweed, and lotus leaf), berberine, Yiqi Qinghua Recipe (composed of Astragalus membranaceus, *Salvia miltiorrhiza*, fried Atractylodes macrocephala, tangerine peel, lotus leaf, *Gynostemma pentaphyllum*, Poria cocos, and corn whisker), Biejiajian Pill (composed of turtle shell glue, donkey hide gelatin, rat worm, dung beetle, bupleurum, Scutellaria baicalensis, and other 17 drugs) (Qiu et al., 2017). Recent researches have reported that the *Mallotus furetianus* extract (MFE) and *Gynostemma pentaphyllum* saponins (GPs) can effectively treate NAFLD and improve gut microbiota structure and composition. Furthermore, MFE and GPs can reverse the gut microbiota disorder caused by a high-fat diet, restore the diversity of the gut microbiota, increase the relative abundance of *Bacteroides*, and reduce the relative abundance of *Firmicutes* as well as the ratio of *Firmicutes* to *Bacteroides* (Lin et al., 2022). In addition to regulating the gut microbiota, natural drugs can induce anti-oxidative stress and play anti-inflammatory roles by activating the adenylate activated protein kinase signaling pathway in the liver and regulating the peroxisome proliferator-activated receptor activity and expression to improve NAFLD. For example, myristica fragrans extract attenuated inflammation and lipid metabolism disorders via NF-κB and AhR-FAS pathways in mice with NAFLD (Zhao et al., 2022).

Male zooid of *A. pernyi* accumulates several nutrients and physiological activity-related substances for reproduction. Its medicinal efficacy has been recorded in *Compendium of Materia Medica*, implying that male zooid of *A. pernyi* benefits essence, strengthens both the vagina and reproduction capability, delays ejaculation, and prevents hematuria. It also cures various sores and lightens scars. The male tussah moth powder has been reported to improve chronic colitis in mice by regulating the expression of inflammatory cytokines (Li et al., 2020). Our previous research has demonstrated that the extracts of the male zooid of *A. pernyi* (EMZAP) can improve liver fat accumulation, reduce blood lipids and the expression of genes associated with cholesterol metabolism, as well as resist hepatocyte mitochondrial lipid peroxidation and liver fibrosis caused by alcohol consumption (Jia et al., 2009; Zhu L et al., 2021). In addition, it can enhance cellular and humoral immunities in mice and exert a certain antitumor effect (Zhao et al., 2008).

In this study, we evaluated the interventional effect of EMZAP by inducing the NAFLD mouse model with a high-fat diet and analyzed the genes associated with fatty acid synthesis (*SREBP-1c*) and oxidation (*PPARα*, *ACOX-1*, *CPT-1*) to explore the hypolipidemic mechanism. The liver antioxidant level, histopathological staining, and enzyme-linked immunosorbent assay analysis were performed to elucidate the mechanism of its protective effect on the liver. To explore its effect on the gut microbiota diversity, 16S rDNA technology was applied to extract and analyze the mouse genomes from feces. The results can also provide a theoretical basis for the high-value utilization of tussah moth.

Material and methods

Preparation of EMZAP

Male zooid of *A. pernyi* was obtained from the emergence of tussah pupae provided by Zaiqiang Tussah Improved Variety Breeding Cooperative in Rushan (Shandong Province). The newly emerged and unmatched male zooid were selected, their urine was drained, and their wings and feet were pinched, followed by soaking in 95% ethanol and crushing and pressing after 7 days. The material liquid ratio was 4:5. The extraction procedure was repeated twice. The supernatant was obtained was centrifugation at 4200 rpm for 12 min to separate the impurities such as fat and crude protein. Then, the supernatant was mixed and poured into a vacuum distillation unit for concentration. The water temperature in the

concentration tank was kept at 55-58°C with the pressure set to not exceed -0.08 MPa. When the mixture was concentrated to 9% of the original volume, no further concentration was done, and thick porridge-like extracts were obtained. Next, the extracts were centrifuged at 4000 rpm for 8 min, and the upper fat was removed to obtain EMZAP.

Animals and treatments

A total of 40 C57BL/6 male mice (20 ± 2 g) and the standard diet (SD) were provided by the Jinan Jinfeng Experimental Animal Co., Ltd. (SCXK 20190003). The high-fat diet (HFD) purchased from Beijing HFK Bioscience Co., Ltd. The HFD consists of 35% fat, 26% carbohydrate and 26% protein. Mice were housed under conventional and uniform conditions at a controlled temperature (26°C) and a relative humidity of 50 ± 5%. A 12-h light/dark cycle with free access to sterile water was provided. A 5-day acclimatization period was allowed for the mice before the experiments were performed.

The mice were randomly assigned to 4 groups of 10 mice each, as follows: the Control Group, the Model Group with a high-fat diet (HFD), the High-dose Group with HFD + EMZAP, and the Low-dose Group with HFD + EMZAP. The Control and Model groups received normal saline *via* gavage daily, while the High-dose and Low-dose Groups received 0.01 mL/g of EMZAP and 0.005 mL/g *via* gavage every day, respectively. The EMZAP solution was prepared with sodium carboxymethylcellulose at a concentration of 0.8 g/mL. All experimental procedures were conducted in conformance to the accepted principles of animal welfare in experimental science.

After 8 weeks of treatments, mice were fasted overnight (12 h) and then taken out of their cage to collect their feces in a microcentrifuge tube. After that, the mice were exsanguinated and sacrificed by removal of the eyeballs. The feces, blood and tissues were collected, processed as described follows. Feces collected for microbiome survey were immediately snap-frozen in liquid nitrogen and stored at −80°C until further analyses. To obtain serum for ELISA, blood samples were kept static in a tube for 1 hour and centrifuged for 15 min at 2500 rpm. A portion of the liver and the abdominal adipose were soaked in 4% formaldehyde solution used for histopathological analyses. The remaining liver was snap-frozen in liquid nitrogen and stored at −80°C for biochemical analysis.

The Animal Ethics Committee of Shandong Academy of Agricultural Sciences reviewed and approved the animal study.

Relative organ weight

The mice were weighed every day to obtain body weight (BW). The weights of organs were recorded and then calculated using the following formula:

$$\text{Relative organ weight}(g/g) = \text{organ weight}(g)/\text{live BW}(g)$$

Blood analysis

The serum lipids and immune factors (i.e., TNF-α, TGF-β_1, IL-6, and IL-8) were determined by using ELISA kits (Nanjing Jiancheng Biological Engineering Institute).

Histopathological analysis

The livers and abdominal adipose tissues were fixed with formaldehyde for 48 h and then embedded in paraffin. Sections of fixed tissues were stained with Oil Red O and H&E. Finally, the tissues were pathologically evaluated by microscopy.

Liver antioxidant capacity and the expressions of relevant genes

A normal saline solution was used to homogenize the livers of the mice on ice. After grinding, the volume was adjusted to 3 mL and centrifuged at 5000 rpm for 10 min at 4°C. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in the liver were measured according to the commercial assay kits(Nanjing Jiancheng Biotechnology Co., Ltd).

The RNA was extracted from the tissue fluid to conduct fluorescence quantitative PCR after electrophoresis and reverse transcription for the determination of the relevant genes (*SREBP-1c, AdipoR2, PPAR-α, CPT-1, ACOX1*). Primers were designed by Primer Premier 5.0 software. The UNlQ-10 column Trizol total RNA extraction kit was used for RNA extraction. The reverse cDNA conditions were as follows: at 25°C for 20 min, at 50°C for 30 min, and then at 85°C for 5 min. Fluorescence quantitative PCR was performed with the PCR instrument (LightCyclr480 type II) using the SYBRGREEN qPCR master mix. The PCR cycle condition was as follows: initial denaturation at 95°C for 3 min, 45 cycles at 95°C for 5 s (melt), and at 60°C for 30 s (annual/extend). Molecular grade water was used as a no-template control.

Gut microbial diversity analysis

The gut microbial diversity was entrusted to the Shanghai Meiji Biomedical Technology Co., Ltd. Briefly, after the genomic DNA was extracted from the sample, the V3–V4 region of 16S rDNA was amplified by polymerase chain reaction (PCR) using 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Quantitation was performed with the QuantiFluor TM fluorometer. The NEXTFLEX Rapid DNA-Seq Kit was employed to build the library and the MiseqPE300 platform (Illumina) was used for sequencing.

Statistical analysis

The data obtained were processed with the Origin software (version 8.5). The results were analyzed and the descriptive statistics (i.e., mean, and standard deviation) were calculated. The data were expressed as the mean ± standard deviation (x ± s). The statistical significance of multiple comparisons was determined by a one-way analysis of variance (ANOVA). $P< 0.05$ was considered to indicate statistical significance. The microbial diversity data were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com). In addition to sample differentiation, OTU clustering and taxonomic analysis were conducted. Several diversity indices were determined for OTU analysis and sequencing depth was determined based on the results of OTU cluster analysis. A statistical analysis of the community structure at different taxonomic levels was conducted using taxonomic information.

Results

Changes in the weight of body and organ

NAFLD induced weight gain is a common clinical symptom. Therefore, the weight changes were measured in mice during treatment for 8 weeks. In Figure 1A, mice in the four groups continuously gained weight. The weights of EMZAP treatment groups were lower than the Model Group. There was a significant change in body weight between the High-dose Group and Model Group. These results demonstrated that EMZAP could effectively prevent obesity in mice with fatty liver.

NAFLD is characterized by the excessive accumulation of fat, mainly in liver. To test whether organ weight was decreased by treatment with EMZAP against HFD-induced NAFLD, we measured liver and epididymal adipose weight. The relative organ weight was shown in Figure 1B. The Model Group had significantly higher relative weight of liver and adipose than that in Control Group ($P< 0.01$). The results revealed that modeling was successful in mice. The relative weight of liver and adipose in High-dose Group and Low-dose Group were significantly lower than that in Model Group ($P< 0.01$, $P< 0.05$).

EMZAP treatment modulates dyslipidemia in HFD-induced mice

After 8 weeks, the Model Group mice also developed hypertriglyceridemia, demonstrated by the markedly increase serum levels of triglyceride (TG) and total cholesterol (TC) and low-density lipoprotein (LDL), 1.3-fold ($P< 0.01$) and 1.6-fold ($P< 0.01$) and 1.7-fold ($P< 0.01$) the levels in Control Group, respectively (Figure 2). The high-density lipoprotein (HDL) level in Model Group was decreased significantly compared to Control Group ($P< 0.01$).

When the mice were orally gavage EMZAP for 8 weeks, the TC, TG and LDL of mice treated with EMZAP decreased in different degree, the High-dose Group decreased significantly compared to Model Group ($P< 0.01$ or $P< 0.05$). The HDL of High-dose Group markedly increased compared to Model Group ($P< 0.01$).

EMZAP treatment modulates liver oxidative stress injury and serum inflammatory cytokine levels in HFD-induced mice

As shown in Figure 2, A significant difference was found between the Model Group and Control Group in terms of hepatic antioxidant capacity and pro-inflammatory cytokines. HFD induced oxidative stress and inflammation in the liver, as indicated by 14.01–54.38% inhibition of hepatic SOD, GSH-P_X ($P< 0.01$) and CAT ($P< 0.05$) and 25.72–86.31% elevation of hepatic MDA, TNF-α, IL-8 ($P< 0.01$) and IL-6 ($P< 0.05$). Compared with the Model Group, High-dose Group showed noticeably reduced hepatic MDA ($P< 0.01$), TNF-α and IL-8 ($P< 0.05$) activities were reduced by 17.98–30.25%. High-dose Group showed significantly increased hepatic SOD ($P< 0.05$) and CAT ($P< 0.01$) compared to those in the Model Group. The results exhibited that EMZAP could enhance hepatic antioxidant capacity effectively and reduce inflammatory responses in NAFLD mice.

EMZAP treatment alleviates lipid accumulation in HFD-induced mice

In Figure 3, Oil Red O staining results showed that the liver sections of Control Group contained significantly fewer lipid droplets than Model Group. There were fewer lipid droplets in the EMZAP-treatment groups than in the Model Group, especially in the High-dose Group. Correspondingly, the diameters of adipose cells in the High-dose Group and Low-dose Group (0.126/0.135 mm) were smaller than those in the Model Group (0.177 mm), respectively.

EMZAP treatment alleviates expression of genes associated with lipid metabolism in HFD-induced mice

Expressional changes of lipid metabolism genes in the liver, measured by quantitative PCR (Figure 4). *SREBP-1c*, *CPT-1*,

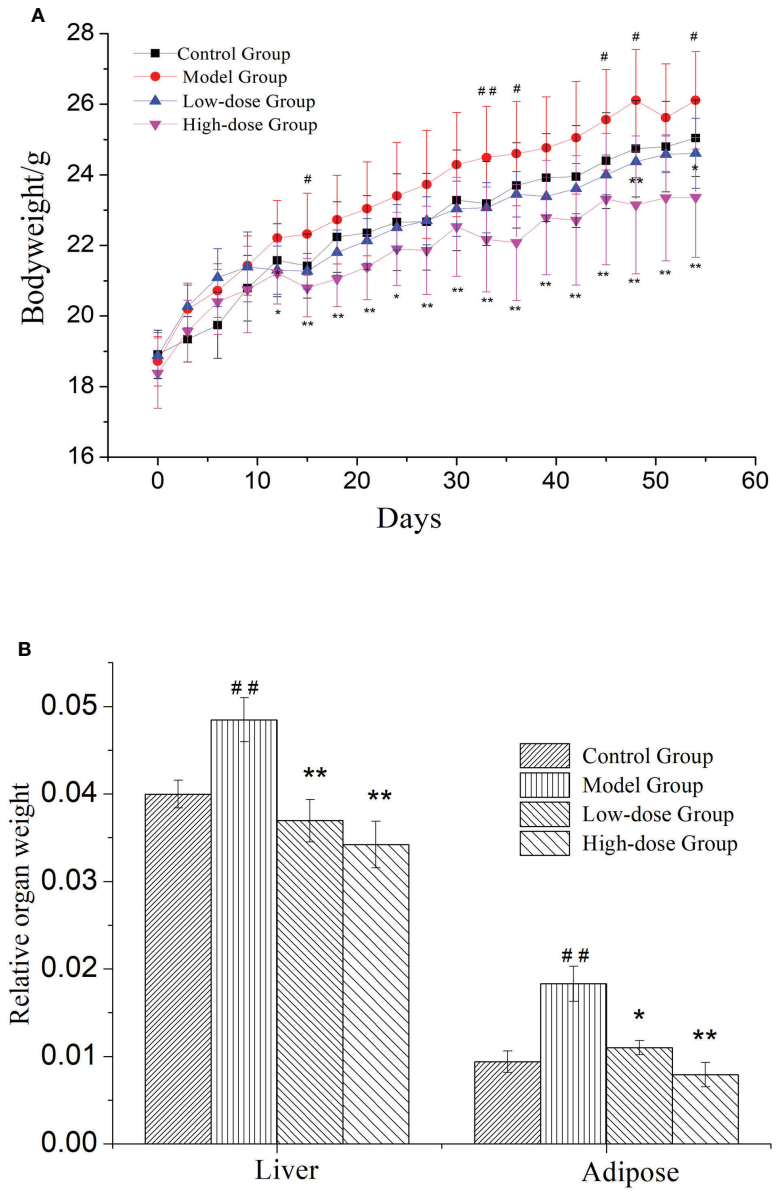

FIGURE 1
Effects of EMZAP on bodyweight **(A)** and relative organ weight **(B)** of mice (n = 10) * $P< 0.05$ and ** $P< 0.01$ vs Model Group, $^{\#}P< 0.05$ and $^{\#\#}P< 0.01$ vs Control Group.

ACOX-1 and *PPARα* expression was upregulated in the liver of Model Group compared to Control Group ($P< 0.05$ and $P>0.05$). The expression of lipid synthesis genes (*SREBP-1c*) in liver of EMZAP treatment groups were decreased significantly compared with the Model group ($P< 0.05$). On the contrary, the expression of *PPARα*, *CPT-1* and *ACOX-1* in EMZAP treatment groups was increased compared with that in the Model Group ($P< 0.05$ and $P< 0.01$). There was no difference in the expression of *Adipo R2* among the groups.

EMZAP treatment modulates the composition of gut microbiota in HFD-induced mice

The V3-V4 region of the 16S rRNA genes in the feces was amplified after EMZAP treatment in HFD-induced mice. We observed that Sobs and Shannon indices did not change significantly among all the groups, implying that EMZAP treatment did not change the diversity and abundance in gut

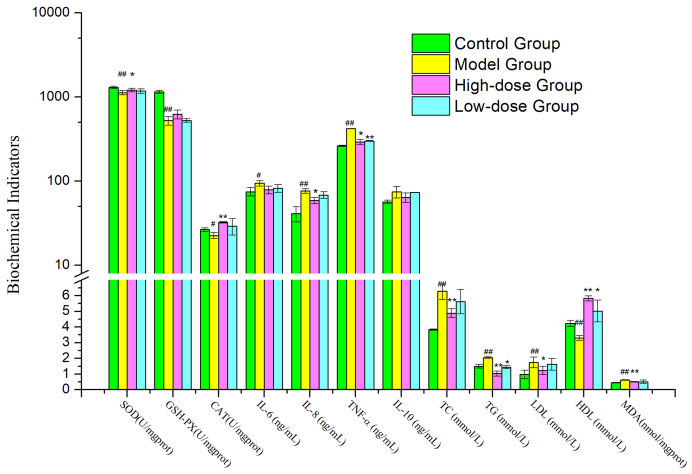

FIGURE 2
Effects of EMZAP on hepatic antioxidant capacity and serum biochemical indicators in mice (n = 10) *P< 0.05 and **P< 0.01 vs Model Group, $^{\#}P$< 0.05 and $^{\#\#}P$< 0.01 vs Control Group.

microbiota (Supplemental Figures 1A, B). In addition, the Shannon dilution curve tended to be flat, indicating that the increase in the sequencing depth did not affect species diversity, as a result, the amount of sequencing was saturated (Supplemental Figure 1B).

A Venn diagram in microbial diversity sequencing showed the number of shared and unique OTUs between samples (Figure 5A). A total of 560 OTUs were identified in all samples. The column chart in Figure 5A showed that the microbial species composition of Model Group was less than that of the Control Group at the OTU level. However, EMZAP could increase the microbial species composition of gut microbiota in High-dose Group and Low-dose Group, which were close to that of Control Group. The Venn diagram shows that the number of OTUs shared among the four groups accounted for 44.6%. The number of different OTUs between Control Group and Model Group accounted for 44.2%, whereas that between High-dose Group/Low-dose Group and Model Group accounted for 25.6% and 24.2%, respectively.

At the phylum level, the *Firmicutes* contents in the Control, Model, High-dose, and Low-dose Groups were 20, 73.39, 70.94, and 68.4%, and the *Bacteroidetes* contents were 74.26, 7.5, 15.39, and 6.62% respectively. The corresponding *Firmicutes*/*Bacteroidetes* (F/B) ratios were 0.28, 10.47, 4.73 and 10.30, respectively (Figure 5B). Compared with the Control Group, the *Firmicutes* content in the Model Group increased, while the proportion of *Bacteroidetes* was significantly decreased. Compared with the Model Group, the *Firmicutes* proportion in the High-dose Group was reduced, while the *Bacteroidetes* content was significantly increased. The F/B ratio in the High-dose Group was decreased compared with that in the Model Group. Most of the recent studies report that more *Firmicutes* than *Bacteroides* can result in more effective intestinal absorption of calories in food, resulting in obesity. The F/B ratios reflect the degree of gut microbiota disorder (Chen et al., 2021; Gao et al., 2021). Based on these results, the High-dose EMZAP can significantly improve the disorder of the gut microbiota.

At the genus level, there were obvious changes in the abundance of gut microbiota. (Figure 5C). The top 10 relative abundances at the genus level were identified to determine the differences in the gut microbiota across all groups. As presented in Table 1, the relative abundance of *norank_f_Muribaculaceae* and *Lactobacillus* significantly decreased (P< 0.01 and P< 0.05), whereas that of *Faecalibaculum*, *Lachnospiraceae_NK4A136_group*, *Helicobacter*, *norank_f_Desulfovibrionaceae*, *unclassified_f_Lachnospiraceae* and *norank_f_Oscillospiraceae* significantly increased in the HFD-fed mice (P< 0.01 and P< 0.05). Following the EMZAP treatment, the relative abundance of *Lactobacillus* significantly increased (P< 0.05) and that of *Faecalibaculum*, *Lachnospiraceae_NK4A136_group* and *Helicobacter* significantly decreased in the High-dose and Low-dose Groups compared with Model Group. The clustering heatmap generated from the species richness at the genus level was shown in Figure 5D. Compared with the Control Group, the Model Group and Low-dose Group had different gut microbiota composition and structure, which was modified by High-dose Group but remained different from the Control Group. According to these results, EMZAP had an effect on the intestinal microflora and reversed dysbacteriosis in the HFD-fed mice.

PCA and hierarchical clustering were used to evaluate the differences and similarities in the development of gut microbiota. The PCA results showed an obvious separation between the Control Group and HFD groups. Compared with the Model Group, the High-dose Group significantly separated

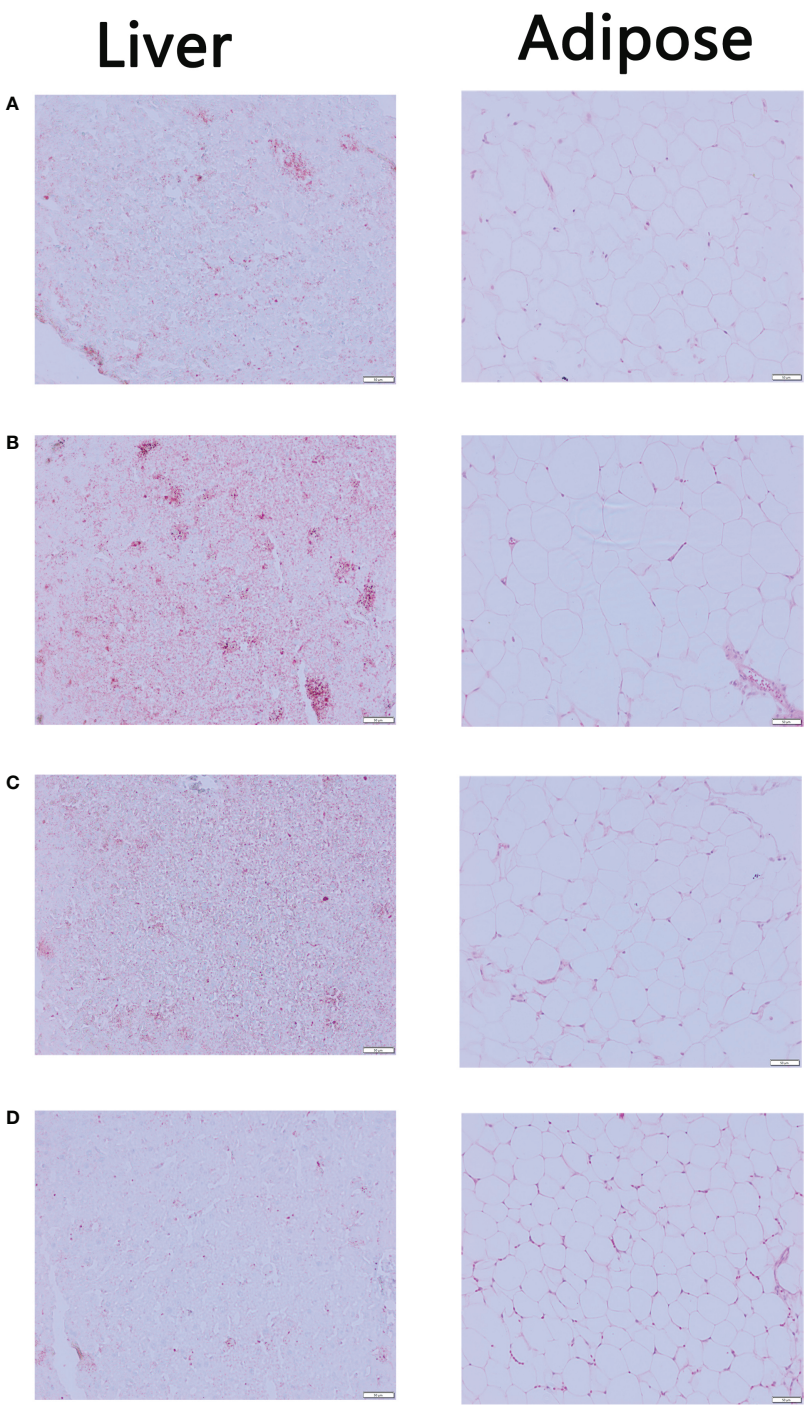

FIGURE 3
The mice liver sections stained by Oil red O and adipose sections stained by HE (×200) **(A)** Control Group **(B)** Model Group **(C)** Low-dose Group **(D)** High-dose Group.

(Figure 5E). In line with the expectations, the EMZAP intervention had a significant impact on the composition of the gut microbiota and could change the microbial composition in a dose-dependent manner. Furthermore, we compared the similarity and differences in the relationships between multiple samples using hierarchical clustering. As seen in Figure 5F, the four groups of gut microbiota could be classified into two groups based on the cluster tree analysis as follows: group I (Control

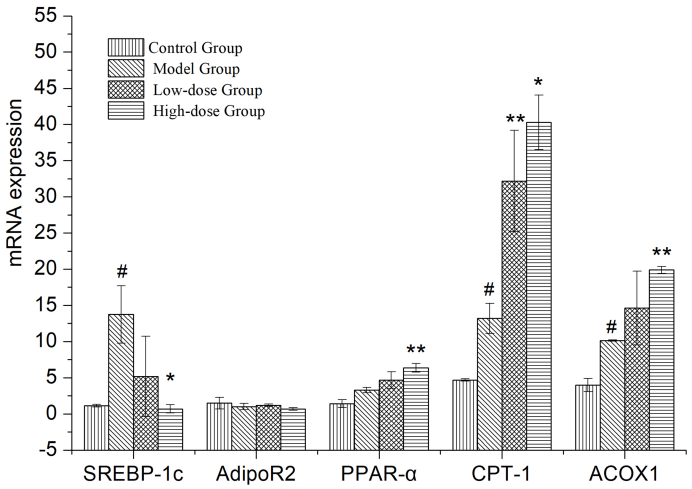

FIGURE 4
Effects of EMZAP on the expressions of relevant genes in the mice liver (n = 10) *P< 0.05 and **P< 0.01 vs Model Group, $^{\#}P$< 0.05 and P< 0.01 vs Control Group.

Group) and group II (HFD groups), and EMZAP treatment groups (High-dose/Low-dose Group) remained close to the group I, which was consistent with the results of PCA analysis. Results showed that EMZAP had significant effects on the intestinal flora of mice, and could propagate the intestinal flora abundance at the species level.

EMZAP treatment modulates key intestinal microflora in HFD-induced mice

In this study, the intestinal microbial community structure in different groups were analyzed. The multilevel difference discriminant analysis (LEfSe) of different groups were showed in Figure 6A, the High-dose Group obtained the most differential microorganisms. Differences of the microbiota genus in different groups were shown in Figures 6B–D. Results showed that Model Group had more abundance of *Faecalibaculum*, *Lachnospiraceae_NK4A136_group*, *norank_f_Desulfovibrionaceae*, *unclassified_f_Lachnospiraceae*, *Helicobacter*, *norank_f_Oscillospiraceae*, *Oscillibacter*, *Colidextribacter*, *norank_f_Lachnospiraceae* than those in the Control Group. Compared with the Model group, mice of the Control Group were characterized by a higher amount of *norank_f_Muribaculaceae* (Figure 6B). Notably, supplementary of high-dose EMZAP markedly increased the relative abundance of *Bacteroides*, *Alistipes*, *Lactococcus*, *Streptococcus* and *Acetivibrio_ethanolgignens_gruop*, but decreased the relative abundance of *Oscillibacter*, *Coriobacteriaceae_UCG-002*, *Romboutsia*, *Intestinimonas* and *unclassified_f_Christensenellaceae* in the HFD-fed mice (Figure 6C). Compared with the Model Group, the Low-dose Group was characterized by higher abundance of *Helicobacter*, *Acetivibrio_ethanolgignens_gruop* and *UBA1819* but lower amount of *Faecalibaculum*, *Lachnospiraceae_NK4A136_group*, *Coriobacteriaceae_UCG-002*, *Odoribacter*, *Intestinimonas* and *GCA-900066575* (Figure 6D). In addition, the mice treated with EMZAP showed a considerable decrease in the relative abundance of *Oscillibacter*, *Faecalibaculum* and *Lachnospiraceae_NK4A136_group*, which was also infrequent in Control Group.

Discussion

In the global context, NAFLD was becoming increasingly common, and it can progress to cirrhosis. There were a number of health problems associated with the increasing incidence of NAFLD (Chen et al., 2020). In our previous study, we found that EMZAP could reduce liver damage and improve liver function (Zhu J et al., 2021). In this paper, we showed that EMZAP was effective in reducing HFD-induced NAFLD by inhibiting lipid accumulation, reducing oxidative stress and inflammation, and increasing intestinal flora proliferation and changed its composition.

The increase in the organ body ratio indicates organ congestion, edema, hyperplasia, and hypertrophy, whereas a decrease indicates organ atrophy and other degenerative changes. NAFLD was a metabolic disease caused by excessive accumulation of fat in the abdomen and liver, which may result from HFD (An et al., 2021). The results of our present study

TABLE 1 Changes of the gut microbiota in the top 10 relative abundances at the genus level.

Microorganisms	Control Group	Model Group	High-dose Group	Low-dose Group
norank_f_Muribaculaceae	0.656 ± 0.116	0.044 ± 0.0314$^{\#\#}$	0.105 ± 0.075	0.035 ± 0.0121
Faecalibaculum	0.00163 ± 0.00293	0.211 ± 0.0553$^{\#\#}$	0.235 ± 0.192	0.0995 ± 0.0539*
Lachnospiraceae_NK4A136_group	0.0383 ± 0.0534	0.146 ± 0.0206$^{\#}$	0.0855 ± 0.0148*	0.0263 ± 0.0126**
Dubosiella	0.0638 ± 0.0715	0.0368 ± 0.0255	0.0713 ± 0.0296	0.064 ± 0.0406
Helicobacter	0.0183 ± 0.00974	0.0423 ± 0.0122$^{\#}$	0.0163 ± 0.0023*	0.112 ± 0.0723
norank_f_Desulfovibrionaceae	0.0008 ± 0.0001	0.0875 ± 0.0419$^{\#}$	0.066 ± 0.00432	0.065 ± 0.014
Lactobacillus	0.046 ± 0.0156	0.011 ± 0.00787$^{\#}$	0.0381 ± 0.0001*	0.0538 ± 0.0491
unclassified_f_Lachnospiraceae	0.0103 ± 0.0082	0.052 ± 0.0108$^{\#\#}$	0.044 ± 0.0299	0.0475 ± 0.0183
Blautia	0.00115 ± 0.0012	0.0148 ± 0.0104	0.015 ± 0.0109	0.114 ± 0.119
norank_f_Oscillospiraceae	0.00867 ± 0.00153	0.039 ± 0.0121$^{\#\#}$	0.0368 ± 0.0141	0.0483 ± 0.0114

*P< 0.05 and **P< 0.01 vs Model Group, $^{\#}$P< 0.05 and $^{\#\#}$P< 0.01 vs Control Group.

indicated that relative weight of liver and adipose in the treatment groups were smaller than those of the Model Group (Figure 1B). When combined with the blood lipid levels (Figure 2), it can be concluded that EMZAP reduced the blood lipid levels, incidence of atherosclerosis, obesity, and liver hypertrophy, thereby slowing down the occurrence and development of NAFLD in mice. The pathological sections stained with oil red O and HE also indicated that EMZAP could reduce fat accumulation in the liver and the abdomen (Figure 3).

Apart from lipogenesis and dyslipidemia, the pathogenesis of NAFLD was closely linked to oxidative stress and inflammation (Culafic et al., 2019). A close positive correlation exists between the total antioxidant capacity of the body's defense system and health (Zhang et al., 2021). Our previous studies reveal that EMZAP could increase antioxidant capacity and reduce oxidative damage in liver. (Zhu et al., 2021). The results of Figure 2 showed that the SOD, GSH-Px, and CAT activities in liver were markedly reduced in the HFD-fed mice, whereas the MDA levels in the liver were significantly rised. Moreover, the HFD also significantly increased the TNF-α, IL-6, IL-8, and IL-10 levels. The SOD, GSH-Px, and CAT activities levels in the EMZAP-treatment Groups were significantly higher than those in the Model Group, whereas the MDA, TNF-α, IL-6, IL-8, and IL-10 levels were lower than those in the Model Group. According to the results, EMZAP reduced oxidative stress and inflammation, which resulted in improved NAFLD in mice.

SREBP-1c mainly regulates the synthesis of fatty acid, which is a transcriptional regulator that maintains the liver lipid homeostasis. *PPARα* is a ligand-activated nuclear transcription factor that is widely expressed in the liver. It mainly regulates the oxidation and transportation of fatty acids, as well as lipid storage (Shen et al., 2021). *ACOX-1*, a downstream gene regulated by *PPARα*, encodes for an oxidation-related enzyme in fat cells and fatty acids. Meanwhile, it is the starting enzyme of the β-oxidation system in the peroxisome. *CPT-1* is the rate-limiting enzyme in the oxidation process of fatty acid β. It is located in the outer membrane of mitochondria, which serves as the key regulatory site of fatty acids entering the mitochondria (Pi et al., 2021). The High-dose Group significantly reduced the synthesis of fatty acids and maintained the same oxidation and transportation of fatty acids as that in the Model Group simultaneously. The Low-dose Control Group could accelerate the oxidation and transportation of fatty acid and maintain the same amount of fatty acid synthesis as that in the Model group simultaneously. This finding suggested that EMZAP could prevent NAFLD by regulating the fatty acid metabolism. In addition, as an insulin-hypersensitive hormone, *Adipo R2* could increase and promote fatty acid oxidation and glucose absorption of the skeletal muscle cells, thereby significantly enhancing the inhibitory effect of insulin on gluconeogenesis and inhibiting the glucose production in the liver. It is an important regulator of the regulation network of lipid metabolism and blood glucose homeostasis (Aksoy et al., 2019). EMZAP does not affect the expression of *Adipo R2*, indicating the lack of significant effect on glucose metabolism in the liver.

A change in gut microbiota played a significant role in the development of NAFLD. There is a possibility that this imbalance of gut microbiota is directly related to lipid disorders and steatosis in the liver. (Zhang et al., 2019). When compared with healthy individuals, the composition of intestinal flora was significantly different in NAFLD patients. In addition, 90% of the human intestinal flora was *Firmicutes* and *Bacteroidetes*. The ratio of two dominant phyla has been used as a marker of microbial dysregulation in some studies. Changes in this ratio have also been found in several metabolic disorders such as type 2 diabetes (T2D) and NAFLD. Some studies have shown that there was a strong positive correlation between *Firmicutes/Bacteroidetes* ratio and hepatic steatosis (Jasirwan et al., 2021; de Vos et al., 2022). In this study, α-Diversity and species composition analyses revealed that the EMZAP mainly affects the composition, instead of the abundance of the gut microbiota in mice. The Model Group showed the highest ratio

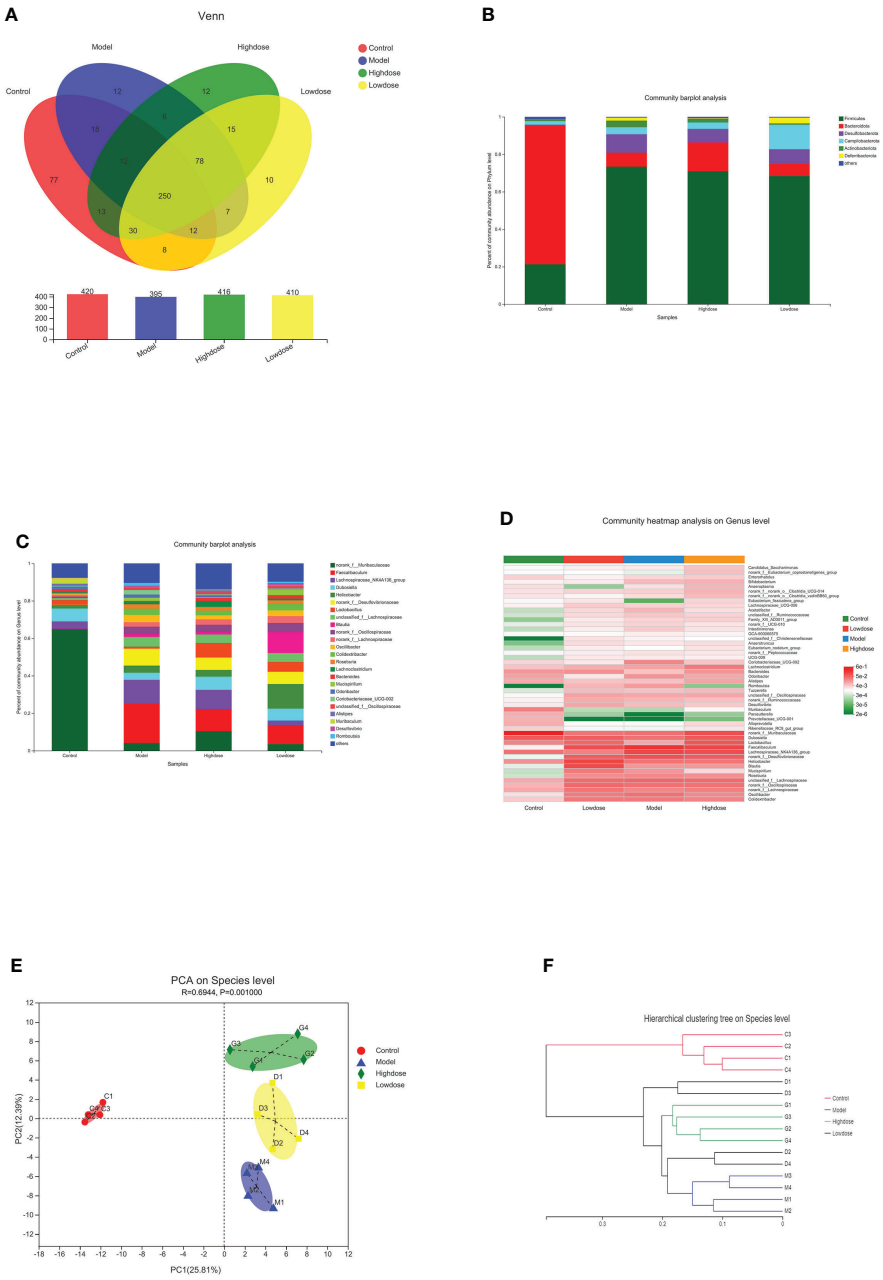

FIGURE 5
Composition of gut microbiota. **(A)** Venn diagram. **(B)** Community barplot analysis on phylum levels. **(C)** Community barplot analysis on genus levels. **(D)** Cluster heatmap of species richness at the genus level. **(E)** PCA on species level. **(F)** Hierarchical clustering tree on species level.

of F/B and the highest degree of gut microbiota disorder. The EMZAP treatment could significantly reduce the degree of disorder. In addition, when treated with EMZAP, the relative abundance of beneficial strains, including those of *Lactobacillus, Bacteroides* and *Alistipes* increased significantly. Meanwhile, the relative abundance of inimical bacteria, including those of *Faecalibaculum*, *Lachnospiraceae_NK4A136_group*, *Oscillibacter* and *Helicobacter*, decreased significantly. The PCA map at the species level also exhibited that the ethanol extract treatment could restore the gut microbiota disorder induced by a high-fat diet in mice. This finding can be attributed to the fact that high fat reduces the species composition of gut microbiota in mice, resulting in gut microbiota disorder. Nonetheless, EMZAP treatment could restore the microbial composition and reduce the degree of disorder.

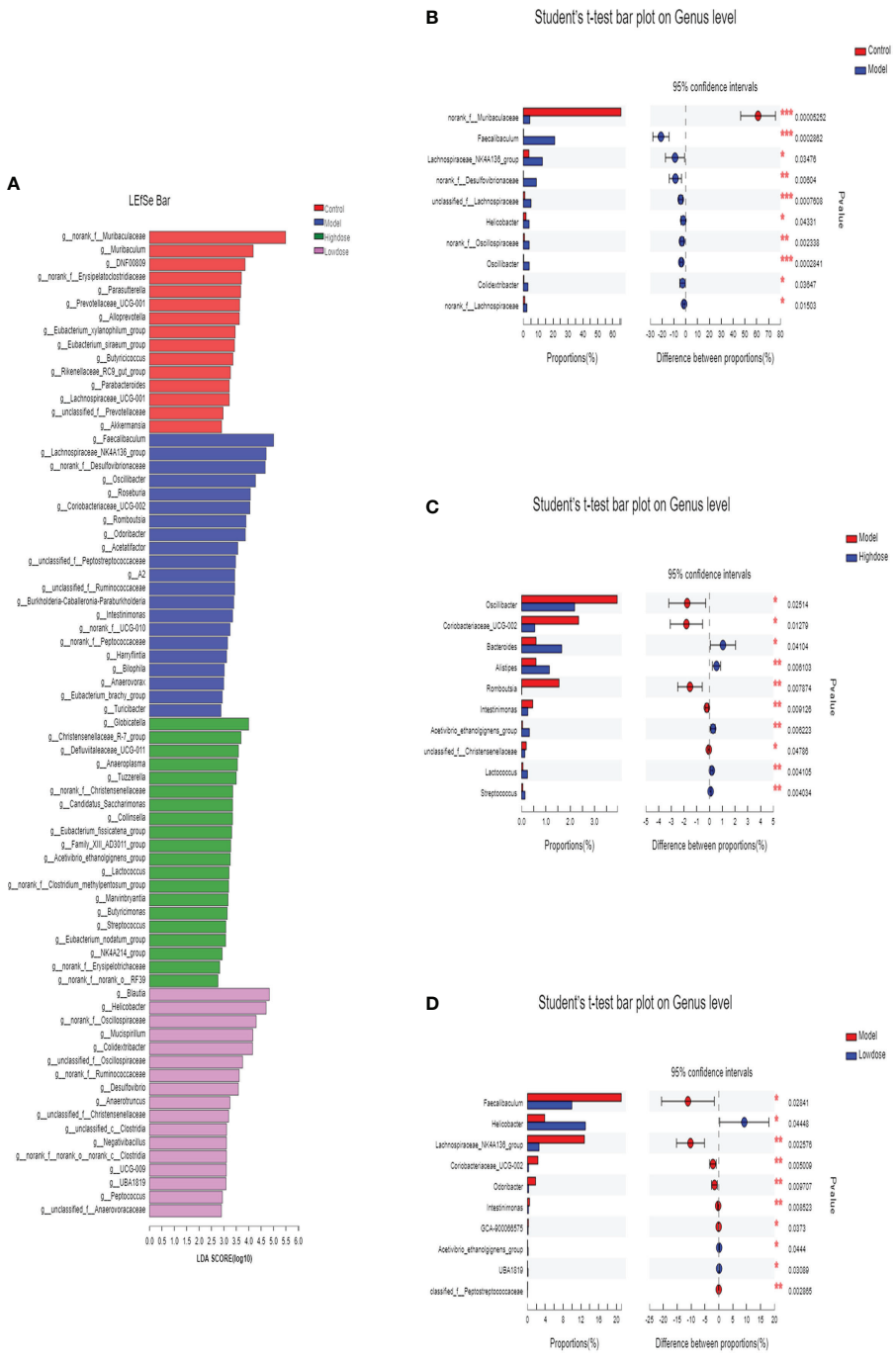

FIGURE 6
Differences of the microbiota genus in different groups. **(A)** LEfSe. Enriched taxa with an LDA score >2.0 was shown in the histogram. **(B)** Control (red) versus Model (blue). **(C)** Model (red) versus High-dose (blue). **(D)** Model (red) versus Low-dose (blue). *P < 0.05 , **P < 0.01 and ***P <0.001.

Conclusions

The present results demonstrated that EMZAP exerts a certain therapeutic effect on NFALD. In the gavage experiment with mice, EMZAP alleviated weight gain caused by a high-fat diet, reduce liver and fat indexes, and reduce the levels of blood lipids to slow down the occurrence of fatty liver. Pathological section analyses showed that EMZAP could significantly reduce the accumulation of fat in the liver and the abdomen of mice. According to the experimental results, the following 3

mechanisms may be responsible for EMZAP-induced modulation in NFALD: 1) reducing the pro-inflammatory factors in serum (i.e., IL-6, IL-8, TNF-α, and TGF-β_1) improved the activity of liver antioxidant enzymes and the antioxidant capacity of the liver and reduced the occurrence of the inflammatory reaction in mice. 2) by regulating the expression of genes associated with fatty acid metabolism (*SREBP-1c, PPARα, ACOX-1*, and *CPT-1*) in the liver, it regulates the synthesis and oxidative decomposition of fatty acids to prevent NAFLD. 3) by regulating the intestinal microenvironment, maintains the gut microbial diversity within the normal range. Furthermore, EMZAP increased the richness of the beneficial intestinal microorganisms.

To summarize, this study evaluated the interventional effect and the mechanism of EMZAP on NFALD in mice. This study not only provides a theoretical basis for the comprehensive development and utilization of tussah and sericulture industries but also indicates innovative ways for the prevention and treatment of NAFLD.

Data availability statement

The data presented in the study are deposited in the SRA database repository, accession number PRJNA890373. The data will be accessible with the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA890373.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of Shandong Academy of Agricultural Sciences. Shandong Academy of Agricultural Sciences.

Author contributions

LZ and GG conceived the study and raised the funding. LZ, NW and XJ designed the study. LZ, NW and XLJ designed the study. LZ, NW, ZQF and XQS performed the experiments. LZ analyzed the microbiome data and wrote the original manuscript and drafted it with substantial contributions from all other authors. All authors contributed to the article and approved the submitted version.

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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/fcimb.2022.1059647/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The Sobs index **(A)** and Shannon curve **(B)**.

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The impact of dietary fibers on *Clostridioides difficile* infection in a mouse model

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Diets rich in fiber may provide health benefits and regulate the gut microbiome, which affects the immune system. However, the role of dietary fiber in *Clostridioides difficile* infection (CDI) is controversial. Here, we investigated the use of fermentable fibers, such as inulin or pectin, to replace the insoluble fiber cellulose to explore how dietary fiber affects *C. difficile*-induced colitis in mice through intestinal microecology and metabolomics. Using *C. difficile* VPI 10463, we generated a mouse model of antibiotic-induced CDI. We evaluated disease outcomes and the microbial community among mice fed two fermentable fibers (inulin or pectin) versus the insoluble fiber cellulose. We analyzed and compared the gut microbiota, intestinal epithelium, cytokine levels, immune responses, and metabolites between the groups. Severe histological injury and elevated cytokine levels were observed in colon tissues after infection. Different diets showed different effects, and pectin administration protected intestinal epithelial permeability. Pectin also steadily increased the diversity of the microbiome and decreased the levels of *C. difficile*-induced markers of inflammation in serum and colonic tissues. The pectin group showed a higher abundance of *Lachnospiraceae* and a lower abundance of the conditionally pathogenic *Enterobacteriaceae* than the cellulose group with infection. The concentration of short-chain fatty acids in the cecal contents was also higher in the pectin group than in the cellulose group. Pectin exerted its effects through the aryl hydrocarbon receptor (AhR) pathway, which was confirmed by using the AhR agonist FICZ and the inhibitor CH2223191. Our results show that pectin alters the microbiome and metabolic function and triggers a protective immune response.

KEYWORDS

***Clostridioides difficile*, dietary fiber, pectin, microbiota, short-chain fatty acids**

Introduction

Clostridioides difficile infection (CDI) causes more than 70% of healthcare-associated gastrointestinal infections, with outcomes ranging from diarrhea, colitis, and severe toxic megacolon (Smits et al., 2016). The stable gut microbial community is a natural barrier against *C. difficile* (Ling et al., 2022). CDI may be caused by disruption of the resident intestinal microbiota. Antibiotic therapy is essential for treating bacterial infections. Most notably, repeated exposure of the intestinal microbiota to antibiotics eliminates commensal bacteria from the intestinal ecology and provides opportunities for pathogens, such as *C. difficile*, to colonize and proliferate. The metabolites of the intestinal flora, including short-chain fatty acids (SCFAs) and bile acids (Kochan et al., 2018), have been associated with *C. difficile*-induced disease. Therefore, diet may influence the incidence and severity of CDI. The gut microbiota and *C. difficile* metabolic interactions determine *C. difficile* fitness (Ng et al., 2013; Buffie et al., 2015; Yan et al., 2021), and reductions in the levels of dietary microbiota-related metabolites cause colon inflammation (Earle et al., 2015).

The Western diet includes high consumption of fatty foods and low consumption of naturally fiber-rich grains, fruits, and vegetables. Low dietary fiber intake may lead to impaired intestinal health and an increased prevalence of chronic inflammatory diseases. Dietary fiber can be broadly classified as insoluble (e.g., cellulose) or soluble (e.g., pectin, inulin), and the soluble fiber is readily fermented by intestinal bacteria to produce SCFAs (Singh et al., 2019). The transformation of dietary fiber into available nutrients is one of the main benefits that the gut microbiota provides to the host. The mechanisms underlying the association of dietary fiber with the development of intestinal inflammation and immune-related diseases have not been fully studied. A diet low in dietary fiber alters the gut microbiota and its metabolism, thereby disrupting host-microbiota interactions (Sonnenburg and Sonnenburg, 2014). Studies have reported conflicting results regarding the relationship between diet and CDI. Some have shown that dietary fiber can alleviate antibiotic-induced CDI (Hryckowian et al., 2018). Other studies suggest that dietary fiber may promote antibiotic-related ecological dysregulation and long-term *C. difficile* carriage (Bhute et al., 2022). Pectin may play a role by regulating the intestinal microbiota composition and T-cell responses (Bernard et al., 2015; Ishisono et al., 2019; Wu et al., 2019). Understanding the effects and potential mechanisms through which dietary carbohydrates influence CDI may offer useful insights into pathogenesis.

Here, we evaluated the effects of soluble dietary fibers (pectin and inulin) on CDI and described the flora diversity and metabolic structure. Thus, we tested whether soluble fiber is more beneficial than unfermentable fiber during CDI due to its ability to act as an SCFA precursor. We report that the dietary soluble fiber pectin ameliorates colitis in a *C. difficile*-related model. The effect of the pectin diet was also explored by targeting the aryl hydrocarbon receptor (AhR) pathway through which it may exert its protective effect.

Methods

Model of infection

C. difficile strain VPI 10463 (ATCC 43255) was cultivated in Difco cooked meat medium (BD Diagnostic Systems, USA) in an anaerobic atmosphere. C57BL/6 male mice (6-8 weeks) were housed and fed a standard laboratory diet for one week (Figure 1A). The mice were then randomly divided: two groups were fed a cellulose control diet (CNC=8 and CCDI=12), and the remaining groups were fed a pectin diet (PCDI=12, PNC=8) and an inulin diet (ICDI=12, INC=8). Table S1 showed the details of the three isocaloric diets (Dyets Inc., Bethlehem, PA, USA, Cat D211015, D211016, and D211017). After 2 weeks of feeding, the three groups (CCDI, PCDI, and ICDI) were modeled for *C. difficile* infection as previously described (Chen et al., 2008). The experimental scheme consisted of 5 days of antibiotic cocktail water including kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml) and vancomycin (0.045 mg/ml). Clindamycin (10 mg/kg, ip, D -1) was administered after the next 2 days of normal water intake. The animals in the CNC group were injected intraperitoneally with phosphate-buffered saline (PBS), which was the vehicle control for clindamycin. On D0, all animals except those in the CNC group received 10^8 CFU of *C. difficile*. The mice were observed, and disease symptoms and diarrhea were recorded. The mice were euthanized on D6.

Histopathological analysis

The colon samples were embedded in paraffin and cut into 4 μm sections. Then, they were stained with hematoxylin and eosin (H&E), and histopathological scores were assessed according to a previous method (Chen et al., 2008). Goblet cells and the mucus layer were observed and evaluated by Alcian blue periodic acid Schiff (AB-PAS) staining. For immunofluorescence and immunohistochemical staining, the embedded colon sections were immunostained with antibodies against ZO-1, F4/80, and Ly6G according to the manufacturer's protocol.

Measurement of serum cytokine and endotoxin levels

Serum endotoxin lipopolysaccharide (LPS) levels were quantified using the limulus amebocyte lysate (LAL) (Hycult

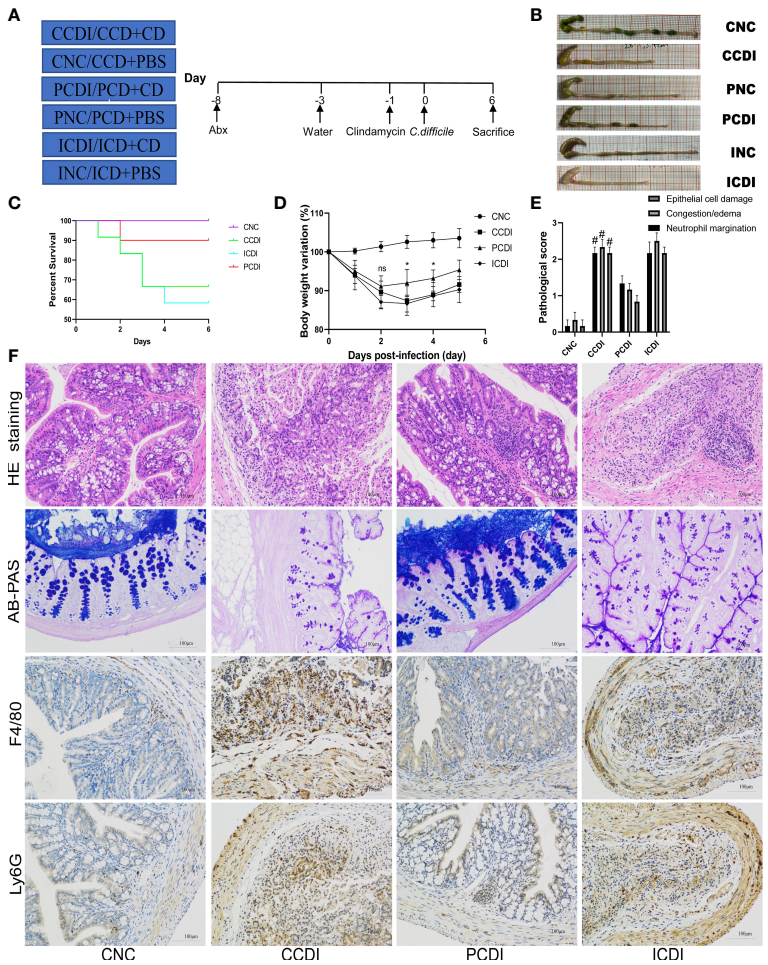

FIGURE 1
A pectin diet protects mice from *C. difficile* infection. **(A)** Experimental design diagram; CDI modeling after two weeks of dietary intervention. **(B)** Representative images of the colon. **(C)** Body weight change curves of the mice. **(D)** Survival curves of different groups. **(E, F)** Pathological analysis and histopathological scoring of colon tissue of the mice by H&E staining, AB-PAS staining and immunohistochemistry (F4/80 and Ly6G). #, CCDI vs PCDI, $P<0.05$; ns, differences are not significant. CNC, cellulose diet with control group; CCDI, cellulose diet with *C. difficile* infection; PNC, pectin diet with control group; PCDI, pectin diet with *C. difficile* infection; INC, inulin diet with control group; ICDI, inulin diet with *C. difficile* infection.

Biotech, USA) assay kit. The concentration of LPS-binding protein (LBP) was measured with an ELISA kit from Abcam (Cambridge, MA, United States). The levels of serum cytokines, including G-CSF, IL-1α, IL-6, TNF-α, IL-1β, and MIP-1α, were analyzed by a cytokine assay kit (Bio-Rad, CA, USA).

qRT–PCR analysis

Colon tissue RNA was extracted using the RNeasy Plus Mini kit (Qiagen, CA, USA). RNA was reverse transcribed into cDNA using PrimeScript RT kits (Takara Biomedicals, Japan). mRNA expression was then repeatedly determined using the ViiA7 real-time PCR system (Applied Biosystems, Massachusetts, USA) with Premix Ex Taq (Takara Biomedicals). All gene expression levels were normalized to β-actin expression levels. Primer information is provided in Table S2.

Sequencing of 16S rRNA

Fecal samples were collected prior to sacrificing the mice. The DNeasy Powersoil Pro Kit was used to extract DNA (Qiagen, Hilden, Germany). PCR amplification of the 16S rRNA gene was performed using modified primers. Sequencing was performed on the Illumina MiSeq platform (Gu et al., 2020). The raw tags were filtered according to QIIME, and chimeric sequences were removed by comparison with the Silva database. Operational taxonomic units (OTUs) were determined as sequences with at least 97% identity.

Metabolic profiling

The cecal contents were collected and stored at -80°C. Metabolomic samples were prepared as described previously (Bian et al., 2020). Metabolites were analyzed by gas chromatography-mass spectrometry (GC–MS) using an Agilent 7890A GC system coupled with an Agilent 5975C inert mass selective detector system (Agilent Technologies, Santa Clara, CA). Metabolites were identified using Lumingbio's untargeted GC–MS database. Partial least squares discrimination analysis (PLS-DA), orthogonal PLS-DA (OPLS-DA), and projection (VIP) values were used to calculate the significance of variables.

The SCFAs in the cecal contents were detected by GC–MS analysis of 20 mg of dry weight contents according to a previously described experimental procedure (Bian et al., 2020).

AhR agonist and antagonist treatments

For the AhR agonist intervention, mice were administered 6-formylindolo [3,2-b] carbazole (FICZ; Sigma, Germany) by intraperitoneal injection (1 μg/mouse) once a week. For AhR antagonist intervention, mice were administered 2-methyl-2H-pyrazole-3-carboxylic acid (CH223191; Sigma, Germany) by intraperitoneal injection (10 μg/mouse) once a week (Monteleone et al., 2011).

Statistical analysis

Data analysis was performed using GraphPad Prism v9.0.0. The data are presented as the mean ± the standard error of the mean (SEM). For the determination of statistical significance, a one-way analysis of variance (ANOVA) followed by Tukey's test was used. P values < 0.05 indicated statistical significance.

Results

A pectin diet provides protection against *C. difficile* infection

To explore the effect of dietary fiber on CDI, we fed mice a diet (Table S1) containing 10% cellulose (CNC and CCDI groups), or three-quarters of the fiber was changed to inulin (INC and ICDI groups) or pectin (PNC and PCDI groups) (Figure 1A). After 2 weeks of feeding, CDI modeling was performed. All infected mice (CCDI, ICDI, and PCDI groups) showed typical symptoms of infection, while the uninfected mice in the control group (CNC, INC, and PNC) remained healthy, and no mice died or showed signs of infection during the experiment. Typical clinical symptoms of infection were significant weight loss (Figure 1D) and diarrhea. These effects were further exacerbated in the ICDI group, which showed weight loss and more diarrhea than in the CCDI group, but only mild symptoms were observed in the PCDI group. Survival rates differed significantly between the pectin-fed mice and the animals in all other infectious groups, and pectin protected the mice (Figure 1C). Proximal colon tissue showed a better appearance and colon length in the PCDI group than in the CCDI group (Figure 1B). Histopathological analysis showed significantly greater pathology in all infected mice than in uninfected mice (Figure 1F). Histological analysis showed that CCDI group and ICDI group mice exhibited typical features of colitis, including a thickened colon wall, distorted crypt structures, and infiltration of inflammatory cells in the mucosa. The pathology and scores in the PCDI group were significantly better than those in the CCDI and ICDI groups (Figure 1E). Goblet cells secrete mucus to protect the colon against pathogens. AB-PAS staining showed that the CCDI group exhibited significantly less mucus secretion and fewer goblet cells than the normal group (Figure 1F). The pectin dietary intervention significantly alleviated the *C. difficile*-induced reduction in mucus and goblet cells. Immunofluorescence staining of macrophages (F4/80+) and neutrophils (Ly6G+) in colon tissue was also performed, demonstrating that infection could cause the infiltration of macrophages and neutrophils in colon tissue and that pectin reduced this inflammatory phenomenon (Figure 1F).

Pectin ameliorates intestinal barrier injury induced by *C. difficile* infection

C. difficile produces toxins and following *C. difficile* infection, the intestinal barrier is defective. Intestinal barrier disruption leads to increased intestinal permeability and endotoxin translocation. To assess the bacterial translocation caused by increased intestinal permeability, we further examined the serum LAL and LBP levels (Figures 2C, D), and *C. difficile* infection led to increased serum LAL and LBP levels in the CCDI group, while the pectin diet decreased the endotoxin response of the organism. Thus, serum immunoreactivity to the bacterial product LPS was increased in both the CCDI and ICDI groups of mice. We also examined the results of immunofluorescence staining for the intestinal barrier protein ZO-1 and evaluated the expression of intestinal barrier indicators (ZO-1 and Occludin) by qPCR. We found that the intestinal barrier was compromised in the CCDI group, while pectin (PCDI group) protected *C. difficile*-infected mice against damage to the intestinal barrier (Figure 2A) and rescued the mRNA levels of tight junction proteins (Tjps) in colonic tissue (ZO-1 and Occludin, P<0.05; Figure 2B), while no alleviation was observed in the inulin group. These results indicate that pectin protects mice from infection *via* mucosal barrier enhancement.

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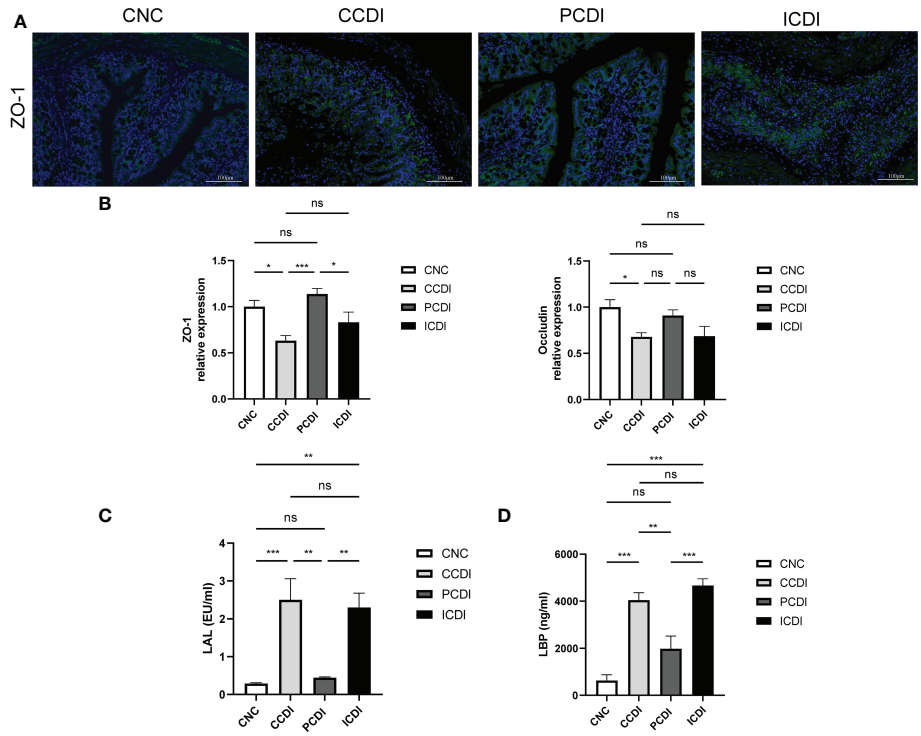

FIGURE 2
Pectin enhances intestinal barrier function. **(A)** Immunofluorescence staining of colon tissue. **(B)** Relative mRNA levels of ZO-1 and Occludin in colon tissues measured by qPCR. **(C, D)** Levels of the inflammatory markers LAL and LBP in mouse serum. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, differences are not significant. LAL, limulus amebocyte lysate; LBP, lipopolysaccharide-binding protein.

Pectin attenuates the serum and intestinal inflammatory response in *C. difficile* infection

The production of toxins by *C. difficile* causes inflammation in the intestine (Kordus et al., 2022). We assessed the immune status by measuring the levels of cytokines reflecting immune cell signaling activity. The mRNA levels of serum cytokines and intestinal inflammatory factors were examined in *C. difficile*-infected mice fed different diets. The results showed that the levels of serum cytokines, such as G-CSF, IL-1α, IL-6, TNF-α, IL-1β, and MIP-1α, were higher in the CCDI group than in the CNC group (Figure 3A). Interestingly, the levels of inflammatory factors (IL-1α, IL-6, TNF-α, and MIP-1α) were decreased in the PCDI group ($P<0.05$). We also assessed the mRNA levels of inflammatory factors in intestinal tissues and showed that the expression of inflammatory biomarkers (TNF-α, IL-1α, and IL-1β) was higher in the CCDI group (Figure 3B) than in the CNC group, while the pectin diet improved the intestinal inflammation levels. In conclusion, these results suggest that colon inflammation induced by *C. difficile* infection was alleviated in the PCDI group but not significantly alleviated in the ICDI group.

Different fibers have different effects on the gut microbiota

Considering the different effects of inulin and pectin on the degree of colitis, we compared the microbiota composition that might underlie or correlate with such differences. To analyze the diet-induced changes in the intestinal flora, we used 16S rRNA sequencing to compare the stool samples (CNC=8, PNC=8, INC=8, PCDI=8, CCDI=8, and ICDI=7). Through comparison of the α-diversity (Chao1 and Shannon index), we found that *C. difficile* infection decreased gut microbial diversity, while pectin increased diversity (Figure 4A). β-diversity (PCoA based on unweighted UniFrac) indicated differences in gut flora structure between groups (Figure 4B).

To identify the characteristic microorganisms, we performed linear discriminant analysis effect size (LEfSe) analysis. LEfSe showed that the CCDI group exhibited higher abundances of opportunistic pathogenic bacteria, such as *Enterobacteriaceae*, *Peptostreptococcaceae*, and *Enterococcaceae*, and lower abundances of *Lactobacillaceae*, *Bifidobacteriaceae*, *Muribaculaceae*, *Rikenellaceae*, *Akkermansiaceae*, and *Desulfovibrionaceae* at the family level than the CNC group (Figure 4C and Figure S1). The

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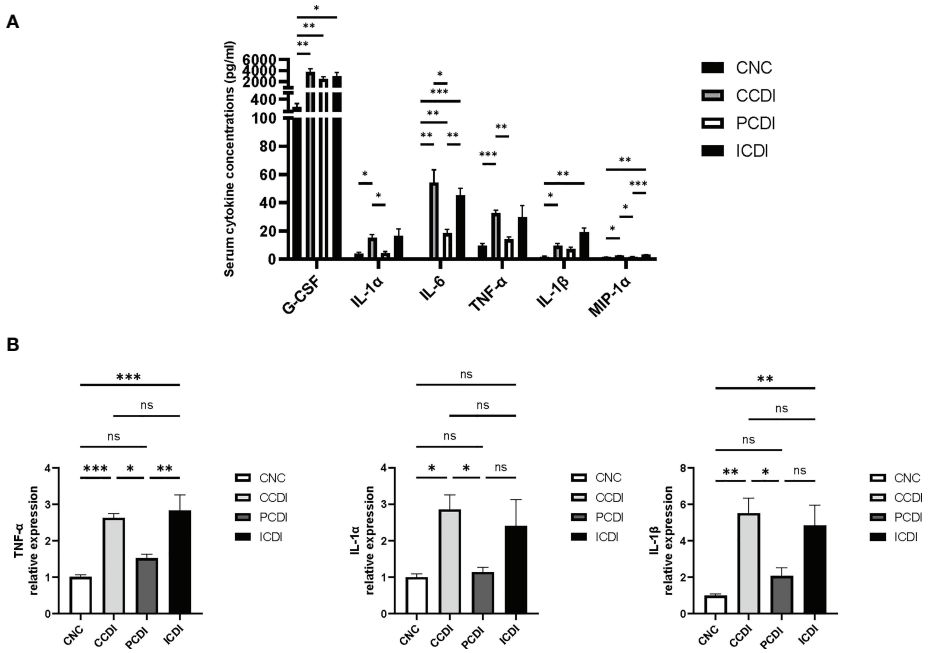

FIGURE 3
Pectin attenuates the systemic and intestinal inflammatory responses in mice with CDI. **(A)** Serum cytokine levels in the mice. **(B)** mRNA expression levels of TNF-α, IL-1α, and IL-1β in mouse colonic tissues. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, differences are not significant.

relative abundance of *Enterobacteriaceae* was lower in the PCDI group than in the CCDI group (Figure 4D). We observed higher abundances of *Lachnospiraceae* and *Blautia* in the PCDI group than in the CCDI and the ICDI groups; these bacteria reduce intestinal inflammation and promote intestinal health (Figure 4D and Figure S2). Bacteria known to readily metabolize fibers into SCFAs, including *Lachnospiraceae*, are preferentially enhanced by pectin (Riviere et al., 2016). Our results show that the pectin diet increased the abundance of these SCFA-producing bacteria over the inulin diet.

Distinct fibers differentially impact the composition of metabolites

To explore the relationship between changes in intestinal metabolites and *C. difficile* intestinal damage, we performed metabolomic analysis using GC–MS analysis of the cecal contents obtained from the four groups. A total of 632 metabolites were identified. The PLS-DA plot showed that each group's metabolomic profile was distinct (Figure 5A), indicating that the metabolomic composition of these groups was different. OPLS-DA plots showed the differences in metabolomic composition between the CCDI group and the CNC group and between the PCDI group and the CCDI group (Figures 5B, C).

Using VIP>1 (based on the OPLS-DA model) and $P<0.05$ between groups, we further explored the different metabolic profiles in the PCDI and CCDI groups. The heatmap showed that the characteristic metabolites between the PCDI and CCDI groups were mainly related to carbohydrates, amino acids, lipids, etc. (Figure 5E). The differential metabolic pathways included the biosynthesis of unsaturated fatty acids, the cAMP signaling pathway, glycine, serine and threonine metabolism, and inflammatory mediator regulation of TRP channels. The characteristic metabolites in the two groups are shown (Figure 5D). The PCDI group was associated with relatively higher levels of the amino acids and bile acids required to inhibit the germination and growth of *C. difficile*, such as chenodeoxycholic acid, than the CCDI group. The PCDI group also showed reduced levels of linolenic acid and aconitic acid. Dehydroabietic acid, adenine, adipic acid, and 2-ketobutyric acid levels were relatively increased in the PCDI group (Figure 5D). We also compared and analyzed the different metabolic profiles that may lead to different efficacies of pectin and inulin diets. Compared to the ICDI group, the PCDI group reduced carbohydrates (e.g. trisaccharide, maltotriose, erythrose, gluconic acid, and galactitol) along with amino acids (valyl-glycine, alanyl-threonine, L-proline, and valyl-valine). In contrast, the PCDI group increased L-aspartic acid, tartaric acid, spermidine, L-phenylalanine, and cholic acid (Figure S3).

Pectin is known to affect the SCFAs involved in T-cell immunity (Smith et al., 2013). To explore whether the protective effect of dietary fibers against infection is related to the major products of fiber metabolism of the microbiome, such as SCFAs, we examined the SCFAs, such as acetic acid, propionic

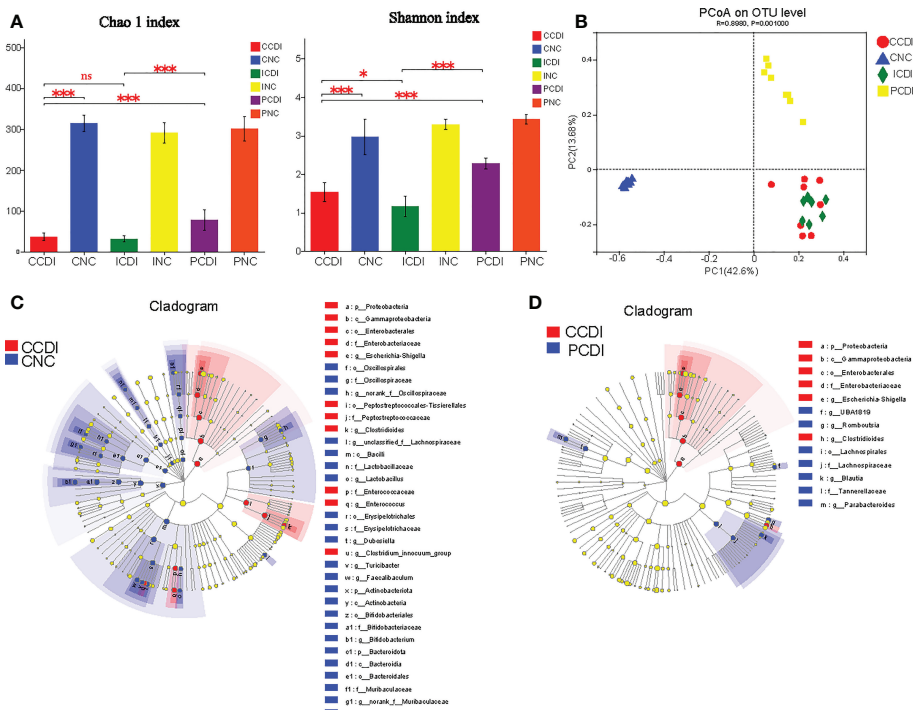

FIGURE 4
Pectin alleviates the dysbiosis of intestinal flora in CDI. **(A)** Chao1 and Shannon indices of intestinal flora. **(B)** The PCoA plot based on unweighted UniFrac shows the β-diversity of the gut microbiome. **(C, D)** LEfSe cladogram. Blue represents the CNC or PCDI group, and red represents the CCDI group. *, $P<0.05$; ***, $P<0.001$; ns, differences are not significant. LEfSe, linear discriminant analysis effect size.

acid, isobutyric acid, butyric acid, 2-methylbutyric acid, and valeric acid, in mouse cecal contents. In the CCDI group, *C. difficile* infection reduced the levels of acetic acid, propionic acid, butyric acid, and 2-methylbutyric acid ($P<0.05$, Figure 5F). The concentrations of acetic acid, propionic acid, and butyric acid were higher in the PCDI group than in the CCDI group ($P<0.05$, Figure 5F). The levels of acetic acid were higher in animals fed the pectin diet than in those fed the inulin diet (Figure 5F). Furthermore, we conducted a correlation analysis between SCFAs and *Lachnospiraceae* (Figure S4). The results exhibit a good linear correlation between the relative abundance of *Lachnospiraceae* and SCFAs (such as acetic acid and butyric acid).

Pectin protects against *C. difficile* infection by activating the AhR pathway

Previous studies have shown that catabolic substances produced by the microbiota can modulate interleukin (IL)-22 production and boost T-cell immunity by activating AhR (Ye et al., 2017; Lamas et al., 2018), which plays a role in mucosal immunity (Zelante et al., 2013). Previous studies have shown that pectin increases the production of metabolites by the microbiota to activate AhR (Monteleone et al., 2011), thereby improving intestinal barrier function. Our gut microbiota analysis also showed that pectin treatment increased *Lachnospiraceae* abundance in the intestine, which is also involved in the activation of the AhR pathway by metabolic substances (Vacca et al., 2020; Zhang et al., 2021). We next determined the role of the AhR pathway in the pectin treatment of *C. difficile*-induced severe colitis. IL-22, secreted by CD3+ T cells and ILC3s in the intestine, has been shown to protect the host from infection as a downstream gene of the AhR pathway (Parks et al., 2015). We evaluated the expression levels of the relevant indicators (AHR and IL-22) in colon tissues of different groups by qPCR (Figures 6A, B). The results showed that the AhR and IL-22 mRNA levels were lower in the CCDI group than in the CNC group, while the pectin group showed increased levels.

We also explored the targeted therapeutic strategies of the AhR pathway in CDI using AhR agonists and inhibitors. The body weight in the *C. difficile*+pectin+AhR antagonist group was lower than that in the *C. difficile*+pectin group (Figure 6C), indicating that the AhR antagonist exacerbated the infection and clinical symptoms in the pectin diet-fed mice. The death of the pectin-treated mice was accelerated early after *C. difficile* induction because of the AhR antagonist (Figure 6D), but the effect of pectin pretreatment on the survival of the *C. difficile*-

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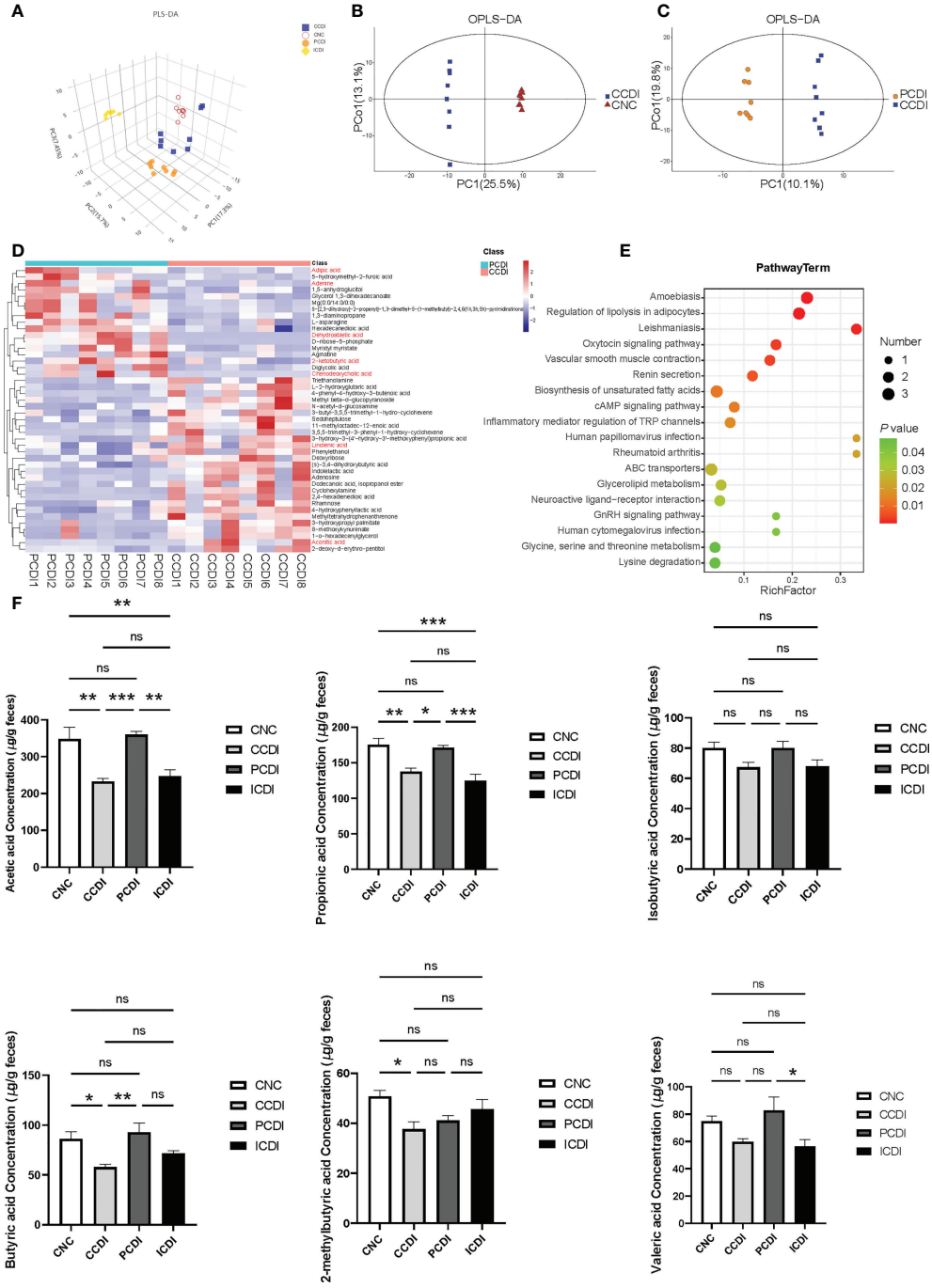

FIGURE 5
Pectin attenuates *C. difficile*-induced dysregulation of the intestinal metabolome. **(A)** Three-dimensional PLS-DA score plots of metabolome profiles for the four groups. **(B, C)** OPLS-DA plots between groups. **(D)** Heatmap for the selected metabolites in the CCDI and PCDI groups. **(E)** Pathway of the differentially abundant metabolites between the CCDI and PCDI groups. **(F)** SCFA levels in the cecal contents of mice. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, differences are not significant. PLS-DA, Partial least squares discrimination analysis; OPLS-DA, orthogonal PLS-DA. The red color showed valuable differential metabolites.

infected mice could not fully counteract the outcome. Next, the effect of the AhR agonist on *C. difficile* infection was evaluated. Body weight loss was lower in the *C. difficile*+AhR agonist-treated mice than in the *C. difficile*-treated mice (Figure 6F). AhR agonists increased the survival rate of infected mice (Figure 6G). We also assessed the expression of intestinal barrier indicators such as ZO-1, and the results showed that the AhR antagonist diminished the protective effect of pectin

(Figures 6E, H). In conclusion, these findings suggest that the roles of gut microbiota in infection after pectin treatment are partly mediated by the AhR pathway. These results confirm that the AhR-dependent pathway contributes to pectin diet-mediated protection against *C. difficile* infection in mice.

Discussion

The microbiota in the gut plays a crucial role in host health and provides resistance to a variety of intestinal pathogens (Sassone-Corsi and Raffatellu, 2015; Khan et al., 2022). The depletion of the gut microbiota caused by antibiotics can be exploited by pathogens such as *C. difficile* (Kriss et al., 2018). Diet affects the composition and function of the gut microbiota. Adequate intake of dietary fiber may play a beneficial role in enhancing intestinal immunity by regulating the gut microbiota (Makki et al., 2018). The study showed the effects of dietary composition on the physiology and pathogenesis of *C. difficile* in an animal model of antibiotic-induced CDI. Our study extensively assessed the functions of two soluble fibers, pectin and inulin, on a mouse model and the response of microbial communities to a diet with a widely varying nutrient composition following antibiotic treatment. Pectin was shown to improve the clinical outcomes of infection compared to inulin, and the mechanism underlying the relief in *C. difficile* infection may relate to anti-inflammatory effects, protection of the mucosal barrier, and maintenance of intestinal flora and metabolism homeostasis. Our study showed that inulin did not exhibit effective protection. However, a previous study showed a protective effect of inulin against *C. difficile* infection (Hryckowian et al., 2018). The different roles played by carbohydrates in *C. difficile* infection may be related to the type of carbohydrate and its concentration. The ability to

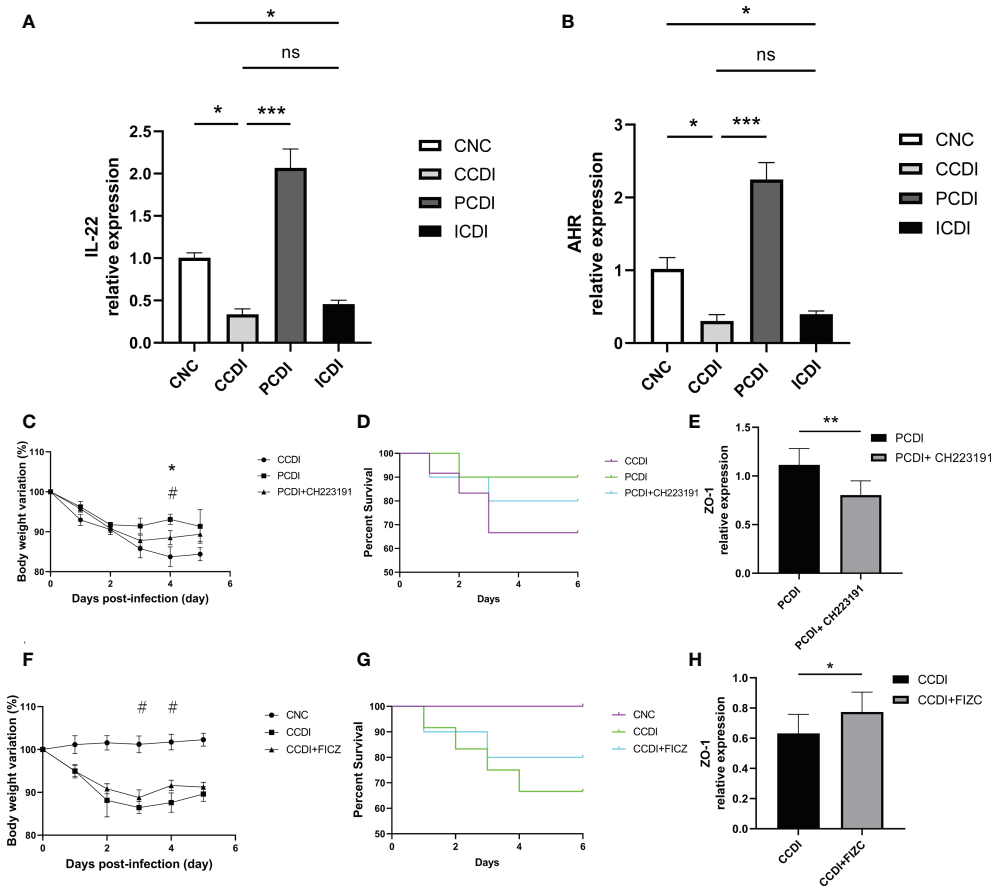

FIGURE 6
Pectin protects mice with CDI from intestinal infection by enhancing AhR pathway activation. **(A, B)** Relative mRNA levels of IL-22 and AhR in colon tissue. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, differences are not significant. **(C, D)** Body weight changes and survival curves of the mice with CDI treated with the AhR antagonist CH223191. *, CCDI vs PCDI+ CH223191, $P<0.05$; #, PCDI vs PCDI+ CH223191, $P<0.05$. **(E)** Relative mRNA levels of ZO-1 in colon tissues. **(F, G)** Body weight changes and survival curves of the mice with CDI treated with the AhR agonist FICZ. #, CCDI vs CCDI+FIZC, $P<0.05$. **(H)** Relative mRNA levels of ZO-1 in colon tissues.

target the microbiome through diet would enable microbiome-based modulation to ameliorate *C. difficile* infection.

Carbohydrate-rich diets (especially high-fiber diets) improve gut health, which has been well studied and is thought to be associated with SCFA production by intestinal flora (Maslowski et al., 2009; Singh et al., 2014). Several studies have shown that dietary fiber alleviates *C. difficile* infection (Hryckowian et al., 2018). *C. difficile* is directly affected by diet through germination, growth, and spore formation as well as indirectly by ecological interactions. In addition, diet may directly alter pathogenic factors expressed by *C. difficile*. Several studies have shown that pectin beneficially affects immunity and prevents inflammation and disease, and it can improve colon cancer by modulating signaling pathways activated by oxidative stress and inflammation (Tan et al., 2018). Toxins produced by *C. difficile* are thought to be the main virulence factors. These toxins induce inflammation, intestinal damage, and diarrhea (Kordus et al., 2022). Our experimental results showed that a pectin diet protected the intestinal barrier, reduced the cellular permeability of intestinal epithelial cells, and reduced the elevated LBP and LPS levels due to the impaired intestinal barrier caused by *C. difficile* infection. The etiology and pathogenesis of *C. difficile* infection are complicated, and an imbalance of pro- and anti-inflammatory cytokines has been linked to *C. difficile* development and progression, leading to persistent inflammatory response in the colon (Pawlowski et al., 2010). Our experimental results showed significantly higher cytokine levels in the CCDI group, and the pectin diet improved this outcome.

The gut microbiome contributes to the disease susceptibility and outcome of CDI and impacts innate and adaptive immunity (Li and Chen, 2022). Consistent with previous studies (Schubert et al., 2015; Ross et al., 2016; Wu et al., 2022), *C. difficile* infection is associated with a flora imbalance and decreased diversity of intestinal microbes. Our results showed that *C. difficile* reduced microbiome richness and diversity, whereas pectin improved microbiome flora diversity. There is clearly a link between microbiome status and disease regulation. Microorganisms can take bile acids from the liver into the intestine and convert them into secondary bile acids. The primary to secondary bile acid ratio is critical and affects the growth of *C. difficile*. *C. difficile* also produces toxins that penetrate the intestinal barrier, while SCFAs, produced by other intestinal microbes, help tighten the intestinal barrier. The maintenance of the homeostasis of the intestinal flora is closely related to the number of bacteria that produce SCFAs, which contribute to the maintenance of the homeostasis of the intestinal environment. Other methods to decrease *C. difficile* survival involve nutritional competition for ecological niches in the intestinal environment and other indirect mechanisms. *C. difficile* infection leads to an increase in the abundance of intestinal opportunistic pathogens such as *Escherichia-Shigella* and *Enterococcus* (Gu et al., 2016). Opportunistic pathogens promote intestinal inflammation and are triggered to induce disease by intestinal inflammation. In contrast, a pectin diet increases the level of beneficial intestinal flora, such as *Lachnospiraceae*, which are the main producers of butyrate in the intestine (Baxter et al., 2019). Correlation analysis showed a good correlation between *Lachnospiraceae* and SCFAs in our results. The pectin group, but not the inulin group, increased the abundance of *Lachnos*piraceae, which may contribute to the observed differences in treatment outcomes between the two diets. *Lachnospiraceae* also produce indole derivatives, which are tryptophan-converting metabolites that activate AhR (Vacca et al., 2020). Rorγt^+ Tregs, which are highly expressed in the intestinal tract, may be stimulated by these tryptophan metabolites (Ye et al., 2017). We also used AhR agonists and inhibitors to demonstrate the involvement of the AhR pathway in the role of a pectin diet against *C. difficile* infection.

Numerous intestinal commensal metabolites, including amino acid derivatives, carbohydrates, and vitamins, modulate a variety of host immune cell subsets through different mechanisms (Li et al., 2022). Based on these findings, we hypothesized that a fermentable fiber diet would negatively affect *C. difficile* adaptation in two interrelated ways. First, a fermentable fiber diet promotes the privileged growth of members of the microbiota that utilize fiber (e.g., *Bacteroides*). Second, SCFAs produced by fermentable fiber metabolism negatively impact *C. difficile* adaptation, likely due to the accumulation of key metabolic pathway end products, such as reduced acetate production and butyrate production (Ferreyra et al., 2014). Dietary fiber and its products, particularly SCFAs, have been proven to benefit inflammatory diseases (Maslowski et al., 2009; Trompette et al., 2014). The expression of TcdB and TcdA is modulated by many factors, such as nutrients, population sensing, and other environmental indices. SCFAs act as signals from the microbiome to ferment *C. difficile*, and this competitive and unsuitable intestinal environment can lead to increased toxin production. *C. difficile* strains are inhibited by SCFAs based on concentration (Hryckowian et al., 2017). Butyrate can affect toxin expression (Karlsson et al., 2000). Butyrate also protects against CDI by protecting the intestinal barrier and alleviating inflammation through overexpression of hypoxia-inducible factor 1 (HIF-1), the host pathways that may independently affect inflammation and *C. difficile* burden and toxin (Kelly et al., 2015; Fachi et al., 2019). Moreover, gut microbes play a role in bile acid metabolism, affect *C. difficile* (Buffie et al., 2015) and may interact with dietary influences. These metabolic processes convert primary bile acids and conjugated bile acids (e.g., taurocholic acid), which promote *C. difficile* germination, into unconjugated primary bile acids and secondary bile acids (e.g., cholic acid and deoxycholic acid), which are either less effective germination agents or even inhibit this process (Ridlon et al., 2006). Our results showed that the pectin group modulated bile acids such as chenodeoxycholic acid, which may also be one of the mechanisms underlying the effects of pectin on CDI. Bile acids are an important signal for *C. difficile* spore germination; however, the bile acid signal alone is not sufficient. Amino acids such as glycine are another signal necessary for *C. difficile* spore germination. Glycine is an important germinator of *C. difficile* spores (Neumann-Schaal et al., 2019). Glycine, serine, and

threonine metabolism is one of the differential metabolic pathways after pectin treatment. By comparing the metabolomes of the pectin and inulin groups, the different metabolites, such as l-phenylalanine, had an inhibitory effect on *C. difficile* spore growth (Pickering et al., 2018). And valine is also a co-emergent source of *C. difficile*. More work will focus on exploring how dietary factors may influence the dynamics of infection.

The gut microbiota has been identified as a potential modifiable nongenetic factor. Diet can affect the microbiota, supporting the hypothesis that changes in diet may affect the occurrence and development of *C. difficile* infection. Recently, studies evaluated the consumption of fiber foods rather than refined fiber, and we examined the effect of refined fibers (pectin and inulin) on *C. difficile* infection. We observed that dietary pectin prevented the development of *C. difficile*-induced colitis, whereas inulin promoted its development, probably at least in part by promoting the activation of the AhR pathway. Although fiber has many beneficial effects on the gut, it may also have negative effects, which may depend on the genetic and physiological status of the host. Accordingly, pectin can be conditionally beneficial, depending on preexisting intestinal conditions and the specific fiber. According to these results, specific dietary fibers may have benefits or risks in the case of *C. difficile* infection. Limitations of this experiment include the fact that our study focused on the effects of pectin and inulin on *C. difficile* infection, used cellulose as a control group, and did not set an infectious group with a standard lab diet, which will be verified in our subsequent experiments.

Our results support the idea that certain dietary fibers have the potential to attenuate inflammation, and the utilization of dietary fiber flora and associated end products of dietary fiber metabolism, such as SCFAs, are associated with reduced *C. difficile* adaptations. Current microbiota-centered therapies for *C. difficile* infection, such as fecal microbiota transplantation and probiotics, are mediated by the introduction of exogenous microorganisms. Prebiotics, such as pectin, is a promising approach to improve the human microbiota. Our studies suggest that different fibers can have different effects, which may also be due to the different microbial fermentations of different fibers. A better understanding of the fibers may contribute to the optimization of fiber-based personalized treatments for *C. difficile* infection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA 873028.

Ethics statement

The animal study was reviewed and approved by the Animal Experimental Ethical Inspection of The First Affiliated Hospital, Zhejiang University School of Medicine.

Author contributions

LJL and QX designed the experiments. QW and QX analyzed the data. ZW wrote the manuscript. Revision of the manuscript by other authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1028267/full#supplementary-material

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Probiotic *Pediococcus pentosaceus* ameliorates MPTP-induced oxidative stress *via* regulating the gut microbiota–gut–brain axis

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Recent evidence demonstrated that functional bacteria were involved in the regulation of Parkinson's disease (PD). However, the mechanism of probiotics in improving PD was unclear. Here the antioxidant effect and the mechanism of probiotics *Pediococcus pentosaceus* (PP) on PD were studied by regulating the gut–brain axis. In this study, male C57BL/6J mice were injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneally to establish a PD model and were then treated with PP for 4 weeks. Subsequently, a series of neurobehavioral tests to evaluate the motor function of the mice was performed. Additionally, degeneration of dopaminergic neurons, accumulation of α-synuclein, the production of an oxidative stress response, and the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway-related proteins were evaluated. Moreover, the gut microbial composition and the level of metabolite γ-aminobutyric acid (GABA) were assessed. The results showed that PP treatment could improve MPTP-induced motor deficits, the degeneration of dopaminergic neurons, and the accumulation of α-synuclein. Moreover, PP treatment significantly increased the levels of SOD1, Gpx1, and Nrf2, while it decreased the levels of Keap1 in the brain of MPTP-induced mice. Notably, PP treatment improved the gut microbial dysbiosis and increased the level of GABA in MPTP-induced mice. These findings indicated that PP might represent a promising candidate, due to the metabolite of GABA, that could be used for the treatment of PD.

KEYWORDS

Parkinson's disease, gut microbiota, *P. pentosaceus*, oxidative stress, Nrf2 signaling, γ-aminobutyric acid

Introduction

Parkinson's disease (PD), the second largest neurodegenerative disease with an increasing prevalence (Dorsey et al., 2018; Marino et al., 2020), is dominated by motor symptoms attributed to the aggregation of α-synuclein and the injury of dopaminergic neurons in the substantia nigra. Current disease-modifying therapies, basically dopamine replacement, have been used in the treatment of PD, but these therapies lack the ability to restrain the degeneration and death of dopaminergic neurons (Raza et al., 2019; Armstrong and Okun, 2020). The neurodegeneration of PD involved many mechanisms, among which oxidative stress played an undeniable role in the loss of dopaminergic neurons by inducing unbalanced redox homeostasis, ultimately leading to neuronal oxidative stress (Puspita et al., 2017; Trist et al., 2019). The nuclear factor erythroid-2 related factor 2 (Nrf2), a key transcription factor in cellular response to oxidative stress, could maintain intracellular redox homeostasis by stimulating the expression of antioxidant and cytoprotective genes (Rojo de la Vega et al., 2018). The Nrf2 pathway can effectively scavenge reactive oxygen species (ROS) and free radicals and protect the dopaminergic neurons from oxidative damage (Wei et al., 2020). Nrf2 exerted its redox-regulating capacities by uncoupling with endogenous inhibitor Keap1 and releasing Nrf2, which was migrated to the nucleus and bound to the promoter region of antioxidant response element (ARE) (Zgorzynska et al., 2021) so as to regulate numerous cytoprotective genes, thus mitigating the oxidative damage and dysfunction of dopaminergic neurons (Ishii et al., 2019). Therefore, regulating the Nrf2 signaling pathway is the key to reduce the oxidative stress of PD.

γ-Aminobutyric acid (GABA), a kind of inhibitory neurotransmitter, could maintain intracellular redox homeostasis and protect neurons from oxidative damage (Ngo and Vo, 2019). Mounting evidence showed that the concentration of GABA was decreased in many neuropsychiatric diseases (Zheng et al., 2017; Cortès-Saladelafont et al., 2018), and there was a negative correlation between the GABA level in the cerebral cortex and the severity of PD symptoms (van Nuland et al., 2020). It was reported that GABA treatment can increase the expression of Nrf2 protein in the nucleus of myoblasts and regulate the level of glutathione and glycogen synthase kinase-3β phosphorylates as well as the activity of catalase (CAT), superoxide dismutase (SOD), and ROS scavenging (Choe et al., 2021) and prevent the H_2O_2-induced transfer of Nrf2 into the nucleus, thus further alleviating oxidative stress injury and restoring the redox homeostasis of cells (Tang et al., 2018). Moreover, GABA can reverse the H_2O_2-induced mRNA expression and protein expression of Keap1 and Nrf2 and protect endothelial cells from oxidative stress damage by regulating the Nrf2 signaling pathway (Zhu et al., 2019). PD patients were often observed to have gut microbiota dysbiosis (Hill-Burns et al., 2017; Aho et al., 2019), accompanied by degeneration of dopaminergic neurons and decrease of dopamine level in the brain (Koutzoumis et al., 2020). Gut functional bacteria can interact with the host through the production of functional metabolites, which contributes to an in-depth understanding of the impact of gut functional bacteria on the host. Interestingly, many intestinal commensal bacteria, such as *Lactobacillus* and *Bifidobacterium*, can produce GABA (Dinan et al., 2013). This randomized, double-blind trial found that treatment with probiotics (GABA-producing bacteria) for 4 weeks significantly improved the gastrointestinal symptoms in patients with PD. Remarkably, many special probiotics have been proven to inhibit brain dysfunction and motor disorder and increase the expression of antioxidant enzymes in brain tissue, which may prevent the pathological development of PD. Bravo et al. reported that *Lactobacillus rhamnosus* can act on GABA nervous system (Bravo et al., 2011) and improve stress- and anxiety-like behaviors along with the regulation of GABA receptor expression in mice brain (Bravo et al., 2011). *Pediococcus pentosaceus* (PP), belonging to GAB- producing bacteria, was identified as a potential probiotic (Jiang et al., 2021) and had many beneficial effects, including anti-oxidation, anti-inflammatory effect, and ability in reversing abnormal gut microbiota (Jiang et al., 2021; Yu et al., 2021; Dong et al., 2022). So far, the effect of PP against the oxidative stress of PD is still unclear.

In the present study, we explored the beneficial effects of PP against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced motor dysfunction *via* regulating the gut–brain axis. Behavioral tests, such as pole test, rotational test, and beam walking test, were evaluated. In addition, the degeneration of dopaminergic neurons was evaluated by measuring the expression of TH and the accumulation of α-synuclein. The activity of antioxidant enzymes and the expression levels of the Nrf2 signaling pathway and downstream-related proteins in brain tissue were detected to evaluate the changes of oxidative stress in the brain of PD mice. Meanwhile, the level of GABA in brain tissue was detected, and the composition and the structural changes of the gut microbiota were evaluated by 16s rRNA sequencing. Our results reveal that GABA-producing bacteria PP might be a novel dietary supplementation of probiotic for inhibiting oxidative stress associated with PD.

Materials and methods

Animals

Male C57BL/6J mice (6–8 weeks old and 20–25g in weight) were purchased from Hangzhou Ziyuan Experimental Technology Co, Ltd, Hangzhou, China. The mice were housed in constant temperature (23 ± 2°C) and humidity (55 ± 5%) in a room with 12-h light/dark cycle. Food and water were available

ad libitum. All animal procedures were performed in accordance with the guidelines of the Animal Ethics Committee of Wenzhou Medical University.

Experimental design

To evaluate the role of PP in PD, all mice were randomly divided into three groups ($n = 8$ each group), namely: Con group, MPTP group, and MPTP + PP group. The experiment was carried out after the mice were adapted to the environment for 5 days. To generate PD models, the mice were intraperitoneally injected with 25 mg/kg MPTP (MACKLIN, China) once a day for 1 week. Then, in the MPTP + PP group, the mice were intragastrically treated with 200 μl *P. pentosaceus* WMU002 (CGMCC 24884) provided by the China General Microbiological Culture Collection Center. The suspension contained 1×10^9 colony-forming units/ml, which was given as 0.2 ml once a day for 4 weeks after the MPTP treatment. For the mice in the Con and MPTP groups, this was replaced with normal saline. The behavior tests in each group were performed 24 h after the last administration. TThen, the colonic contents of the mice were collected for 16s rRNA sequencing. Moreover, the brain tissues were obtained for immunohistochemistry and western blot. A diagram to describe these procedures was shown in Figure 1A.

Balance beam test

The balance beam test was used to test the balance ability, muscle strength, and motor coordination of mice. The balance beam was a square with a length of 0.8 m and a width of 14 mm.

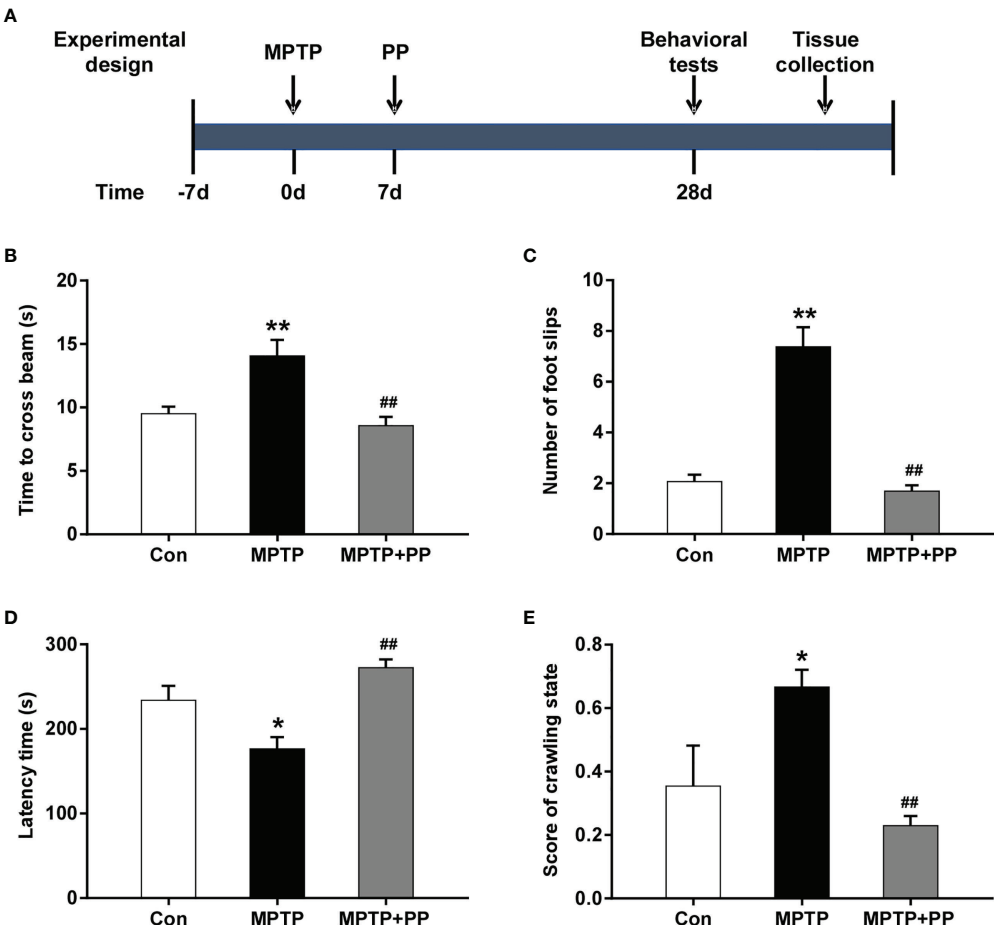

FIGURE 1
Pediococcus pentosaceus treatment inhibited the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced motor dysfunction. **(A)** Schematic diagram of the animal experiments. **(B)** Time to cross the beam in the balance beam test. **(C)** Number of foot slips in the balance beam test. **(D)** Latency time in the rotarod test. **(E)** Score of crawling state in the pole test. Statistical comparison by one-way ANOVA with *post-hoc* comparisons of Dunnett's multiple-comparisons test. Data are presented as means ± SEM. $^{*}P < 0.05$ *vs*. Con group, $^{**}P < 0.01$ *vs*. Con group, $^{\#\#}P < 0.01$ *vs*. MPTP group.

Each mouse was placed on one end of the balance beam and guided to crawl to the black box for 2 days. On day 3, the mouse was placed horizontally on the beam to cross in 5 min; the number of foot slips (sliding of the front or rear paws on the smooth surface of the beam) and the time taken for the mice to cross were recorded.

Rotarod test

Rotarod test was used to evaluate the ability of anti-fatigue and coordinated exercise of mice. The Rotarod (RL04-YLS-4D, Haifuda, Beijing, China) was equipped with automatic timers and falling sensors. The mice were trained once a day for 3 days before the experiment. On day 4, the mice were placed on an accelerated rotating cylinder, the speed of which was slowly increased from 4 to 40 revolutions per minute in 5 min. The end of the test was determined such that when the mice fell off or grabbed the device and rotated continuously for two turns without trying to walk; the latency time of the mice was measured. The test was repeated three times with an interval of at least 30 min, and the average time of latency was analyzed.

Pole test

Pole test was used to detect the coordination ability of mice. A pole with a length of 40 cm and a diameter of 1.5 cm was fixed on a base and wrapped with non-sticky gauze for the mice to easily clamp. The mice were trained before the test to make sure that they could climb down the pole. The test ended with both hind limbs of the mice reaching the base. The total time of climbing down from the pole and the state of the mice during climbing down were measured. Each mouse was subjected to three consecutive tests, and the average was calculated for statistical analysis. The scoring criteria for the state of the mice in climbing the pole were as follows: 0, used the limbs to climb down the pole smoothly; 0.5, step-by-step spiral downward crawl, but with a sliding behavior of the hind legs; 1.0, paused several times to climb down but held on tightly to the pole; 1.5, slid down the pole and fell off; and 2.0, could not grab the pole, drop directly. After each experiment, the facility was sprayed with alcohol to get rid of the odor.

Nissl staining

The mice were fixed in formaldehyde, embedded with paraffin, and cut into 5-μm sections for pathological staining. The slices of substantia nigra were dewaxed with xylene and rehydrated by gradient ethanol. Then, the slices were dyed with 1% tar violet dye (C0117, Beyotime, Shanghai, China) for 30 min, washed in distilled water, separated with 70% alcohol, and dehydrated by gradient alcohol. The slices were finally fixed in xylene and sealed with neutral resin. The stained tissues were observed under a microscope.

Immunohistochemistry

The slides of substantia nigra were repaired for antigen and blocked for peroxidase. Then, the non-specific antigen of the slides was blocked with 5% fetal bovine serum for 30 min and washed with phosphate-buffered saline (PBS) three times for 5 min. The primary antibodies, including α-synuclein (1:200, BS3429, Bioworld, Minnesota, USA) and TH (1:200, AB137869, Abcam, Cambridge, UK), were incubated with the slides, respectively, at 4℃ overnight. On the second day, the slides were incubated with secondary antibody (PV-6001, ZSGB-BIO, Beijing, China) for 30 min at room temperature and washed with PBS three times for 5 min. Subsequently, the slides were added with chromogen DAB (ZLI-9018, ZSGB-BIO, Beijing, China) for target antigen detection, and the staining time was controlled under a microscope. The slides were then stained with hematoxylin dye for 2 min and dip-rinsed in hydrochloric alcohol for 2 s. After fixing by xylene and sealing with neutral resin, the brown granules in the brain tissues observed under the microscope were considered to represent a positive immune response.

Western blot

The brain tissues of the nigrostriatum were homogenized, and the total proteins of the samples were extracted by radioimmunoprecipitation assay lysis buffer (P0013B, Beyotime, Shanghai, China) and centrifuged at 12,000 revolutions/min for 20 min, and the supernatant was taken. The protein concentration was measured with BCA kit (P0010S, Beyotime, Shanghai, China) and adjusted to the same level. After heat denaturation, the protein samples were added to 12% SDS-PAGE gel for electrophoresis, transferred to polyvinylidene fluoride membrane, and sealed with 5% skimmed milk powder solution for 2 h. Subsequently, the membrane was incubated overnight in a primary antibody of synuclein-α (1:1,000, BS3429, Bioworld, MN, USA), TH (1:1,000, BS1432, Bioworld, MN, USA), Keap1 (1:1,000, BS6783, Bioworld, MN, USA), Nrf2 (1:1,000, BS1258, Bioworld, MN, USA), SOD1 (1:1,000, BS6057, Bioworld, MN, USA), GPx1 (1:1,000, BS61511, Bioworld, MN, USA), and β-actin (1:1,000, AP0060, Bioworld, Minnesota, USA) at 4℃ and then transferred to diluent containing horseradish peroxidase-labeled secondary antibody (1:5,000, A0208, Beyotime, Shanghai, China) for further incubation. The membrane was added with chemiluminescent solution, and the gray values of each protein were recorded and analyzed by Image J software. β-actin was used as the internal reference.

ELISA assay

The content of GABA in brain tissue was determined by ELISA (FT-T4051, Fantai, Shanghai, China). Brain tissue of the nigrostriatum and lysate were mixed at a ratio of 1:9, added with phenylmethylsulfonyl fluoride (ST506-2, Beyotime, Shanghai, China), and homogenized by a homogenizer, and then the supernatant was taken. The specific experimental operations were carried out in accordance with the instructions. The absorbance (optical density value) of each sample was measured by a microplate reader at a wavelength of 450 nm.

Gut microbiota analysis

The colonic contents of mice were collected and frozen at -80℃, and DNA was extracted with DNA extraction kit (QIAamp DNA stool mini kit, Qiagen, Hilden, Germany) for real-time fluorescence quantitative polymerase chain reaction (PCR), which amplified the V3–V4 region of the bacterial 16s rRNA gene. The purified amplicons were sequenced by Illumina MiSeq system, and the high-quality sequences were clustered according to 97% similarity by *de novo* UCLUST algorithm to obtain operational taxonomic unit (OTU). Then, the α-diversity index was assessed, and the difference of OTUs was analyzed by Mann–Whitney nonparametric test. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify differential marker species by LDA algorithm.

Statistical analysis

All data were expressed as the mean ± standard error of mean (SEM) and were analyzed by using GraphPad Prism 7 software (La Jolla, CA, USA), in which one-way ANOVA was used for the overall variance analysis, and Dunnett's multiple-comparisons test was used to compare the differences between groups. The composition difference of gut microbiota was analyzed by Kruskal–Wallis rank sum test, and *post-hoc* test was performed to compare the differences among the three groups by Tukey–Kramer. P <0.05 was considered statistically significant.

Results

PP treatment inhibited the MPTP-induced motor dysfunction

In the balance beam test, the time to cross the beam and the number of foot slips in the MPTP group were significantly increased compared with the Con group ($P < 0.01$, Figures 1B, C), while these were reversed after PP treatment ($P < 0.01$, Figures 1B, C). In the rotarod test, the latency time of the MPTP group was significantly shorter than that of the Con group ($P < 0.05$, Figure 1D), while the latency time of the MPTP + PP group was longer than that of the MPTP group ($P < 0.01$, Figure 1D). In the pole test, the score of the crawling state in the MPTP group was significantly increased compared with the Con group ($P < 0.05$, Figure 1E), whereas the score in the MPTP + PP group was significantly decreased compared with the MPTP group ($P < 0.01$, Figure 1E). These results suggested that PP could improve the MPTP-induced motor dysfunction.

PP treatment suppressed the MPTP-induced neuronal degeneration

The results of Nissl staining showed that the number of neurons in the MPTP group was less than that in the Con group, while the number of neurons in the MPTP + PP group was more than that in the MPTP group (Figure 2A), suggesting that PP treatment reduced the neuronal damage of PD. Then, we assessed the level of α-synuclein in substantia nigra by immunohistochemistry and Western blot. The results showed that the accumulation of α-synuclein in the MPTP group was significantly increased compared with that in the Con group, while the α-synuclein level was decreased after PP treatment ($P < 0.01$, Figures 2B–E). Moreover, the TH level was measured to evaluate the injury of dopaminergic neurons. The results showed that the TH level of the substantia nigra in the MPTP group was significantly lower than that in the Con group, while the TH level in the MPTP + PP group was significantly higher than that in the MPTP group ($P < 0.01$, Figures 2C, D, F). These results suggested that PP treatment could attenuate the dopaminergic neuronal degeneration of PD.

PP treatment reversed the MPTP-induced decreased level of GABA

Subsequently, we measured the concentration of GABA in the mouse brain. The result showed that the GABA level was decreased in MPTP mice than that in the Con group, while the GABA level in the brain was increased after PP treatment ($P < 0.05$, Figure 2G), suggesting that PP could reverse the decrease of GABA in PD.

PP treatment improved the MPTP-induced oxidative stress and regulated the levels of Keap1 and Nrf2

The activities of the antioxidant enzyme SOD1 decreased significantly in the MPTP group compared with those in the Con group, while the SOD1 level in the MPTP + PP group was significantly increased than that in the MPTP group ($P < 0.01$, Figures 3A, B). Similarly, the activities of the antioxidant enzyme

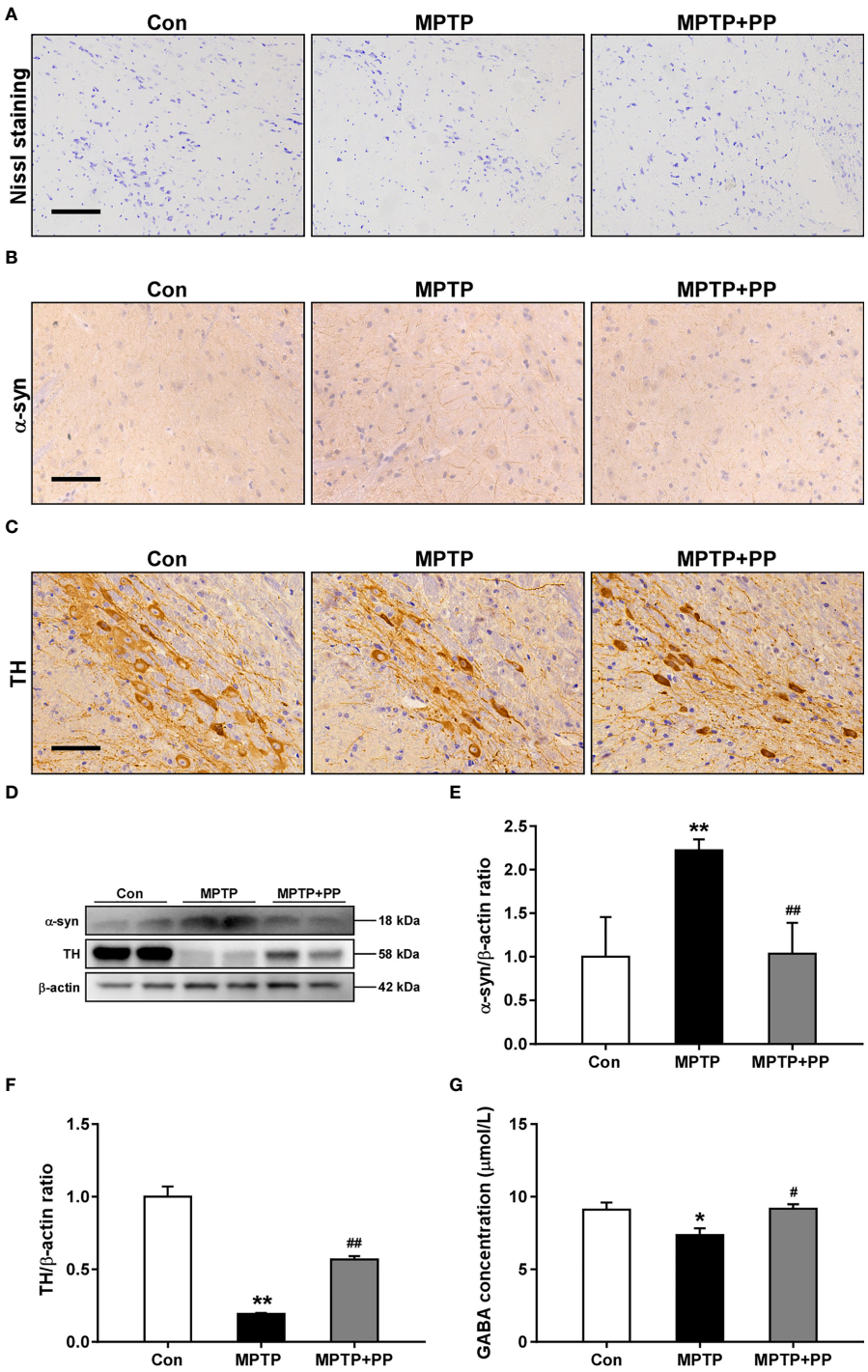

FIGURE 2
Pediococcus pentosaceus treatment attenuated the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neuronal degeneration and the decreased level of γ-aminobutyric acid (GABA). **(A)** Representative images of Nissl staining for Nissl body in neurons. Magnification, ×400; scale bar = 100 μm. **(B)** Representative immunohistochemistry images of α-synuclein in substantia nigra. Magnification, ×400; scale bar = 100 μm. **(C)** Representative immunohistochemistry images of TH in substantia nigra. Magnification, ×400; scale bar = 100 μm. **(D)** Representative Western blotting images of α-synuclein (α-syn) and TH. **(E)** Quantitative analysis of α-syn. **(F)** Quantitative analysis of TH. β-actin was used as the internal reference. **(G)** Quantitative analysis of the GABA level in the brain. Statistical comparison by one-way ANOVA with *post-hoc* comparisons of Dunnett's multiple-comparisons test. Data are presented as means ± SEM; n = 4–6 per group. $^{*}P < 0.05$ *vs*. Con group, $^{**}P < 0.01$ *vs*. Con group, $^{\#}P < 0.05$ *vs*. MPTP group, $^{\#\#}P < 0.01$ *vs*. MPTP group.

GPx1 decreased significantly in the MPTP group compared with the Con group ($P < 0.01$, Figures 3A, C), which was reversed after PP treatment ($P < 0.05$, Figures 3A, C). Furthermore, we measured the expression level of Nrf2 signal pathway-related proteins, including Keap1 and Nrf2. The results showed that the expression level of Keap1 was significantly increased in the MPTP group compared with the Con group ($P < 0.05$, Figures 3D, E), while the level was significantly decreased in the MPTP + PP group compared with the MPTP group ($P < 0.01$, Figures 3D, E). On the contrary, the Nrf2 level was decreased significantly in the MPTP group ($P < 0.01$, Figures 3D, F), whereas the level was elevated after PP treatment ($P < 0.05$, Figures 3D, F). These results suggested that PP treatment could improve oxidative stress by regulating the abnormal Nrf2 signaling pathway in PD.

PP treatment reversed the MPTP-induced abnormal microbiota composition

We used the Shannon index and the Simpson index to assess the changes in gut microbiota α-diversity of mice. The results showed that the Shannon index in the MPTP group was

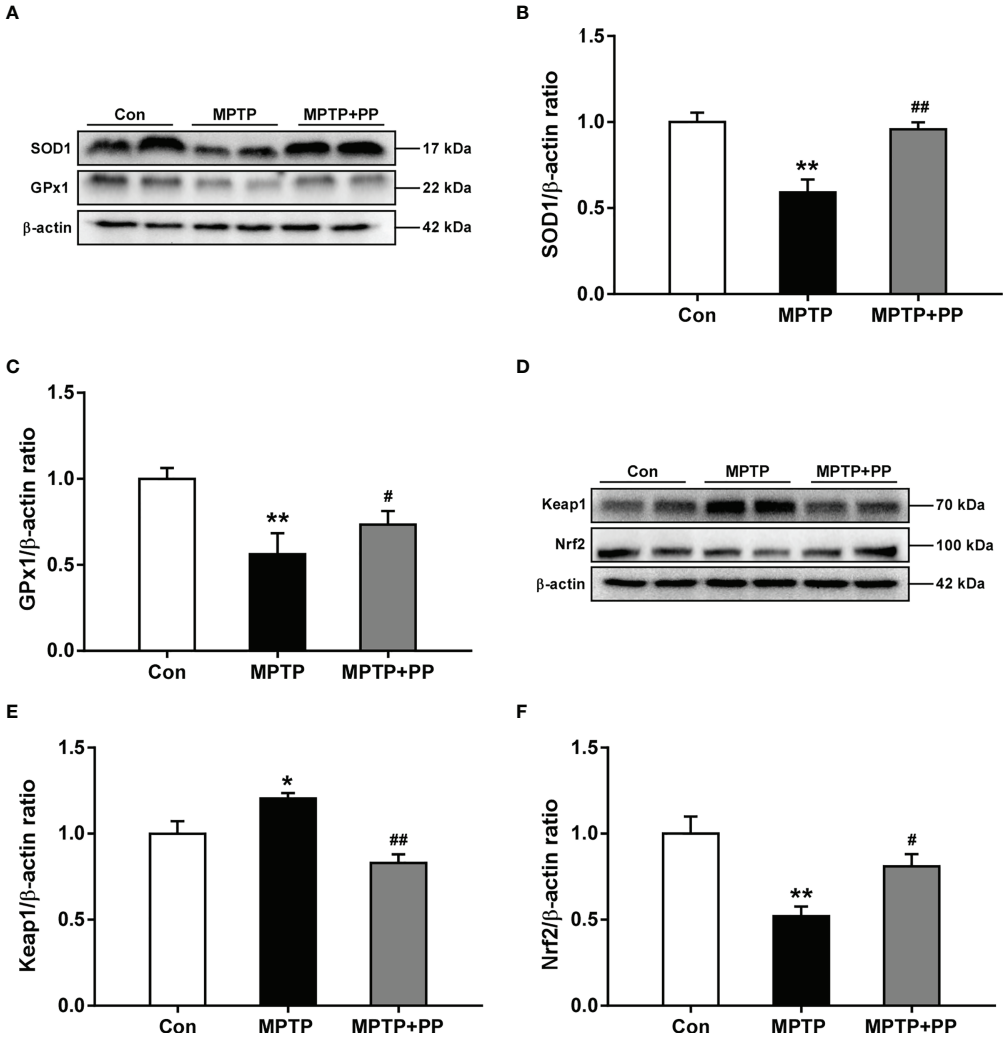

FIGURE 3
Pediococcus pentosaceus treatment improved the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced oxidative stress and regulated the levels of Keap1 and Nrf2. **(A)** Representative western blotting images of SOD1 and GPx1. **(B)** Quantitative analysis of SOD1. **(C)** Quantitative analysis of GPx1. **(D)** Representative western blotting images of Keap1 and Nrf2. **(E)** Quantitative analysis of Keap1. **(F)** Quantitative analysis of Nrf2. β-actin was used as internal reference. Statistical comparison by one-way ANOVA with *post-hoc* comparisons of Dunnett's multiple-comparisons test. Data are presented as means ± SEM; $n = 4–6$ per group. $^{*}P < 0.05$ *vs.* Con group, $^{**}P < 0.01$ *vs.* Con group, $^{\#}P < 0.05$ *vs.* MPTP group, $^{\#\#}P < 0.01$ *vs.* MPTP group.

significantly decreased compared with the Con group, while the index in the MPTP + PP group was significantly increased compared with the MPTP group ($P < 0.01$, Figure 4A). On the contrary, the Simpson index in the MPTP group was significantly higher than the Con group, while the index in the MPTP + PP group was significantly decreased than the MPTP group ($P < 0.01$, Figure 4B), suggesting that PP could significantly improve the decline of microbial diversity of PD. Meanwhile, the principal coordinate analysis on amplicon sequence variant (ASV) level showed the changes of microbial β-diversity, which was significantly different among the three groups (Figure 4C). The Venn diagram of microbiota showed that 53 ASVs were shared among the three groups, whereas 208 ASVs were shared only between the Con group and the MPTP + PP group (Figure 4D). The microbial community composition was primarily composed of *Staphylococcaceae*, *Muribacullaceae*, and *Lachnospiraceae* (Figures 4E, F). Then, we measured the different bacteria in each group. At the phylum level, the relative abundance of *Firmicutes* and *Proteobacteria* in the MPTP group was increased than the Con group, whereas the relative abundance of which was decreased in the MPTP + PP group compared with the MPTP group ($P < 0.01$, Figures 5A, C). In contrast, phylum *Bacteroidota* in the MPTP group was decreased compared with the Con group and was increased in the MPTP + PP group ($P < 0.01$, Figure 5B). At the family level, the relative abundance of *Muribaculaceae* ($P < 0.01$, Figure 5D), *Lachnospiraceae* ($P < 0.05$, Figure 5E), and *Defluviitaleaceae* ($P > 0.05$, Figure 5H) in the MPTP group was decreased than the Con group, whereas the relative abundance of *Muribaculaceae* ($P < 0.01$, Figure 5D), *Lachnospiraceae* ($P < 0.05$, Figure 5E), and *Defluviitaleaceae* ($P < 0.05$, Figure 5H), respectively, was increased in the MPTP + PP group compared with the MPTP group. Additionally, the relative abundance of *Erysipelotrichaceae* ($P > 0.05$, Figure 5F) and *Enterococcaceae* ($P < 0.01$, Figure 5G), respectively, at the family level in the MPTP group was increased compared with the Con group, whereas the relative abundance of *Erysipelotrichaceae* ($P < 0.05$, Figure 5F) and *Enterococcaceae* ($P < 0.01$, Figure 5G) at the family level was decreased after PP treatment. At the genus level, the relative abundance of norank_f_*Muribaculaceae* ($P < 0.01$, Figure 5I) and *Lachnospiraceae* ($P < 0.05$, Figure 5J) in the MPTP group was decreased than that of the Con group, whereas the relative abundance of norank_f_*Muribaculaceae* ($P < 0.01$, Figure 5I) and *Lachnospiraceae* ($P < 0.01$, Figure 5J) was increased in the MPTP + PP group compared with the MPTP group. Moreover, the relative abundance of *Dubosiella* ($P < 0.05$, Figure 5K) and *Enterococcus* ($P < 0.01$, Figure 5L) at the genus level in the MPTP group was increased compared with the Con group, whereas the relative abundance of *Dubosiella* ($P < 0.05$, Figure 5K) and *Enterococcus* ($P < 0.01$, Figure 5L) was decreased after PP treatment. Subsequently, we further identified the specific bacterial taxa among the three groups by LEfSe analysis. The cladogram represented the microbial structure and predominant bacteria among the three groups from the family level to the genus level (Figure 6). These results suggested that the microbial composition in PD was significantly changed, while PP could improve the abnormal microbial composition of PD.

Discussion

Oxidative stress was involved in the pathogenesis of PD. Recently, the anti-PD effect of probiotics was related to the antioxidant effect of its metabolites and the regulation of the gut–brain axis. In this study, we demonstrated that PP (a GABA-producing bacteria) treatment significantly improved the MPTP-induced motor dysfunction, neuronal degeneration, and oxidative stress in mice. Moreover, PP treatment could reverse the abnormal gut microbiota, increase the level of GABA, and regulate the levels of the Nrf2 pathway-related proteins in the brain of MPTP-induced mice. The neuroprotective mechanism of PP on PD might be related to the regulation of the metabolite GABA–gut–brain axis.

Motor dysfunction was characteristic of PD (Feng et al., 2020). PD patients showed typical symptoms of motor dysfunction (Sarasso et al., 2021), which was caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (Surmeier, 2018) and widespread α-synuclein aggregated in the form of Lewy bodies (Raza et al., 2019). Mounting studies showed that the loss of dopaminergic neurons and the α-synuclein level increased in PD animal models (Su et al., 2019; Zhao et al., 2021). In this study, we proved that PP treatment could improve the MPTP-induced motor disorders and decrease the MPTP-induced dopaminergic neuronal loss and α-synuclein accumulation, suggesting that PP could improve the neurodegeneration of PD.

Oxidative stress played an important role in the progression of PD, aggravated the destruction of oxidoreductive homeostasis, and induced the progressive neurodegeneration of dopaminergic neurons. It was reported that PD patients exhibited obvious oxidative stress, manifested as the increased concentrations of DNA oxidative damage and lipid peroxidation markers in the blood as well as the decreased levels of antioxidant substances, such as catalase and glutathione (Wei et al., 2018). A large number of studies had shown that inhibiting the excessive oxidative stress could improve PD (Lv et al., 2020; Yan et al., 2021). In this study, PP treatment significantly increased the levels of antioxidant enzymes SOD and GPx in PD. Similarly, *P. pentosaceus* ZJUAF-4 increased the activity of SOD, while it reduced the production of ROS and MDA and relieved oxidative stress injury in mice induced by quinoline (Hao et al., 2021). Additionally, *Lactobacillus pentosus*, one of the GABA-producing bacteria, significantly decreased the intracellular ROS and inhibited particulate matter-induced cell death (Lee and Park, 2021). *Lactobacillus paracasei* PS23 could also upregulate the expression level of antioxidant genes, improve the activity of SOD, and alleviate the motor and

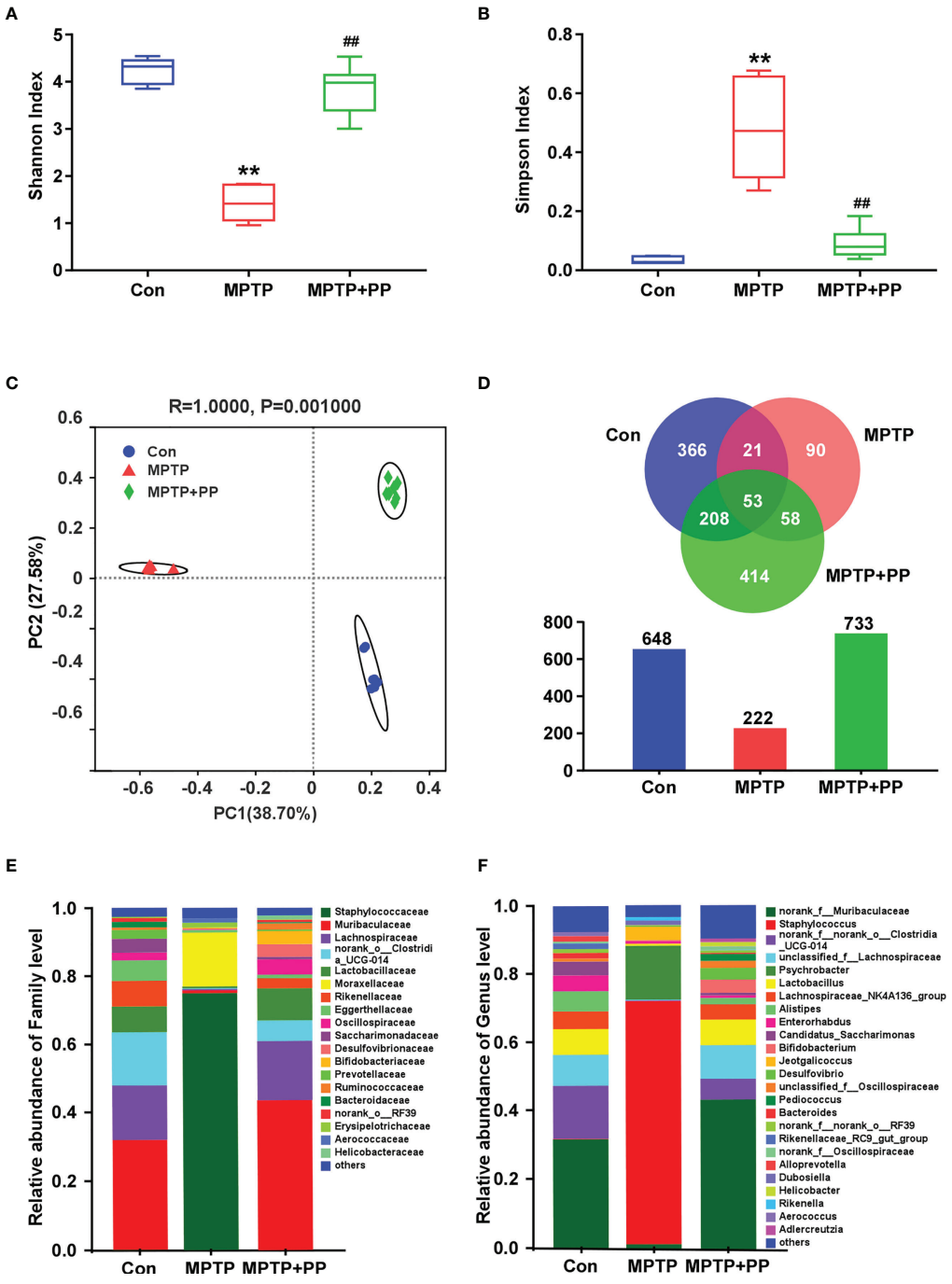

FIGURE 4
Pediococcus pentosaceus treatment reversed the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced abnormal microbiota composition. **(A)** Shannon index of gut microbial diversity. **(B)** Simpson index of gut microbial diversity. **(C)** Principal coordinate analysis plots of gut microbial diversity. **(D)** Venn diagram and quantitative analysis of microbial community composition among three groups. **(E)** Relative abundance of gut microbiota at the family level among three groups. **(F)** Relative abundance of gut microbiota at the genus level among three groups. Statistical comparison by Kruskal–Wallis rank sum test with *post-hoc* comparisons of Tukey–Kramer. Data are presented as median + interquartile range. $^{**}P < 0.01$ *vs.* Con group, $^{\#\#}P < 0.01$ *vs.* MPTP group.

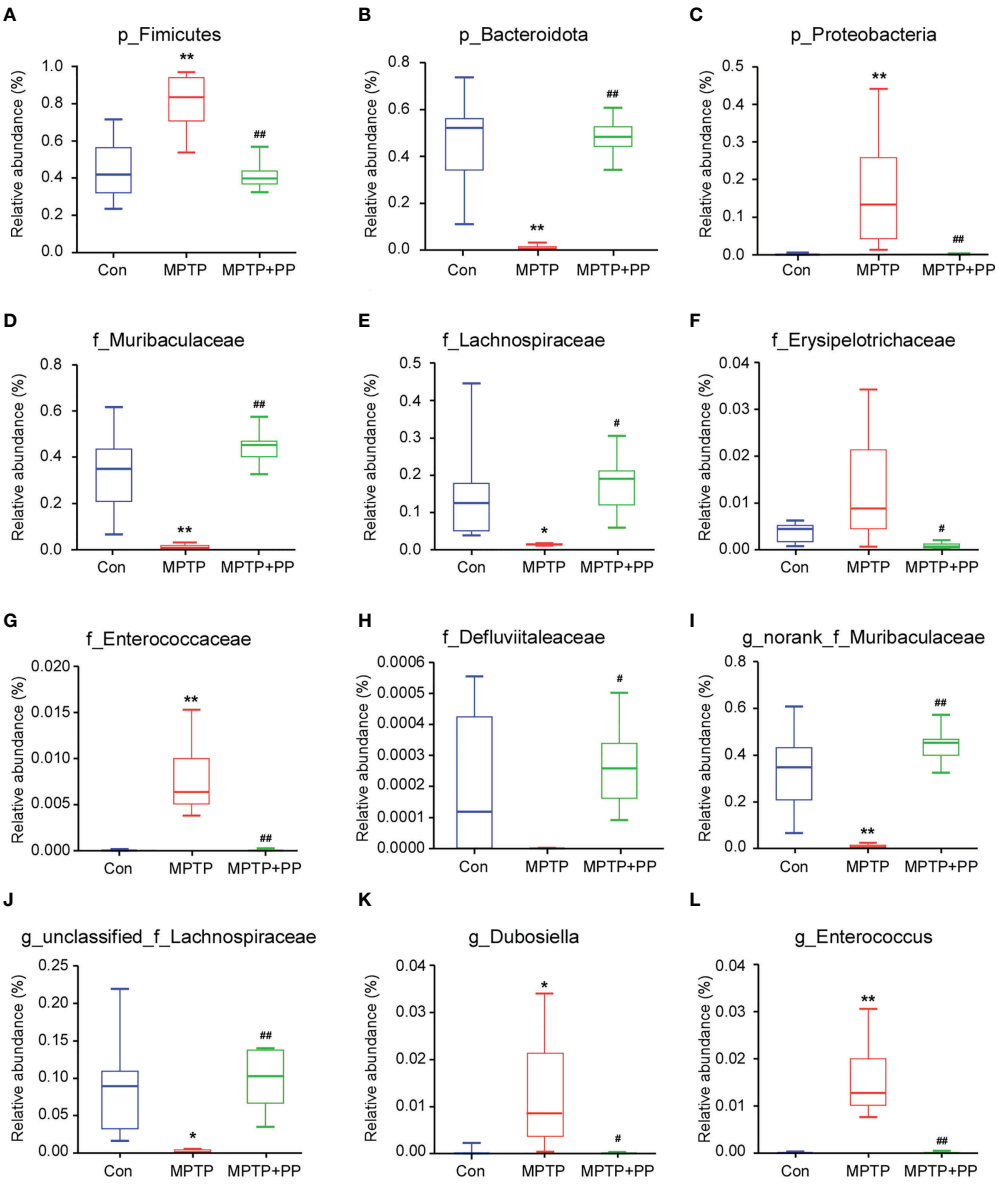

FIGURE 5
Pediococcus pentosaceus treatment reversed the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced change of the relative abundance of different bacteria. **(A)** p_*Firmicutes*, **(B)** p_*Bacteroidota*, **(C)** p_*Proteobacteria*, **(D)** f_*Muribaculaceae*, **(E)** f_*Lachnospiraceae*, **(F)** _*Erysipelotrichaceae*, **(G)** f_*Enterococcaceae*, **(H)** f_*Defluviitaleaceae*, **(I)** g_norank_f_*Muribaculaceae*, **(J)** g_unclassified_f_*Lachnospiraceae*, **(K)** g_*Dubosiella*, and (**L**) g_*Enterococcus*. Statistical comparison by Kruskal–Wallis rank sum test with *post-hoc* comparisons of Tukey–Kramer. Data are presented as median + interquartile range. $^{*}P < 0.01$ *vs.* Con group, $^{**}P < 0.01$ *vs.* Con group, $^{\#}P < 0.01$ *vs.* MPTP group, $^{\#\#}P < 0.01$ *vs.* MPTP group.

anxiety behavior in aging mice (Cheng et al., 2022). Our results indicated that PP was able to alleviate the oxidative stress of PD.

Nrf2, a redox-regulated transcription factor, was critically involved in the regulation of oxidative stress in PD (He et al., 2020; Parga et al., 2021). Normally, Nrf2 bound to Keap1 in the cytoplasm, while in excess of ROS, Nrf2 disconnected from Keap1, migrated to the nucleus, and combined with ARE, which resulted in the upregulation of cytoprotective and antioxidant enzymes that protected against oxidative stress (Fão et al., 2019). It was reported that Nrf2 transcription and protein expression were increased in PD patients compared with the control, and the level of Nrf2 transcription was directly related to the course of the disease (Petrillo et al., 2020). Accumulating evidence showed that the activation of the Nrf2 signaling pathway was significantly inhibited in MPTP-induced mice (Lee et al., 2018; Mohamed et al., 2021), while inducing Nrf2 activation could mitigate the degeneration of dopaminergic neurons and provide neuroprotection for PD (Zhang et al., 2021). Our results showed

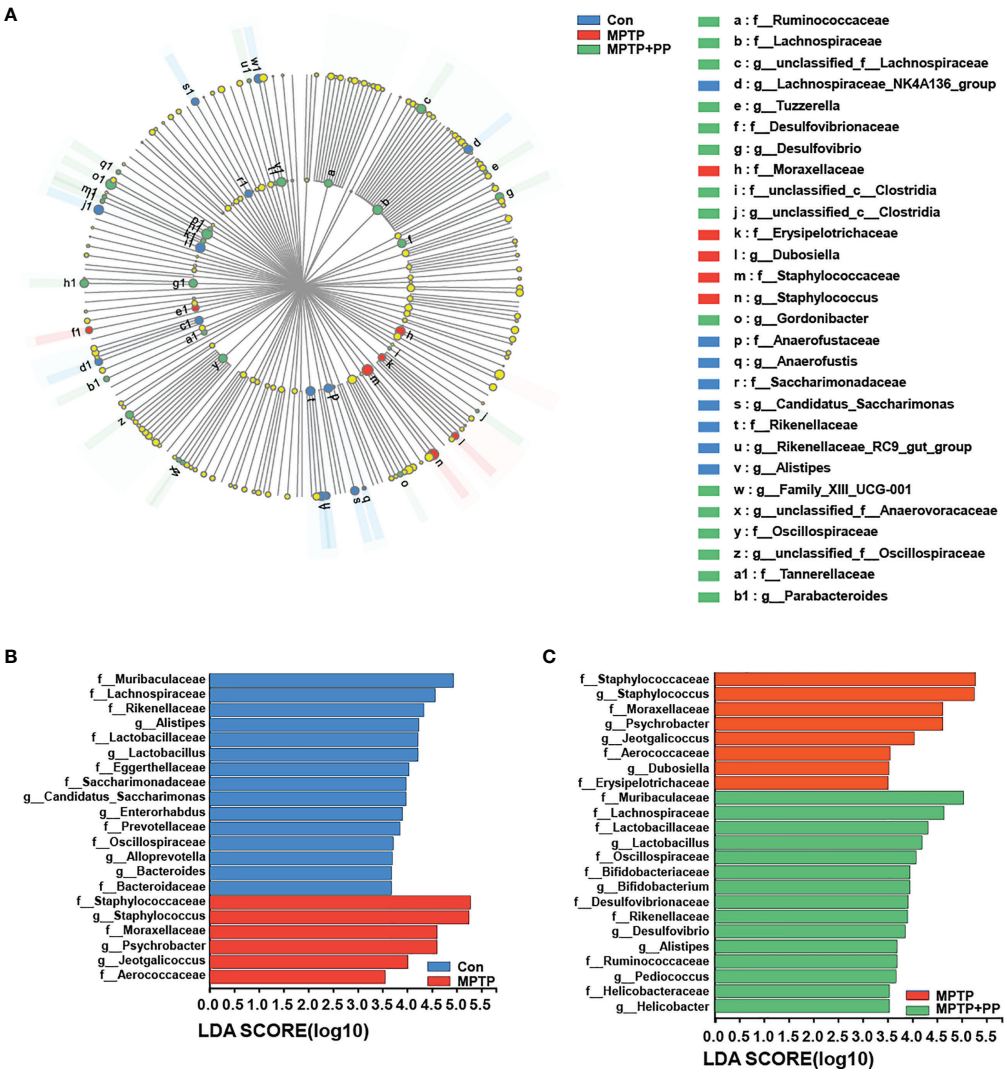

FIGURE 6
Pediococcus pentosaceus (PP) treatment changed the methyl-4-phenyl-1,2,3,6-tetrahydropyridine MPTP)-induced abnormal composition of specific bacterial taxa. **(A)** LEfSe multilevel species hierarchy tree cladogram representing the microbial structure and the predominant bacteria among the three groups. **(B)** Microbial structure and the predominant bacteria between the Con group and MPTP group; linear discriminant analysis (LDA) > 3.5. **(C)** Microbial structure and the predominant bacteria between the MPTP group and the MPTP + PP group; LDA > 3.5.

that PP treatment could increase the level of Nrf2, thus reducing the expression level of Keap1, which was consistent with former studies. PP treatment increased the Nrf2 level and its downstream genes as well as restored redox homeostasis in quinoline-induced mice (Hao et al., 2021). In addition, *Lactobacillus plantarum* KSFY06 downregulated the Keap1 expression and upregulated the Nrf2 mRNA expression in D-galactose-induced mice (Li et al., 2021). *Lactobacillus plantarum* DP189 could upregulate the Nrf2 level and the mRNA level of antioxidant enzymes (Wu et al., 2021), which further improved the neurodegeneration in PD (Wang et al., 2022). Our results suggested that PP could regulate the Nrf2 pathway in exerting an antioxidant role in PD.

The composition and the structure of the gut microbiota in PD patients and animals were disturbed (Sun et al., 2018; Lin et al., 2019). In this study, gut microbiota diversity was decreased, and the relative abundance of *Firmicutes* and *Proteobacteria* was increased in PD, which was reversed by PP treatment. Consistent with our results, a higher abundance of *Firmicutes* and phylum *Proteobacteria* in the fecal samples of PD mice was observed (Sun et al., 2018; Zhou et al., 2019). In this study, the decline of *Bacteroidota* was restored after PP treatment. Previous studies confirmed that *Bacteroidota* was reduced in PD patients compared with the matched controls (Unger et al., 2016). The abundance of *Bacteroidota* was significantly positively correlated with gut barrier function and

cognitive behavior index but negatively correlated with hippocampal inflammation (Shi et al., 2020). In this study, PP reversed the low abundance of *Muribaculaceae*, *Lachnospiraceae*, and *Defluviitaleaceae* as well as the overabundance of *Erysipelotrichaceae*, *Enterococcaceae*, *Dubosiella*, and *Enterococcus* of PD. It was demonstrated that *Lachnospiraceae* and the related genera were decreased in PD patients (Barichella et al., 2019; Vascellari et al., 2020), while *Enterococcaceae* was increased in patients and mice of PD (Lai et al., 2018; Pietrucci et al., 2019), and a lower level of *Lachnospiraceae* and a higher level of *Enterobacteriaceae* were also correlated with the aggravation of motor impairment and disease severity (Barichella et al., 2019; Pietrucci et al., 2019). Our results suggested that PP could regulate the homeostasis of gut microbiota.

GABA was an inhibitory neurotransmitter which could effectively reduce the excitability of neurons and maintain the redox homeostasis of cells (Sarasa et al., 2020). A study indicated that an abnormal GABA level was positively correlated with the severity of motor symptoms in PD (O'Gorman Tuura et al., 2018). A clinical study showed that the level of GABA in the brain of PD patients was significantly lower than that of healthy controls (Song et al., 2021), which resulted in the dopaminergic pathology of PD. GABA treatment increased the expression level of Nrf2 and the activity of antioxidant enzymes, such as CAT and SOD, effectively reducing the consumption of glutathione and the level of ROS and thereby reducing oxidative stress (Zhu et al., 2019; Choe et al., 2021). Excessive GABA in astrocytes caused the loss of TH, which showed a decrease in the discharge of dopaminergic neurons, while blocking the astrocytic GABA synthesis could reverse this effect (Heo et al., 2020). GABA is generally considered as a key candidate mediator, which is a metabolite of the functional bacteria. GABA might be involved in the communication of the gut–brain axis and influence brain function. We believed that PP might possess many mechanisms in the neuroprotection of PD, among which GABA might be a very important mechanism. The level of GABA affected by symbiotic gut microbiota could lead to behavioral and cognitive changes (Strandwitz, 2018; Zheng et al., 2019), which were consistent with our results. Furthermore, oral supplementation of *Lactobacillus plantarum* strain SNK12 upregulated the GABA level and improved the habitual ability and stress behavior in stress-induced mice (Tsukahara et al., 2019). Long-term treatment with *Lactobacillus rhamnosus* JB-1 influenced the mRNA expressions of GABA receptors in the brain, regulated the gut–brain axis in a vagal-dependent manner, and reduced the depression- and anxiety-like behaviors of pressure-induced mice (Bravo et al., 2011). The treatment of probiotics and prebiotics also effectively restored the level of GABA and normalized the levels of lipid peroxidation and antioxidant glutathione in the brain of rats with cerebral poisoning (Al Suhaibani et al., 2021), indicating that GABA produced by the gut microbiota had great potential in regulating oxidative stress associated with neurodegenerative diseases. Given that gut microbiota has been shown to be influenced by PP and that the level of cerebral GABA was altered significantly, the GABA/Nrf2 pathway might be changed by the communication between PP and the gut–brain axis. The neuroprotective mechanism of PP on the oxidative stress of PD through the metabolites of the GABA–gut–brain axis needs to be studied further.

Collectively, our results illuminated that PP treatment had an antioxidant effect in MPTP-induced mice, and its beneficial mechanism was referred to in the GABA/Nrf2 pathway *via* adjusting the gut–brain axis. PP was able to be a promising candidate used for PD treatment due to the metabolite of GABA.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository and accession number can be found below: NCBI, SUB11968017. The SRA records can be accessible with the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA873227.

Ethics statement

The animal study was reviewed and approved by the First Affiliated Hospital of Wenzhou Medical University.

Author contributions

JL and YaZ conceived and designed the experiments. SP, HW, SY, YK, HY, YuZ, XC, and WC performed the experiments and conducted the statistical analyses. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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The role of gut microbiota in gout: Is gut microbiota a potential target for gout treatment

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Numerous studies have demonstrated that gut microbiota is essential for the host's health because it regulates the host's metabolism, endocrine, and immune systems. In recent years, increasing evidence has shown that gut microbiota plays a role in the onset and progression of gout. Changes in the composition and metabolism of the gut microbiota, result in abnormalities of uric acid degradation, increasing uric acid generation, releasing pro-inflammatory mediators, and intestinal barrier damage in developing gout. As a result, gout therapy that targets gut microbiota has drawn significant interest. This review summarized how the gut microbiota contributes to the pathophysiology of gout and how gout affects the gut microbiota. Additionally, this study explained how gut microbiota might serve as a unique index for the diagnosis of gout and how conventional gout treatment medicines interact with it. Finally, prospective therapeutic approaches focusing on gut microbiota for the prevention and treatment of gout were highlighted, which may represent a future avenue in gout treatment.

KEYWORDS

gout, hyperuricemia, gut microbiota, probiotics, prebiotics

1 Introduction

Gout is a common disease characterized by the deposition of monosodium urate (MSU) crystals in joint and non-joint structures (Dalbeth et al., 2021). The inflammatory response of host tissue to deposit monosodium urate (MSU) crystals induces clinical symptoms (Dalbeth et al., 2019). Globally, gout is highly prevalent. Adults in China have

a gout prevalence rate of 1.1%, compared to 3% to 4% in the United States and 1% to 4% in Europe (Dehlin et al., 2020). Genetic diversity, environmental exposure, gene-environment interaction, and intrinsic risk factors (including age, gender and weight) contribute to the risk of developing gout (Major et al., 2018). In addition, gout and hyperuricemia have many common comorbidities, such as cardiovascular disease, chronic kidney disease, diabetes, metabolic syndrome and neurodegenerative diseases (Bardin and Richette, 2017).

The human digestive system contains trillions of species, including bacteria, fungi, archaea, viruses, and protozoa, which comprise the gut microbiota, a complex ecological community (Human Microbiome Project, 2012). The taxonomic diversity of the gut microbiota impacts the integrity of the epithelial barrier, preservation of intestinal metabolism, and immunological homeostasis (Parker et al., 2020). The gut microbiota influences healthy physiological function and disease susceptibility through its collective metabolic activity and host interaction (Lozupone et al., 2012). With the advancement of sequencing technology and the creation of new bioinformatics, it has been discovered that gut microbiota composition changes and metabolism disruptions are connected to the pathogenesis of numerous diseases, such as autoimmune disease (Jiao et al., 2020), mental illness (Jarbrink-Sehgal and Andreasson, 2020), cerebrovascular disease (Xu et al., 2020), and disorders of the central nervous system (Vuotto et al., 2020).

Emerging evidence revealed a link between gut microbiota and arthritis diseases, including gout (Chu et al., 2021). Therefore, this review aims to summarize gut microbiota function in gout pathogenesis and illustrate gut microbiota as a potential target of gout treatment.

2 Gut microbiota and physiologic acid uric metabolism

In humans and higher primates, urate is the final oxidation product of purine catabolism (Cabau et al., 2020). It is synthesized mainly in the liver, intestines and tissues, such as muscles, kidneys, and vascular endothelium (El Ridi and Tallima, 2017). Purine synthesis begins with 5-phosphoribosyl-alpha-1-pyrophosphate (PRPP) leading to hypoxanthine nucleotide formation (Dewulf et al., 2022). Hypoxanthine is converted to xanthine, which is then transformed into uric acid (UA) by either xanthine oxidase (XO) or xanthine dehydrogenase (Sun et al., 2022). Approximately 700 mg of UA is produced daily by such processes (Yanai et al., 2021). Renal and gut excretions accounts for around two-thirds and one-third of urate excretion, respectively (Mandal and Mount, 2015). Urate homeostasis is primarily influenced by renal proximal tubule cells, which express several transporters that either reabsorb urate or are involved in urate excretion (Eckenstaler and Benndorf, 2021).

During the evolution of humanoid primates, the pseudogenization of the uricase gene caused humans and other mammals to lose uricase activity. It left them unable to oxidize further urate to the more water-soluble compound, allantoin (Chang, 2014).Therefore, serum uric acid (sUA) levels in humans are three- ten times higher than in organisms that preserve uricase (Kratzer et al., 2014).

Unlike humans, bacteria can degrade uric acid by urate oxidase (uricase), and specific bacterial strains also exhibit xanthine dehydrogenase (XDH) inhibitory activity (Alam et al., 2011). *Lactobacillus* species break down inosine and guanosine to inhibit uric acid biosynthesis during purine metabolism (Alvarez-Lario and Macarron-Vicente, 2010; Wu et al., 2020). Recently, *Lactobacillus gallinii* has been shown to reduce purine levels in the gut, and its fermentation products have urate-lowering effects (Li et al., 2014). In addition, *Lactobacillus gasseri* strains can reduce purine absorption in the gut (Yamada et al., 2016). However, not all gut microbiota is protective. Xi et al. demonstrated that *Escherichia-Shigella* secretes xanthine deaminase, converting hypoxanthine and xanthine into uric acid and elevating serum uric acid levels (Xi et al., 2000).

The gut microbiota also plays a role in uric acid excretion. Studies have shown that two short-chain fatty acids (SCFAs) (propionate and butyrate) provide adenosine triphosphate (ATP) to the intestinal wall cells and promote UA excretion (Nieuwdorp et al., 2014). In addition, a recent study found higher *Escherichia coli* levels in greater uric acid decomposition (Liu et al., 2020).

3 Dysbiosis and gout

3.1 Description of the microbiome in gout patients

The abnormal secretion of interleukin-1β (IL-1β) stimulated by MSU causes the acute onset of gout, which occurs by activating the innate immune system through the recognition of Toll-like receptor (TLR) or NOD-like receptor (NLR) (Dalbeth et al., 2021). The release of a large amount of IL-1β by activating of NLRP3 (NOD-, LRR-, and pyrin domain-containing 3 inflammasome) is the central process of MSU-mediated gout acute attack (Martinon et al., 2006).

A recent study reported that *Phascolarctobacterium* and *Bacteroides* were enriched in gout patients and identified a "core microbiota" for the gout group encompassing three *Bacteroides* species (Mendez-Salazar et al., 2021). *Bacteroides* is a gut enterotype reported to promote urate conversion into allantoin, and might be involved in serum urate level regulation in humans (Lim et al., 2014).

GUO et al. observed that the gut microbiota of gout was characterized by significantly-impaired butyric acid synthesis

(Guo et al., 2016). Vieira et al. found that SCFAs were necessary to assemble inflammasome and produce IL-1β (Vieira et al., 2015). Another study explored the effects of a high-fiber diet and acetate on inflammation in gout mice models. The regression of neutrophil inflammation was found to be related to a decrease of nuclear factor κ B (NF-κ B) activity and an increase of anti-inflammatory mediators (including interleukin-10, tumor necrosis factor-β and Annexin A1). Acetate controlled the inflammatory response to the MSU lens by promoting the regression of the inflammatory response (Vieira et al., 2017). The species that make SCFAs have a protective effect on inflammation, and are more abundant in the healthy group (Sheng et al., 2021). These results collectively showed that SCFAs and gut microbiota play a role in controlling inflammatory response to MSU crystals (Cleophas et al., 2017). Additionally, by up-regulating TLR2/4/5 and promoting the release of IL-1β and tumor necrosis factor-α (TNF-α), the increase in the number of inflammation-related microbiota causes immunological diseases and intestinal barrier dysfunction. Increased intestinal permeability, positively linked with serum uric acid level, results from decreased levels of the epithelial tight junction proteins occludin and claudin-1 (Lv et al., 2020).

3.2 Changes of gut microbiota composition and hyperuricemia

3.2.1 Diversity and abundance of gut microbiota in hyperuricemia

The variety and richness of gut microbiota have changed in hyperuricemic individuals and rats, indicating that gut microbiota may have a possible involvement in gout (Liu et al., 2020; Chu et al., 2021). Uric acid is the final product of purine metabolism and alterations in uric acid production or excretion can lead to abnormal serum uric acid levels (Balakumar et al., 2020). The changes in the abundance and composition of gut microbiota increase the serum uric acid level through the dysfunction of uric acid degradation and increased uric acid production (Sheng et al., 2021).

Gout has higher relative abundances of *Prevotella* and *Bacteroides* while lower relative abundances of *Enterobacteriaceae*, which might cause uric acid degradation dysfunction and the buildup of uric acid in gout (Chu et al., 2021). Additionally, the greater serum urate (SU) level was closely connected to the lower relative abundance of *Faecalibacterium* in hyperuricemia (Wei et al., 2021).

A shotgun metagenomic study revealed the microbiota with the allantoinase gene, which can convert uric acid into urea was deficient in gout. In contrast, the microbiota with the xanthine dehydrogenase gene was abundant. The buildup of uric acid may be caused by excessive xanthine dehydrogenase and a relative lack of allantoinase (Guo et al., 2016; Gong et al., 2020).

An increase in UA in blood circulation affects the intestinal environment, causing changes in the gut microbiota (Wang et al., 2022). A metagenomic study reveals that hyperuricemia causes an imbalance in the gut microbiota and alters its composition. It might induce the gut microbiota to translocate into other tissues, particularly the kidney, causing inflammation (Xu et al., 2019). In addition, a recent study has demonstrated that the abundance of inflammation-related microbiota in hyperuricemia and increased uric acid levels are associated with the impairment of intestinal barrier, which disrupts the host-microbiota crosstalk (Lv et al., 2020). While luminal UA can play a protective role in intestinal injury, some studies have shown that the changes in gut microbiota caused by uric acid are beneficial to the body (Wada et al., 2022). However, a study exploring the relationship between blood uric acid levels and gut microbiota in diabetic patients, found that fluctuations in uric acid within the normal range were not associated with changes in gut microbiota (Zhang et al., 2021). Therefore, further studies are needed to explore the causal relationship between the alteration of gut microbiota and hyperuricemia (Figure 1).

3.2.2 Metabolism of gut microbiota in hyperuricemia

Various metabolites, including SCFAs, trimethylamine, amino acid derivatives, and vitamins, are produced by the gut microbiota from dietary components, including significant amounts of micronutrients, fiber, and polyphenols (Parker et al., 2020). Acetate (C2), propionate (C3), and butyrate (C4) are the most prevalent SCFAs in the human body. SCFAs are most extensively researched (Macfarlane and Macfarlane, 2003). The human body relies heavily on SCFAs. Butyric acid protects the human gut by nourishing the mucosa, fosters the development and repair of intestinal villi, boosts intestinal immunity, encourages the growth of good bacteria, and prevents the colonization of pathogens (Louis and Flint, 2009). A study through the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis revealed significant differences in amino acid metabolism (phenylalanine, tyrosine and tryptophan biosynthesis, D-glutamine and D-glutamate metabolism, and phosphate and phosphonate metabolism) and nucleotide metabolism (purine metabolism) between hyperuricemia and healthy controls. The gut microbiota's metabolic dysfunction may influence serum uric acid levels through its impact on host metabolites (Wei et al., 2021). The production of SCFAs (concentrations of acetate, propionate, and butyrate) derived from the gut microbiota in mice positively correlates with the effectiveness of treating hyperuricemia. This finding demonstrates that some beneficial bacteria decrease in HUA mice, including bacteria that produce SCFAs, such as *Clostridium* and *Ruminococcus* (Yu et al., 2018; Xu et al., 2019; Guo et al., 2021).

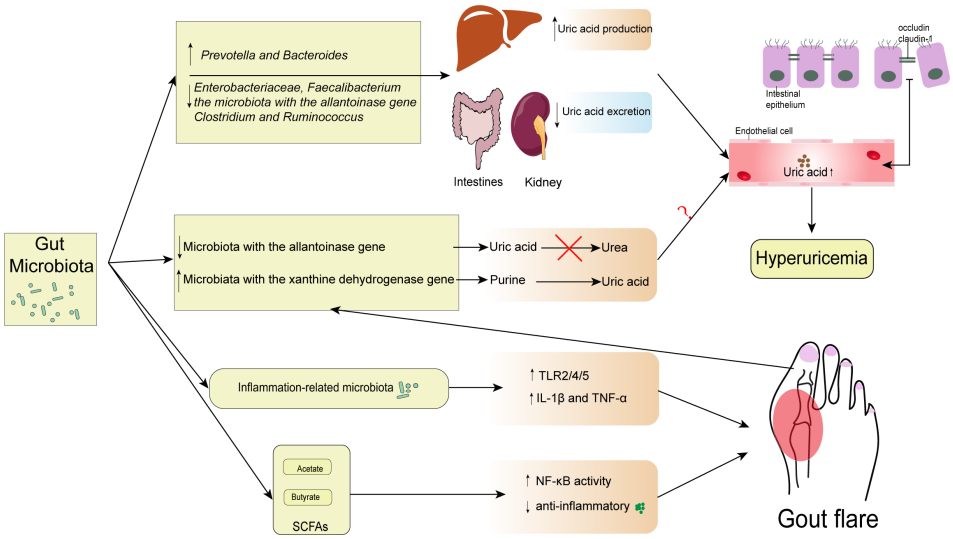

FIGURE 1
Interactions between gut microbiota and gout. The diversity and abundance of gut microbiota change include the increase of *Prevotella* and *Bacteroides* and the decrease of *Enterobacteriaceae*, *Faecalibacterium*, the microbiota with the allantoinase gene, *Clostridium*, and *Ruminococcus*. These changes result in excessive uric acid production in the liver and insufficient uric acid excretion in the kidney and intestine, raising serum uric acid levels above normal. In addition, some microbiota with the allantoinase and the xanthine dehydrogenase gene changed in gout can directly regulate the intestinal uric acid levels. However, the contribution of elevated intestinal uric acid levels to elevated serum uric acid levels remains unknown. Consequently, occludin and claudin-1 levels at tight epithelial junctions can drop when serum uric acid levels rise. Gout is caused by inflammation-related bacteria that upregulate TLR2/4/5 and encourage the release of IL-1 β and TNF- α. However, some SCFAs may have a protective role in inflammation. SCFAs, especially butyrate, are associated with the increased expression of Inhibitory-κκBα (I-κBα), which inhibits the phosphorylation and nuclear translocation of NF-κB p65, and the downstream inflammatory cytokine, MCP-1, and IL-1β expression.

4 Gout diagnosis based on gut microbiota

Due to the causative relationship between the gut microbiota and gout development, the gout- specific gut microbiota may be a diagnostic marker. Lin et al. made a classification model using significantly-enriched bacterial genera between healthy individuals and gout patients. The result showed a high mean area under the working curve (AUC) of up to 0.973 by the receiver operating characteristic (ROC) analysis (Lin et al., 2021). Likewise, a cohort study established a diagnostic model based on 17 kinds of gout-related bacteria and reached 88.9% accuracy (Guo et al., 2016). The metagenomic analysis of gut microbiota by Chu et al. found three genes significantly enriched in the cohort gout. The AUC of the development and validation cohort were 0.91 and 0.80, respectively (Chu et al., 2021).

Furthermore, Yang et al. verified that several bacteria, including *unclassified Enterobacteriaceae*, *Roseburia*, and *Faecalibacte-rium*, have excellent diagnostic value for asymptomatic hyperuricemia (Yang et al., 2021). Therefore, the gut microbiota imbalance characterized by gout may become a non-invasive diagnostic tool for gout and asymptomatic hyperuricemia. It has a promising target for future prevention and intervention.

5 Treatment of gout

5.1 Traditional treatment and gut microbiota changes

Non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and colchicine are the first-line drugs for acute gout (Kiltz et al., 2016). International guidelines describe xanthine oxidase inhibitors and uricosuric drugs as the first- and second-line treatments, respectively, in uric acid-lowering therapy (Engel et al., 2017). Gut microbiota has become an important factor in hyperuricemia and has been shown to affect the response to disease treatment (Yu et al., 2018). A recent study found substantial alterations in the gut microbiota composition and promotion of SCFA formation, particularly acetate, in gouty arthritis patients after treatment (Park and Lee, 2022).

5.1.1 Non-steroidal anti-inflammatory drugs

NSAIDs are classic drugs for treating acute gout that affect pain relief and inflammation (Bindu et al., 2020). However, NSAIDs have several side effects, including gastrointestinal damage. Studies have indicated that NSAIDs can disrupt the gut microbiota equilibrium, multiplying gram-negative bacteria

and decreasing gram-positive bacteria. Subsequently, pathogens activate inflammatory pathways through TLR4 and release inflammatory cytokines (Wang et al., 2021). In addition, NSAIDs can enhance intestinal permeability, making bacteria enter the mucosa (Montalto et al., 2010), leading to further changes in gut microbiota composition.

5.1.2 Colchicine

Colchicine (COL) is a traditional drug for gout that can block tubulin polymerization and prevent inflammasome activatin (Tristan Pascart, 2018). However, colchicine has potential toxicity to human health. Gastrointestinal discomfort is the most common symptom of COL toxicity, including nausea, vomiting and diarrhea (Akodad et al., 2020). Shi et al. found that acute oral COL in mice significantly affected the gastrointestinal structure and substantially changed the gut microbiota's diversity, composition, and function. This is closely related to the down-regulation of intestinal proinflammatory cytokines and the destruction of intestinal integrity in mice. Therefore, it supports the destruction of homeostasis in the intestinal microbiome and might increase the toxic burden of COL (Shi et al., 2020). Another study in the same group identified bacterial biomarkers associated with diarrhea, indicating that the adverse reactions caused by COL were closely related to the gastric microbiological disturbance. By understanding the microbiome's role in adverse COL reactions, the gut microbiota can be targeted, and the effectiveness of COL treatment can be increased (Shi et al., 2021).

5.1.3 Allopurinol

Allopurinol, an inhibitor of xanthine oxidase, is one of the most widely-used uric acid-lowering drugs (Mackenzie et al., 2020). Yu et al. found that the gut microbiota in the allopurinol treatment group changed compared with the control group. The treatment group had increased bifidobacterium and decreased anaerobes, which may be related to the decrease in UA. In addition, Bilophila, the only reduced genus in the allopurinol treatment group (Yu et al., 2018), has been shown to cause systemic inflammation (Feng et al., 2017).

5.1.4 Benzbromarone

Benzbromarone decreases blood uric acid levels and reabsorption by blocking the dominant apical (luminal) uric acid exchanger in the human proximal tubule, URAT-1 (Azevedo et al., 2019). Similar findings were made in another study, which showed that treating with benzbromarone altered the gut microbiota in the group that received it. It led to an increase in *Bifidobacterium* and a decrease in anaerobes *Butyricimonas*. In addition, benzbromarone repaired the lipid metabolism disorder in hyperuricemia rats through gut microbiota intervention (Yu et al., 2018).

5.1.5 Febuxostat

Febuxostat, a nonpurine inhibitor of xanthine oxidase, treats hyperuricemia in gout patients. It inhibits oxidized and reduced forms of xanthine oxidase, reducing uric acid formation (White et al., 2018). Lin et al. detected a restriction of gut microbiota biodiversity in untreated gout patients and febuxostat partially restored this change. Functional analysis revealed that the gut microbiome of gout patients was functionally-enriched for carbohydrate metabolism but had a lower potential for purine metabolism, which was relatively enhanced in gout patients treated with febuxostat (Lin et al., 2021). Another animal study verified that febuxostat could reshape gut microbiota dysbiosis in an animal model, regulate gut-derived metabolites, and inhibit microinflammation *in vivo (*Tu et al., 2020).

5.2 Treatment of gout based on gut microbiota changes

5.2.1 Probiotics and prebiotics

Nowadays, the low rates of urate-lowering therapy initiation and continuation and the side effects of traditional drugs remain challenges for gout treatment. These side effects include gastrointestinal toxicity, tolerance, allopurinol hypersensitivity syndrome, nephrotoxicity, and contraindications of other common comorbidities (Khanna et al., 2012; Becker et al., 2015; Vargas-Santos and Neogi, 2017; Rai et al., 2018). About 40% of gout patients suffer from chronic kidney disease (CKD) (at least stage II) and decreased GFR (Gaffo and Saag, 2008). Non-steroidal anti-inflammatory drugs, colchicine and uricosuric medicine use also are limited (Aslam and Michet, 2017). Therefore, therapies or drugs which are safer and can intervene in gout development are greatly needed.

In recent years, a better understanding of gut microbiota in the pathogenesis of gout and applying natural products in the prevention and treatment of gout have attracted widespread attention. Natural products, probiotics, probiotics and fecal microbiota transplantation (FMT) have been widely studied by new therapeutic methods acting on gut microbiota (Wu et al., 2021; Zhao et al., 2022; Xie et al., 2022). These play a role in treating gout by inhibiting the metabolism of purine and inflammatory factors and bodies, regulating the expression of transporters, and protecting the integrity of intestinal barrier. It can also increase the abundance of intestinal bacteria related to the production of SCFA and promote SCFA production, thus inhibiting the activity of XOD in serum and liver (Ni et al., 2021).

Probiotics are "live microorganisms that, when administered adequately, confer a health benefit on the host (Hill et al., 2014). *Bifidobacterium and Lactobacillus strains* are the most widely-used probiotics in functional food and dietary supplements. However, the next generation of probiotics, such as *Faecalibacterium prausnitzii, Akkermansia muciniphila*, or the

genus *Clostridium*, have shown promising results (Vallianou et al., 2020).

Some *in vitro* experiments have proven that diets containing probiotics can prevent hyperuricemia by regulating the structure and function of intestinal flora. For example, *Lactobacillus fermentans* JL-3 can regulate hyperuricemia-induced intestinal microbiota dysbiosis and effectively reduce the UA level in mice (Wu et al., 2021). DM9218, as a probiotic strain, has the potential to ameliorate fructose-induced hyperuricemia. Animal experiments showed that DM9218 could reduce serum UA level and hepatic xanthine oxidase activity and regulate intestinal dysbiosis induced by high fructose in fructose-fed mice (Wang et al., 2019). In addition, Garcia-Arroyo et al. demonstrated that probiotics containing urate-decomposing bacteria could reduce serum uric acid in hyperuricemic animals. It could also beneficially affect hypertension and kidney disease (Garcia-Arroyo et al., 2018).

A prebiotic published is "a substrate that is selectively utilized by host microorganisms conferring a health benefit (Swanson et al., 2020). Recent studies employing a variety of probiotic molecules have consistently demonstrated an increase in the relative numbers of *Lactobacillus* and *Bifidobacterium* spp. as well as changes in bacterial metabolism, as evidenced, in particular, by increased production of short-chain fatty acids, such as butyrate and propionate (Holscher, 2017). The research of prebiotics in treating gout has become a promising direction.

An animal experiment by Ren et al. found that fisetin reversed changes in *Bacteroides*, and *Firmicutes* in hyperuricemic mice, suggesting that fisetin reduced serum uric acid levels by modulating hyperuricemia-induced changes in gut microbiota. In addition, fisetin could improve renal function in hyperuricemia-induced CKD mice by regulating intestinal microbiota-mediated tryptophan metabolism (Ren et al., 2021). In addition, relevant metabolomics studies have shown that nuciferine may inhibit the pathological process of hyperuricemia by regulating the disturbed metabolic pathways. Furthermore, nuciferine can restore the metabolic changes caused by hyperuricemia by regulating intestinal microbiota composition in rats (Wang et al., 2020). E. prolifera polysaccharides (EPP), one of the most widely distributed green algae belonging to the family Ulvaceae, showed beneficial effects on the serum levels of UA and significantly improved the diversity of gut microbiota, especially the proportions of Alistipes and *Parasutterella*. Further, correlation analysis revealed that the presence of *Parasutterella* might be negatively associated with increased UA (Li et al., 2021).

In addition, it has been shown that co-feeding of β-carotene and green tea powder in gouty mice significantly increased the positive interaction between gut microbes, which may positively in relieve gout symptoms (Feng et al., 2022). Dietary administration of tuna meat oligopeptides (TMOP) alleviates hyperuricemia and renal inflammatory phenotypes. Furthermore, it reprograms the uric acid metabolism pathway. TMOP treatment repairs the intestinal epithelial barrier, reverses the dysbiosis of the gut microbiota, and increased the production of SCFAs. Furthermore, the antihyperuricemic effect of TMOP was transmitted by transplanting fecal microbiota from TMOP-treated mice, mediating the protective effect, at least in part, by the gut microbiota (Han et al., 2020).

In recent years, there have been more studies on the mechanism of various probiotics and prebiotics in treating gout. Table 1 shows a summary of gout treatment targeting intestinal microorganisms. However, most studied conducted animal experiments, and no testing has been done on humans. Future research should focus more on human experiments to explore whether these new treatments, such as prebiotics and probiotics, can relieve the symptoms of gout and achieve the purpose of treatment.

5.2.2 Dietary habits

The microbiota composition can be modified by a variation in the individual's dietary-nutritional habits, especially concerning the quality and quantity of fats, dietary fibers and carbohydrates consumed (Voreades et al., 2014). Dietary factors are also considered a risk factor for gout (Dehlin et al., 2020), thus, several well-established healthy eating patterns, such as the Mediterranean and Diet to Stop Hypertension (DASH) diets, can lower serum urate levels (Yokose et al., 2021). Cohort studies have shown that a typical western diet is associated with a higher risk of developing gout, while adherence to a Mediterranean diet is associated with a lower risk (Rai et al., 2017). Therefore, the Mediterranean and DASH diets have preventive effects on hyperuricemia (Sun et al., 2022).

In addition, studies have shown that an excessive fructose diet can affect gut microbiota composition through a series of damage to the intestinal barrier function of the inflammatory response. Therefore, a new method for gout treatment could be to limit the specific fructose intake and improve the composition of gut microbiota or targeted metabolite (Fang et al., 2022).

5.2.3 Fecal microbiota transplantation

FMT is the transfer of fecal microbial content from a healthy individual into the gastrointestinal tract of a diseased individual (Ooijevaar et al., 2019). The action mechanism is not entirely understood, but restoring a disturbed microbiota underlies the observed effect (Smits et al., 2016). Since gut microbiota imbalance is closely related to gout, FMT may become a new direction for treating gout.

Xie et al. found that the washed microbiota transplantation (WMT) effectively reduced serum uric acid levels, relieved gout symptoms, and improved impaired intestinal barrier function in gout patients (Xie et al., 2022). In addition, a previous study showed that fecal transplantation attenuated hyperuricemia and renal inflammatory phenotypes in mice (Han et al., 2020).

TABLE 1 Mechanism of targeted gut microbiota in the treatment of gout.

	Type	Effect	Mechanism
Inulin (Guo et al., 2021)	Prebiotics	Reduces serum uric acid level, relieves inflammation and repairs intestinal epithelial barrier. Enhances microbial diversity and increases the relative abundance of beneficial bacteria.	Increase ABCG2 expression in the intestinal tract. Down-regulate XOD expression and activity in the liver of KO mice.
Chicory (Bian et al., 2020)	Prebiotics	Reduces serum uric acid level and increases fecal uric acid. Repairs intestinal mucosal injury.	Increases the number of probiotics and reduce the number of pathogenic bacteria to restore intestinal microbiota. Reduces the inflammatory response of the LPS/TLR4 axis by down-regulating the inflammatory pathways of serum LPS and TLR4/NF-κB in the kidney, thus promoting the excretion of uric acid in the kidney
Tuna meat oligopeptides (TMOP) (Han et al., 2020)	Prebiotics	Reduces hyperuricemia and renal inflammatory phenotype.	Reprograms the uric acid metabolic pathway to inhibit NLRP3 inflammasome activation and toll-like receptor 4/bone marrow differentiation factor 88/NF-kappaB (TLR4/MyD88/NF-κB) signal pathway, and the phosphorylation of p65 murine NF-κ B. Repairs the intestinal epithelial barrier. Reverses intestinal flora imbalance and increases short-chain fatty acids production.
Camellia japonica bee pollen polyphenols (CPE-E) (Xu et al., 2021)	Prebiotics	Reduces serum uric acid level and improve renal function.	Inhibits hepatic xanthine oxidase (XOD) activity and regulates the expression of URAT1, GLUT9, OAT1, OCT1 and ABCG2 in the kidney. Changes gut microbiota structure and increases the abundance of beneficial bacteria and the content of short-chain fatty acids. Decreases NLRP3 inflammasome and related inflammatory cytokines.
Sea cucumber hydrolysates (Wan et al., 2020)	Prebiotics	Reduces hyperuricemia and renal inflammation caused by diet.	Inhibits uric acid biosynthesis and promote uric acid excretion. Down-regulates pro-inflammatory cytokine transcription and up-regulates anti-inflammatory cytokine transcription. Inhibits TLR4/MyD88/NF-κB signal pathway. Increases the abundance of beneficial lactobacillus and short-chain fatty acids producers and reduces the abundance of opportunistic pathogens to alleviate intestinal microbiota dysfunction.
β-carotin and green tea powder (Feng et al., 2022)	Prebiotics	Relieves acute gout attack.	Reduces the joint swelling and pain in mice with gout. Reduces serum uric acid and pro-inflammatory cytokines levels. Improves the gut microbiota profile and reduces the metabolic levels of purines and pyrimidines.
Enteromorpha prolifera (Li et al., 2021)	Prebiotics	Reduces hyperuricemia and reverses kidney damage.	Decreases serum uric acid, serum urea nitrogen, serum xanthine oxidase (XOD), and hepatic XOD. Up-regulates UA excretion genes, such as ABCG2, OAT1, and NPT1. Down-regulates UA absorption gene URAT1 was down-regulated. Maintains intestinal flora stability, which is closely related to the regulation of hyperuricemia.
Hexapeptides derived from Apostichopus japonicus hydrolysate (Fan et al., 2022)	Prebiotics	Reduces uric acid biosynthesis and reabsorption.	Inhibits uric acid biosynthesis and reabsorption to reduce serum uric acid. Reduces renal inflammation and inhibits the activation of NLRP3 inflammasome. Decreases the richness and diversity of gut microbiota and changes the composition of phylum and genus levels. Changes miRNA expression in the kidney.
Anserine supplementation (Han et al., 2021)	Prebiotics	Promotes uric acid excretion. Has anti-inflammatory effects.	Increases hypoxanthine phosphate ribose transferase expression. Inhibits the uric acid synthesis by activating uric acid transporter. Inhibits NLRP3 inflammasome and TLR4/MyD88/NF-κB pathway. Regulates the composition and abundance of gut microbiota during hyperuricemia and renal inflammation.
Nuciferine (Wang et al., 2020)	Prebiotics	Relieves hyperuricemia and improves renal function.	Interferes with the gut microbiota and restores the metabolic balance of hyperuricemia rats. Reverses the levels of creatinine and creatine in rats to some extent after nuciferine treatment.
Fisetin (Ren et al., 2021)	Prebiotics	Prevents CKD induced by hyperuricemia.	Regulates tryptophan metabolism and aromatics receptor (AHR) activation mediated by gut microbiota.
Curcumin (Xu et al., 2021)	Prebiotics	Reduces the level of uremic toxin and improves renal inflammation and fibrosis.	Regulates gut microbiota's structure and improves intestinal permeability. Increases beneficial bacteria and reduces pathogens.

(Continued)

TABLE 1 Continued

	Type	Effect	Mechanism
AJOP (Lu et al., 2021)	Prebiotics	Relieves hyperuricemia.	Regulates uric acid metabolism, inhibits NLRP3 inflammasome and NF- κB signal pathway activation, and repairs intestinal epithelial barrier. Globally changes the spectrum of GIT microflora.
Lactobacillus brevis DM9218 (Wang et al., 2019)	Probiotics	Reduce serum uric acid level.	Down-regulates xanthine oxidase expression and activity stimulated by inflammatory cytokines.
Limosilacto-bacillus fermentum JL-3 (Wu et al., 2021)	Probiotics	Attenuates oxidative stress and inflammation induced by UA and regulates UA-induced flora imbalance in hyperuricemia mice.	Degrades UA in the intestine and reduces the amount of UA reabsorbed by intestinal epithelium into circulation. Contains purine-degrading lactobacillus strains and improves defecation activity, thus reducing fecal excretion of UA. Regulates gut microbiota's structure and function.
Qu-Zhuo-Tong-Bi Decoction (QZTBD) (Wen et al., 2020)	Traditional Chinese medicine	Relieves acute gout attack.	Recovers the imbalance of gut microbiota and enhances SCFA formation. Inhibits intestinal barrier function, key glycolysis-related enzymes, and inflammatory factors production.
Fecal microbiota transplantation (Liu et al., 2020; Han et al., 2020; Xie et al., 2022)		Reduces serum uric acid level.	Restores damaged intestinal barrier function.

5.2.4 Traditional Chinese medicine

Traditional Chinese medicine (TCM) has been applied to treat gout since ancient China. Some chemical ingredients isolated from TCM herbs are multi-target and low toxicity, showing advantages and good prospects in gout prevention and treatment (Chi et al., 2020).

An animal study by Lin et al. demonstrated that Si Miao decoction improved gut microbiota dysbiosis associated with gouty arthritis by significantly reducing the abundance of *Prevotella* in the gut microbiota of mice, beneficial to relieve inflammation. In addition, some pathogenic bacteria positively correlated with intestinal inflammatory cytokines were reduced by Si Miao decoction, including *Klebsiella, Brautia, Escherichia-Shigella, and Enterococcus* (Lin et al., 2020).

Qu-Zhuo-Tong-Bi Decoction (QZTBD) has been shown to inhibit the growth of *Larrelidae* _A2, a bacterium enriched in gouty mice, and to improve the abundance of *ranunculus* (a bacterium closely related to SCFAs). QZTBD can exert its therapeutic effects by restoring the gut microbiota composition and SCFA production. QZTBD treatment attenuated intestinal mucosal barrier function and promoted intestinal uric acid excretion through these changes. Furthermore, it inhibited glycolysis and suppressed intestinal proinflammatory cytokines (Wen et al., 2020). Therefore, traditional Chinese medicine targeting intestinal flora in gout treatment may be a promising direction

6 Conclusions

Gut microbiota plays a key role in gout pathogenesis through the changes of diversity, abundance, metabolic pathway, and metabolites, such as SCFAs, resulting in hyperuricemia and gout flare. Hyperuricemia and gout can cause an imbalance in the microbiota in the gut, which can then trigger the development of other metabolic illnesses, creating a vicious cycle. In addition, drugs related to gout treatment can play a therapeutic role by changing the composition of gut microbiota. Gut microbiota examination provides a non-invasive, simple, sensitive, and reliable index in diagnosis. Developing novel and safe new drugs targeted at gut microbiota has become a research focus. Prebiotics, probiotics, traditional Chinese medicine and fecal transplantation therapy are expected to become new methods for gout treatment.

Author contributions

ST: Writing - Original Draft. PZ and MC: Visualization. QC and XC: Resources. ZW: Methodology. XL: Writing- Reviewing and Editing. HW: Conceptualization. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Gut microbiota signature as predictors of adverse outcomes after acute ischemic stroke in patients with hyperlipidemia

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Introduction: The alterations of gut microbiota have been associated with multiple diseases. However, the relationship between gut microbiota and adverse outcomes of hyperlipidemic stroke patients remains unclear. Here we determined the gut microbial signature to predict the poor outcome of acute ischemic stroke (AIS) with hyperlipidemia (POAH).

Methods: Fecal samples from hyperlipidemic stroke patients were collected, which further analyzed by 16s rRNA gene sequencing. The diversity, community composition and differential gut microbiota were evaluated. The adverse outcomes were determined by modified Rankin Scale (mRS) scores at 3 months after admission. The diagnostic performance of microbial characteristics in predicting adverse outcomes was assessed by receiver operating characteristic (ROC) curves.

Results: Our results showed that the composition and structure of gut microbiota between POAH patients and good outcome of AIS with hyperlipidemia (GOAH) patients were different. The characteristic gut microbiota of POAH patients was that the relative abundance of *Enterococcaceae* and *Enterococcus* were increased, while the relative abundance of *Lachnospiraceae*, *Faecalibacterium*, *Rothia* and *Butyricicoccus* were decreased. Moreover, the characteristic gut microbiota were correlated with many clinical parameters, such as National Institutes of Health Stroke Scale (NIHSS) score, mean arterial pressure, and history of cerebrovascular disease. Moreover, the ROC models based on the characteristic microbiota or the combination of characteristic microbiota with independent risk factors could distinguish POAH patients and GOAH patients (area under curve is 0.694 and 0.971 respectively).

Conclusions: These findings revealed the microbial characteristics of POAH, which highlighted the predictive capability of characteristic microbiota in POAH patients.

KEYWORDS

acute ischemic stroke, hyperlipidemia, post-stroke poor outcome, gut microbiota, ROC curve

Introduction

Acute ischemic stroke (AIS) was a leading cause of death and chronic disability worldwide. Stroke survivors frequently had various complications, such as cognitive impairment and physical disability, which had a great impact on the quality of life (Duncan et al., 2021; Paul and Candelario-Jalil, 2021). Recent studies have shown that some risk factors including age, smoking and hyperlipidemia could affect the functional outcome after stroke (Meschia and Brott, 2018; Diener and Hankey, 2020). Hyperlipidemia could result in the neuroinflammation of brain and aggravated ischemic brain injury (Kim et al., 2014), and half of stroke patients were found to have hyperlipidemia (Rother et al., 2008). Hyperlipidemic stroke patients might suffer from functional deterioration after AIS. Kim et al. reported that the elevated plasma cholesterol levels were positively correlated with stroke severity in the hyperlipidemic mice (Kim et al., 2020). Elevated low-density lipoprotein cholesterol (LDL-C) was independently associated with severe stroke in patients with chronic kidney disease (Zhang et al., 2021). Currently, early detection of poor outcome of AIS with hyperlipidemia (POAH) was often challenging. Therefore, it is very urgent to find early biomarkers to evaluate the prognosis of hyperlipidemic stroke patients.

Recent studies have emphasized that the characteristic gut microbiota (GM) are associated with AIS. It was reported that stroke patients showed significant dysbiosis of bacteria with enriched short-chain fatty acids (SCFAs) (Li et al., 2019). Our previous studies showed that Proteobacteria was highly increased in the post-stroke cognitive impairment patients compared with the post-stroke noncognitive impairment patients (Ling et al., 2020). More and more evidence showed that GM have important influences on the occurrence, development and severity of stroke. Zhu et al. reported that GM directly impact cerebral infarct size and adverse outcomes following stroke through GM-derived metabolite trimethylamine-N-oxide (Zhu et al., 2021). GM have been increasingly recognized as vital determinants involved in the development of stroke and hyperlipidemia (Ling et al., 2022). The patients with hyperlipidemia showed abnormal GM composition (Gargari et al., 2018), which would aggravate dyslipidemia (Deng et al., 2019; Gu et al., 2020), while regulating GM could alleviate the abnormality of serum lipid in animal models (Yan et al., 2022). These findings demonstrated that GM might be an important regulator of the prognosis of hyperlipidemic stroke patients.

Recent evidences demonstrate that GM could be regarded as a diagnosis biomarker for many diseases. Our previous studies showed that patients with post-stroke comorbid cognitive impairment and depression exhibited an increased abundance of Proteobacteria, and a decreased abundance of several SCFAs-producing bacteria (Ling et al., 2020). It was reported that the abundance of *Alcaligenaceae* and *Acinetobacter* could remarkably distinguish autism spectrum disorders from the healthy group (Li et al., 2019). GM could distinguish stroke patients from healthy controls and the level of SCFAs appeared to effectively predict the severity and prognosis of stroke to some extent (Sun et al., 2021; Tan et al., 2021). The increased relative abundance of *Finegoldia magna, Bifidobacterium dentium*, and *Clostridium clostridioforme* could be used as a predictor of aging (Chen et al., 2022). Although the diagnostic application of GM has been well studied, the characteristic microbiota in POAH patients remains unclear.

Therefore, the present study was performed to investigate the characteristic GM of POAH patients. We further confirmed the correlation between characteristic GM and clinical parameters, as well as determined the gut microbial signature to predict POAH.

Materials and methods

Study patients

This study was conducted in the Department of Neurology of the Second Affiliated Hospital of Wenzhou Medical University, from September 2020 to July 2021. Inclusion

criteria: patients diagnosed with AIS; admission within 72 hours after stroke onset; previously diagnosed with hyperlipidemia or triglyceride (TG) > 2.28 mmol/L or total cholesterol (TC) > 6.2 mmol/L or high-density lipoprotein (HDL) < 0.91 mmol/L or low-density lipoprotein (LDL) > 3.4 mmol/L. Exclusion criteria: application of antibiotics or probiotics within three months, restriction of diet, concurrent pregnancy, schizophrenia, bipolar disorder, or other serious life-threatening illnesses (heart failure, respiratory failure, or severe renal dysfunction). The modified Rankin Scale (mRS) was applied to assess the post-stroke functional outcome of each patient in a 90-day follow-up after the stroke onset. The included AIS with hyperlipidemia were divided into the good functional outcome group (mRS score < 3) and the poor functional outcome group (mRS score ≥ 3).

Clinical data collection

All hyperlipidemic stroke patients were collected basic information at enrollment, including sex, age, years of education, history of smoking and drinking, presence of hypertension and diabetes, and history of cerebrovascular disease. Hypertension was considered as blood pressure ≥ 140/90 mmHg. Diabetes was defined as fasting blood glucose ≥ 7.0 mmol/L or 2 h blood glucose ≥ 11.1 mmol/L in an oral glucose tolerance test. The blood samples were extracted on an empty stomach after fasting overnight and centrifuged at 1300xg for 10 minutes. The biochemical indicators analyzed included TG, TC, HDL, LDL, creatinine, vitamin B12, folic acid (FOA), uric acid (UA), homocysteine (Hcy), C-reactive protein (CRP), hypersensitive C-reactive protein (hs-CRP), fasting blood glucose (FPG), glycosylated hemoglobin, thyrotropin, free triiodothyronine (FT3), free tetraiodothyronine (FT4), mean arterial pressure (MAP), D-dimer, alanine transaminase (ALT), aspartate transaminase (AST) and troponin. Moreover, computed tomography (CT) and magnetic resonance imaging (MRI) were used to identify new lesions of patient. Stroke severity was evaluated based on the National Institutes of Health Stroke Scale (NIHSS) by professional physicians within 24 hours of admission. Sleep condition was also quantified through Pittsburgh Sleep Quality Index (PSQI) during hospitalization.

GM analysis

Fresh stool samples (200 mg) were obtained, and fed into a labeled 2 ml sterile centrifuge tube and quickly stored in a -80°C freezer. The bacterial DNA was isolated by E.Z.N.A. ® Manual of soil Kit (Omega Bio-tek, Norcross, GA, U.S.), and the concentration and purity of which were detected with NanoDrop2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable regions of the 16s rRNA gene were amplified using PCR with primers 338F: ACTCCTACGGGAGGCAGCAG and 806R: GGACTACHVGGGTWTCTAAT. Next, PCR products were recycled by 2% agarose gel, and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego,USA). Alpha diversity was analyzed through Shannon and ACE. Principal coordinates analysis (PCoA) on the Bray-Curtis dissimilarity index was used for beta diversity analysis. The intestinal typing analysis was performed at the genus level by clustering samples with similar dominant microbiota structures into a class. Moreover, we identified the significant differences in relative abundance at levels of phylum, class, order, family, genus, and species by Wilcoxon rank sum tests based on the obtained community abundance data. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to find significantly enriched taxa and their influence between the two groups using nonparametric Kruskal Wallis (KW) sum rank test, with thresholds of LDA score > 2.

Statistical analysis

Statistical analysis was carried out by SPSS V.22.0 (SPSS, Chicago, USA). Chi-square test and multivariate logistic analysis were used to analyze the categorical variable data. Odds ratio (OR) and 95% confidence interval (95% CI) were figured out. The values of continuous variables were represented as median with quartile or mean with standard deviation (SD) based on the fact whether they were normally distributed, and compared by rank sum test or t-test respectively. The P value < 0.05 was considered to be of significance.

Results

Baseline characteristics of the recruited patients

According to the follow-up mRS results, 231 hyperlipidemic stroke patients were divided into two groups: 58 POAH patients and 173 good outcomes of AIS with hyperlipidemia (GOAH) patients. As showed in Table 1, POAH patients had significantly elevated levels of age, history of cerebrovascular disease, CRP, hs-CRP, NIHSS score, D-dimer and mRS score compared with GOAH patients. Additionally, a reduction of FT3, MAP and ALT was observed in POAH versus GOAH. There were no statistical differences in demographic data, including gender, educational level, history of smoking and drinking, diabetes, hypertension, and hyperlipemia between the two groups. As

TABLE 1 Baseline characteristics of the recruited patients.

Parameter	GOAH group	POAH group	*P*
	(n=173)	(n=58)	
Male (%)	117 (67.6)	35 (60.3)	0.473
Age (years old)	64.03 ± 12.28	70.91 ± 10.77	<0.001
Educational level			0.175
Illiteracy	32	16	
Primary school	64	20	
Junior high school	53	17	
High school and above	24	5	
Smoking	67 (38.7)	17 (29.3)	0.198
Drinking	57 (32.9)	13 (22.4)	0.132
Hypertension	130 (75.1)	46 (79.3)	0.520
Diabetes	67 (38.7)	29 (50.0)	0.133
Hyperlipemia	126 (72.8)	44 (75.9)	0.651
Cerebrovascular disease	28 (16.2)	24 (41.4)	<0.001
Creatinine (μmol/L)	66.30 (55.45-78.65)	62.25 (52.03-76.33)	0.099
Vitamin B12 (pg/mL)	339 (224–436)	350.5 (238-533.25);	0.286
Folic acid (ng/mL)	8.82 (6.77-11.40)	8.65 (5.88-10.53);	0.397
Uric acid (μmol/L)	323.0 (261.0-386.5)	313.5 (241.3-391.5)	0.301
Hcy (μmol/L)	11.40 (9.60-13.99)	11.30 (8.88-14.00)	0.500
CRP (mg/L)	3.30 (2.98-6.05);	4.40 (3.13-13.35)	0.005
Hs-CRP (mg/L)	1.70 (0.94-4.83);	4.39 (1.80-10.00)	<0.001
Triglycerides (mmol/L)	1.68 (1.27-2.23)	1.56 (1.04-2.24)	0.200
Total cholesterol (mmol/L)	4.52 (3.57-5.27)	4.78 (3.61-5.69)	0.263
LDL (mmol/L)	2.94 (2.15-3.67)	3.32 (2.32-3.81)	0.203
HDL (mmol/L))	0.87 (0.77-1.04)	0.91 (0.77-1.19)	0.364
Fasting plasma glucose (mmol/L)	5.49 (4.84-6.64)	5.75 (4.91-7.14)	0.317
Glycosylated hemoglobin (%)	6.11 (5.60-7.02)	6.03 (5.63-7.83)	0.342
Thyrotropin (μIU)	1.91 (1.19-3.02)	1.99 (1.12-2.77)	0.793
FT3 (pg/mL)	2.98 (2.76-3.25)	2.76 (2.53-2.91)	<0.001
FT4 (ng/dL)	1.17 (1.05-1.30)	1.14 (1.07-1.24)	0.959
NIHSS score	2.0 (1.0-3.5)	4.50 (2.75-9.00)	<0.001
PSQI score	5.0 (3.0-8.0)	6.14 (4.0-6.14);	0.105
MAP (mmHg)	139.61 ± 17.03	109.24 ± 11.27	<0.001
D-dimer (mg/L)	0.35 (0.26-0.52)	0.48 (0.33-0.75)	0.003
ALT (μ/L)	17 (13–26)	15.00 (11-21.25)	0.028
AST (μ/l)	18 (15-23)	18.00 (13.75-24)	0.669
Troponin (μmol/L)	0.012 (0.012-0.013)	0.012 (0.012-0.019)	0.396
mRS score	1 (0-2)	3 (3-4)	<0.001

POAH, poor outcomes AIS with hyperlipidemia; GOAH, good outcomes AIS with hyperlipidemia; Hcy, homocysteine; CRP, C-reactive protein; Hs-CRP, hypersensitive C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FT3, free triiodothyronine; FT4, free thyroid hormone; NIHSS, National Institutes of Health Stroke Scale; PSQI, Pittsburgh Sleep Quality Index; MAP, mean arterial pressure; ALT, alanine transaminase; AST, aspartate transaminase; mRS, modified Rankin scale.

shown in Table 2, the multivariate logistic regression analysis of demographic and clinical parameters with significant differences described above. The results indicated that a history of cerebrovascular disease (OR = 4.669, p = 0.008), increased NIHSS score (OR = 1.524, $P < 0.001$), and decreased MAP (OR = 0.842, $P < 0.001$) were the independent risk factors of POAH.

Analysis of GM diversity of POAH

Alpha diversity was evaluated by the Ace index (p = 0.4627, Figure 1A) and Shannon index (p = 0.1218, Figure 1B), exhibited no significant difference between the two groups. β diversity of the POAH differed from the GOAH according to the PCoA scatterplot (p = 0.018, Figure 1C). The Venn and the Bar diagrams exhibited the number of ASVs in the two groups, with 1656 shared ASVs (Figure 1D). The number of unique ASVs in GOAH group was 3097, which was higher than the number 839 in POAH.

Analysis of microbial composition of POAH

As shown in Figure 2, the microbial population of phylum level was mainly composed of Firmicutes, Bacteroidota, Proteobacteria and Actinobacteriota (Figure 2A). The proportion of Proteobacteria was 55% in the GOAH group. At the family level, the bacterial

TABLE 2 Multivariate logistic regression analysis.

Parameter	B (SE)	*P*-value	OR	95%CI
Age	0.02 (0.024)	0.271	1.026	0.980-1.075
Cerebrovascular disease	1.541 (0.022)	0.008	4.669	1.486-14.672
Hs-CRP	0.08 (0.085)	0.325	1.087	0.921-1.284
CRP	-0.01 (0.015)	0.245	0.983	0.955-1.012
FT3	0.343 (0.733)	0.639	1.410	0.335-5.925
NIHSS score	0.42 (0.110)	<0.001	1.524	1.228-1.892
MAP	-0.17 (0.029)	<0.001	0.842	0.795-0.892
ALT	-0.01 (0.014)	0.348	0.987	0.961-1.014

Hs-CRP, hypersensitive C-reactive protein; CRP, C-reactive protein; FT3, free triiodothyronine; NIHSS, National Institutes of Health Stroke Scale; MAP, mean arterial pressure; ALT, alanine transaminase; OR, odds ratio; 95%CI, 95% confidence interval.

composition was primarily dominated by *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Enterobacteriaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Bifidobacteriaceae*, *Preotellaceae*, *Enterococcaceae*, *Veillonellaceae* (Figure 2B). And the abundant of the top ten genera that occupied the most of the total microbiota were *Bacteroides*, *Lactobacillus*, *Streptococcus*, *Blautia*, *Escherichia-Shigella*, *Faecalibacterium*, *Bifidobacterium*, *Klebsiella*, *Enterococcus*, *Subdoligranulum* (Figure 2C).

Analysis of characteristic microbiota of POAH

As shown in Figure 3A, significant bacterial differences in the taxa of the two groups, mainly including *Enterococcaceae*, *Enterococcus*, *Alistipes*, *Rikenellaceae*, *RF_39*, *Turicibacter*, *Acetanaerobacterium*, *Ethanoligenenaceae*, *Hungateiclostridiaceae*, *Sanguibacteroides*, *Staphylococcaceae*, *Staphylococcus* in POAH,

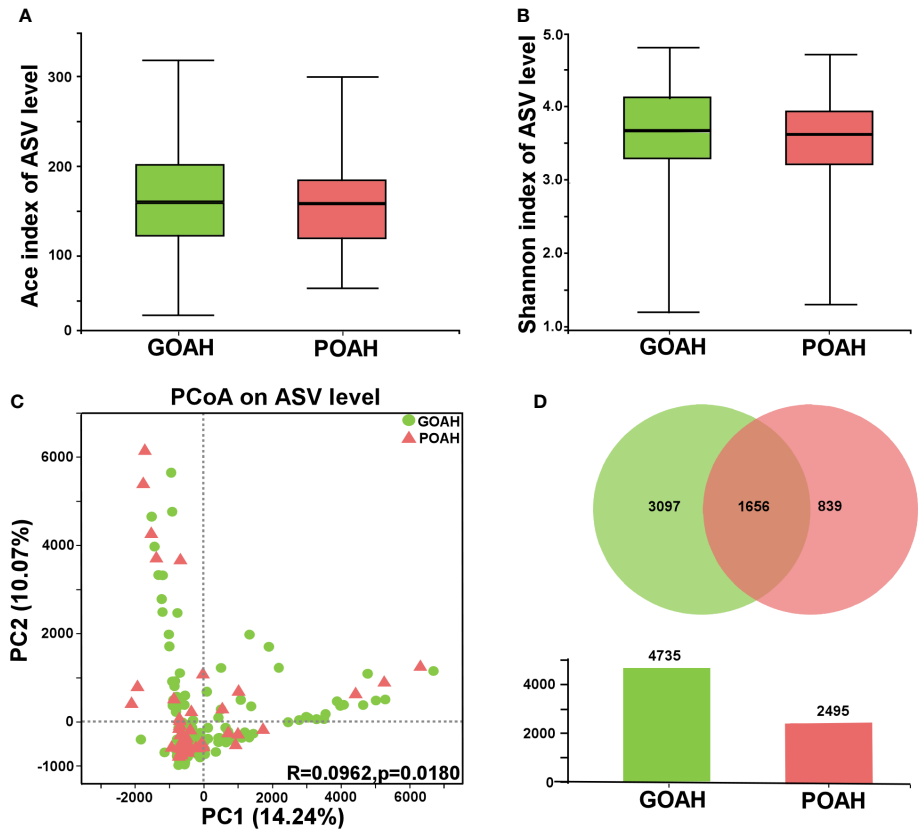

FIGURE 1
Analysis of gut microbiota diversity of POAH. **(A, B)** Alpha diversity indices, including Ace index and Shannon index. **(C)** Principal coordinate analysis (PCoA) diagram of gut microbiota based on the distance matrix of Bray Curtis (PC1 = 14.24%, PC2 = 10.07%). **(D)** Venn and Bar diagrams showed the number of unique ASVs in GOAH group (green) and POAH group (light red) and their shared ASVs (dark red).

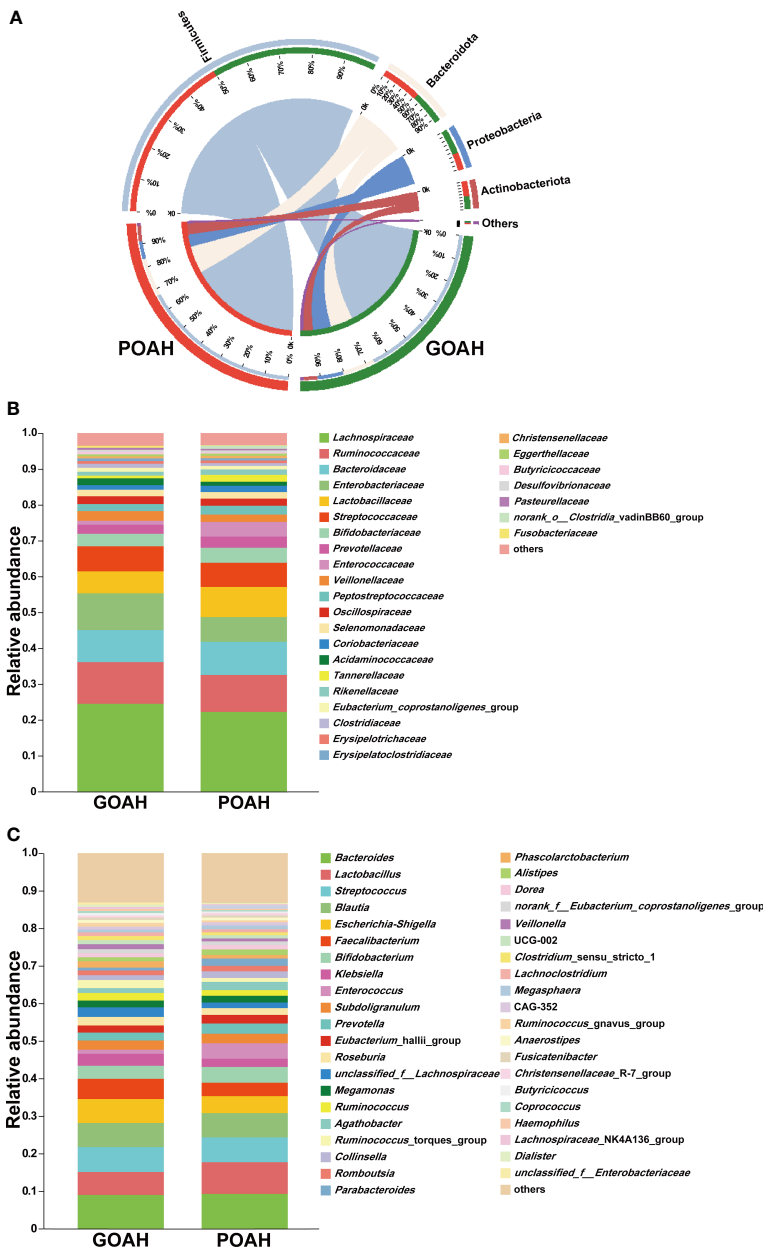

FIGURE 2
Analysis of microbial composition of POAH. **(A)** Microbial composition at the phylum level. The red bands represent the proportion of phyla in the POAH group. The green bands represent the proportion of phyla in the GOAH group. **(B)** Microbial composition at the family level. **(C)** Microbial composition at the genus level.

and Proteobacteria, *Gammaproteobacteria*, *Enterobacteriaceae*, *Enterobacterales*, *Escherichia-Shigella*, *Negativicutes*, *Faecalibacterium*, *unclassified_f_Lachnospiraceae*, Fusobacteriota, *Fusobacteriales*, *Fusobacteriaceae*, *Fusobacteriia*, *Butyricicoccaceae*, *Butyricicoccus*, *Pasteurellaceae*, *Fusobacterium*, *Pasteurellaceae*, *Haemophilus*, *Lachnospiraceae_NK4A136*, *Bacilli*, *Lachnospiraceae_UCG-010*, *norank_f_Lachnospiraceae*, *Micrococcaceae*, *Rothia* and *Micrococcales* in GOAH. As shown in Figures 3B–D, the relative abundance of *Enterococcaceae*, *Alistipes*, *Turicibacter*, *Enterococcus* and RF39 were higher in the POAH group than GOAH group, while the relative abundance of Proteobacteria, Fusobacteriota, *Enterobacteriaceae*, *Escherichia-Shigella*, *Faecalibacterium*, *Lachnospiraceae*, *Butyricicoccus*, *Haemophilus*, *Lachnospiraceae_NK4A136_group*, *Fusobacterium*, *Bacilli*, *Lachnospiraceae_UCG-010* and *Rothia* were lower in POAH group than GOAH group.

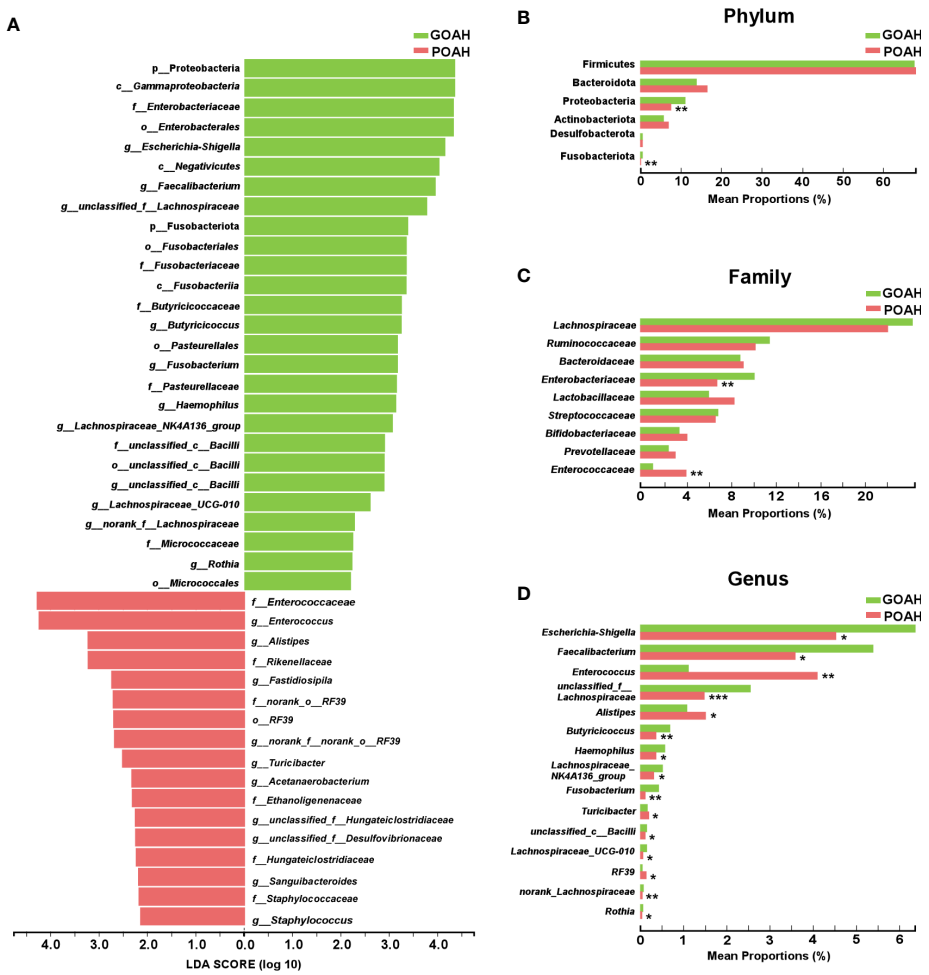

FIGURE 3
Analysis of characteristic microbiota of POAH. **(A)** Distribution diagram of linear discriminant analysis (LDA) scores of gut microbiota. (LDA > 2). **(B–D)** The extended error bar plot showed significant differences in gut microbial abundance at the level of phylum, family and genus. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Analysis of correlation between GM and mRS scores

As shown in Figure 4, *Lachnospiraceae* ($P < 0.01$), *Faecalibacterium* ($P < 0.01$) and *Butyricicoccus* ($P < 0.05$) were negatively correlated with the mRS score, while *Enterococcus* was positively correlated with the mRS score ($P < 0.05$). Spearman correlation heatmap (Figure 5A) indicated significant associations between the three independent risk factors and GM. A history of cerebrovascular disease (CVD) was negatively correlated with *Escherichia-Shigella*, *Lachnoclostridium* and *Ruminococcus_gnavus_group*. An elevated NIHSS score was also associated with a reduction of unclassified_f_*Lachnospiraceae*, *Ruminococcus* and *Haemophilus*. Furthermore, a positive relation was observed in MAP with the abundance of *Faecalibacterium*, *unclassified_f_Lachnospiraceae*, *Roseburia*, *Ruminococcus_torques_group*, *Megamonas*, *Phascolarctobacterium*, *Fusicatenibacter*, and *Butyricicoccus*, and a negative relation with the abundance of *Lactobacillus*, *Enterococcus*.

Analysis of correlation between GM and independent risk factors

We screened out the five genera as biomarkers according to the LDA value, including *unclassified_f_Lachnospiraceae*, *Enterococcus*, *Faecalibacterium*, *Lachnospiraceae_UCG-010*, and *norank_f_Lachnospiraceae*, achieving AUC values of 0.694 (Figure 5B, $P < 0.001$, 95% CI 0.618 - 0.770). Moreover, the predictive model combined with the five genera and the three independent risk factors could also distinguish POAH from GOAH (Figure 5B, $P < 0.001$, AUC = 0.971, 95% CI 0.952 - 0.989).

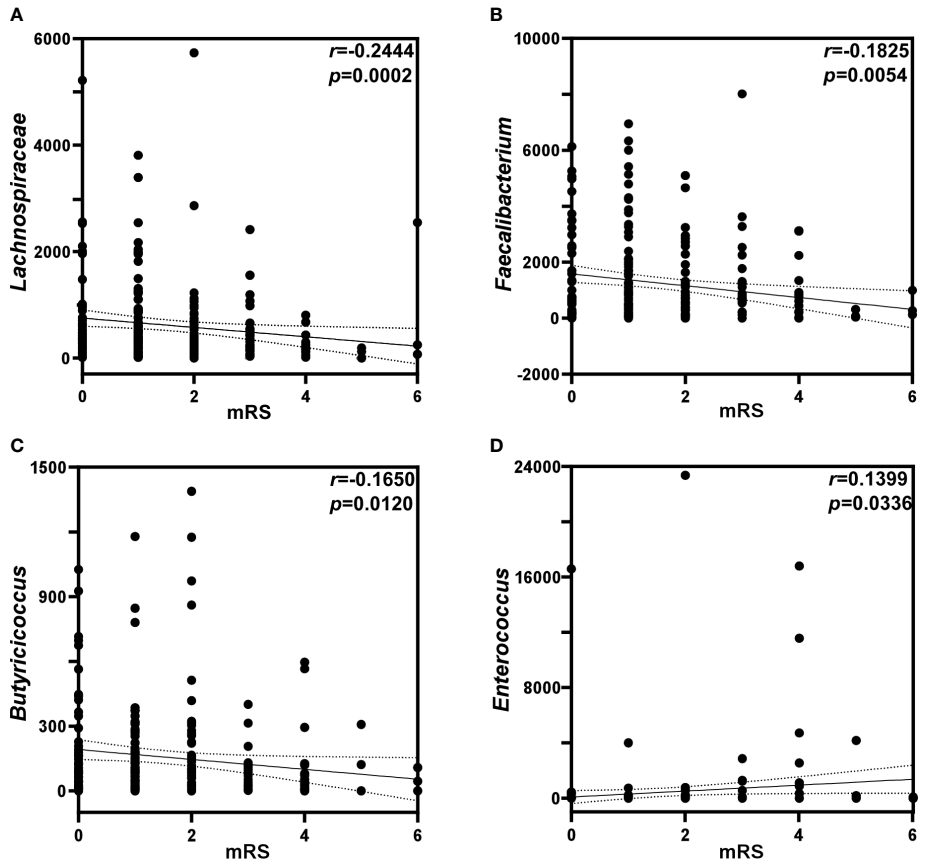

FIGURE 4
Analysis of correlation between gut microbiota and mRS scores. Correlations of mRS scores with the relative abundance of **(A)** *Lachnospiraceae*, **(B)** *Faecalbiacteruim*, **(C)** *Butyricicoccus*, and **(D)** *Enterococcus*. *p*: probability; *r*: Spearman's rank correlation.

Discussion

This study revealed that GM feature of POAH was that the abundance of *Enterococcus* increased while the abundance of bacteria producing SCFAs decreased, which was closely related to independent risk factors, such as cerebrovascular history, NIHSS score, and MAP. Moreover, the characteristic microbiota and microbiota plus with the three independent risk factors could establish a distinction for predicting POAH. These results indicated that GM might provide novel microbial biomarkers for predicting POAH.

Our results showed that the composition and structure of microbiota were different between POAH and GOAH. Previous studies revealed that gut microbial communities in the group with adverse prognosis after stroke were distinct from those in the group with good prognosis, accompanied by an increase in the abundance of Bacteroidota, and Actinobacteriota, and the decreased abundance of Proteobacteria and the Bacteroidetes to Firmicutes ratio (B/F) (Benakis et al., 2016; Singh et al., 2016; Shimizu et al., 2019; Guo et al., 2021). The diversity of GM was affected by many factors, such as lipid homeostasis (Schoeler and Caesar, 2019). The decreased B/F induced dyslipidemia, leading to more severe outcomes, such as obesity and liver steatosis (Hussain et al., 2020). Our results showed that the abundance of *Enterococcus* in POAH was enriched, and positively related to the mRS score, indicating that the abundance of *Enterococcus* might be related to the risk of POAH. It was reported that *Enterococcus* was an opportunistic pathogen in the gastrointestinal tract, and the risen level of *Enterococcus* was relevant to many neurological and metabolic diseases, such as Parkinson's disease, Alzheimer's disease and diabetes (Underly et al., 2015; Li et al., 2017). *Enterococcus* appeared in subjects of the adverse outcome group, manifested as the post-stroke cognitive impairment (PSCI) and post-stroke affective disorder (Huang et al., 2021), which was consistent with our studies. *Enterococcus* could induce the secretion of proinflammatory

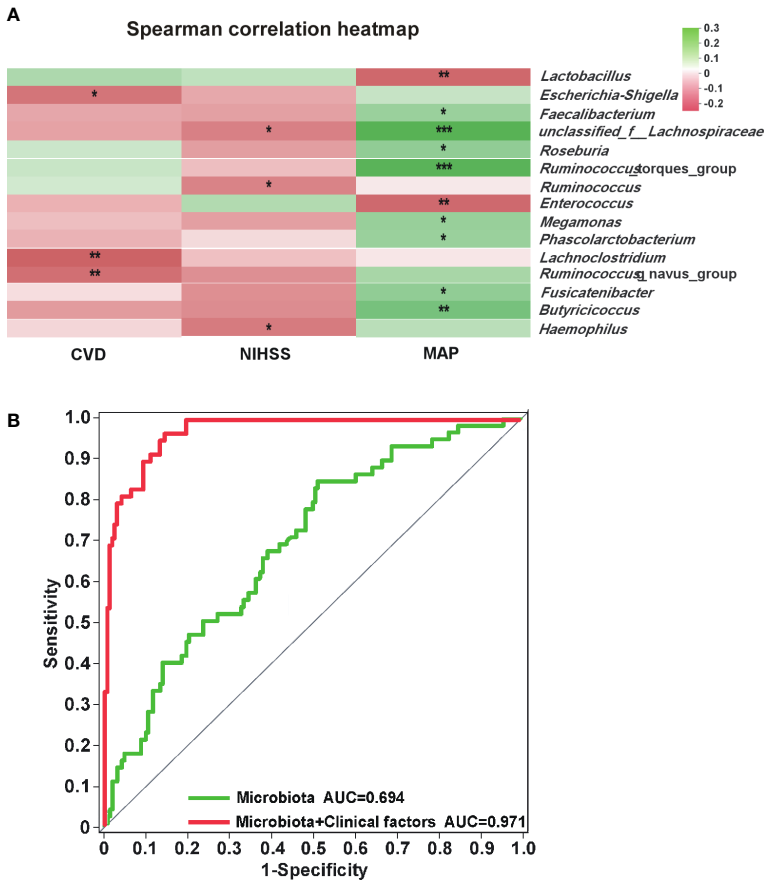

FIGURE 5
Analysis of correlation between gut microbiota and independent risk factors. **(A)** Heatmap of gut microbiota and independent risk factors for POAH. The colors of grids represent the correlation value of Spearman's rank correlation analysis. Green grids mean positive correlations, and red grids mean negative correlations. The deeper green or red indicates higher correlation values. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. **(B)** The green ROC model indicated the predicted value of the composite of five characteristic gut microbiota. The red ROC model was built to evaluate the accuracy based on the complex of five characteristic gut microbiota and three independent risk factors.

cytokines, such as IL-6 (García-Solache and Rice, 2019), and further contribute to systemic inflammation (Stanley et al., 2016; Chen et al., 2019), which led to POAH (Suda et al., 2018). Evidence showed that *Enterococcus faecalis* disturbed the lipid metabolism (Huang et al., 2018; Zhu et al., 2021). Hu X et al. revealed that a higher abundance of *Enterococcus* had a closely related to poor prognosis of hypertriglyceridemia-related acute pancreatitis leading to poor prognosis in hypertriglyceridemia patients (Hu et al., 2021), suggesting that *Enterococcus* might be involved in the prognosis of hyperlipidemic stroke patients.

In this study, there was a significantly lower relative abundance of SCFAs-producing bacteria in POAH group, such as *Lachnospiraceae*, *Faecalibacterium*, *Rothia* and *Butyricicoccus*. Moreover, *Lachnospiraceae*, *Faecalibacterium*, and *Butyricicoccus* were associated with lower mRS score. *Lachnospiraceae*, a primary producer of butyrate, was related to the functional prognosis of diseases (Sorbara et al., 2020). Many studies showed that the abundance of *Lachnospiraceae* was significantly decreased in stroke patients and animal models (Zeng et al., 2019; Lin et al., 2021). The abundance of *Lachnospiraceae* in patients with post stroke cognitive impairment (Ling et al., 2020) and patients with nervous neurocritical illness (Xu et al., 2019) was less. In addition, lower blood lipid could increase the abundance of *Lachnospiraceae* and levels of SCFAs in hyperlipidemia model animals (Gui et al., 2019; Liu et al., 2021). In addition, our results showed that the relative abundance of *Faecalibacterium* in POAH group was significantly lower. *Faecalibacterium* is a butyrate-producing bacteria, belonging to *Lachnospiraceae* family. Previous studies showed that the relative abundance of *Faecalibacterium* had a lower relative abundance in patients with stroke (Silveira-Nunes et al., 2020), transient ischemic attack (Yin et al., 2015) and PSCI (Huang et al., 2021) was lower. Lee et al. reported that

Faecalibacterium prausnitzii ameliorated post-stroke neurological deficits and elevated concentrations of intestinal SCFAs in aged mice with stroke (Lee et al., 2020). *Faecalibacterium prausnitzii* was decreased in fecal samples of hyperlipidemia adolescents (Gargari et al., 2018), and the abundance of *Faecalibacterium prausnitzii* in patients with mild hypercholesterolemia was significantly negatively correlated with TC and LDL (Xu et al., 2021). Furthermore, *Faecalibacterium* was observably elevated in the hyperlipidemia rats after probiotic intake, which could prevent the progression of hyperlipidemia (Shao et al., 2017). Enriched *Faecalibacterium* could reverse the increase of plasma TG level (Tong et al., 2018), and was positively correlated with plasma concentrations of butyric acid (Khan et al., 2018). *Butyricicoccus*, a butyrate-producing clostridial cluster genus, was related to reduced incidence of hyperlipidemia or hypercholesteremia in patients with colorectal cancer (Han et al., 2019). The abundance of *Butyricicoccus* was negatively correlated with the serum levels of LDL, TG and TC of obese patients, which could be used as a biomarker to predict obesity related lipid metabolism abnormalities (Zeng et al., 2019). Recent multiple studies have shown that SCFAs were closely linked to stroke and dyslipidemia. AIS patients, especially those with more severe stroke (Ling et al., 2020), showed a lack of SCFAs-producing bacteria and decreased levels of fecal SCFAs levels, which led to increased risks of post-stroke infection (Haak et al., 2021) and poor functional outcomes (Tan et al., 2021). Furthermore, the feces of young rats transplantation could effectively increase the concentration of SCFAs, and attenuate the neurological deficit and inflammation after stroke in elderly stroke mice (Lee et al., 2020) and in middle cerebral artery occlusion (MCAO) model rats (Chen et al., 2019). In addition, compared with control, subjects with hypercholesterolemia had a lower level of butyrate, which was negatively correlated with LDL (Granado-Serrano et al., 2019). SCFAs played an important role in reducing the risk of cholesterol and coronary heart disease, and valeric acid was negatively correlated with HDL-C in patients with mild hypercholesterolemia (Xu et al., 2021). These results indicated that decreased SCFAs-producing bacteria, such as *Lachnospiraceae*, *Faecalibacterium*, *Rothia* and *Butyricicoccus* and their metabolites SCFAs might participate in the occurrence of POAH.

Our results showed that the characteristic bacteria in POAH patients were closely related to independent risk factors, such as increased, decreased MAP, and history of cerebrovascular disease. The higher NIHSS scores, the greater the risk of disability, the more serious the neurological impairment, and the larger the area of ischemic lesions (Cucchiara et al., 2019; Cucchiara et al., 2020; Wang et al., 2021). A study showed that stroke patients with a history of hyperlipidemia were associated with a higher NIHSS score on day 7 and were less likely to have neurological improvements (Restrepo et al., 2009). Higher MAP could maintain cerebral perfusion and cerebral blood flow velocity in stroke patients. MAP was found to be positively associated with adverse functional outcomes and recurrence risk in stroke patients. It was reported that there was a positive correlation between MAP and the adverse functional outcome and recurrence risk of stroke patients (Ma et al., 2019). Moreover, GM also had a close connection to the clinical parameters. Our results showed that the decrease of unclassified_f_*Lachnospiraceae* was associated with the increase of NIHSS score, and MAP was positively correlated with the abundance of *Faecalibacterium*, *unclassified_f_Lachnospiraceae*, and *Butyricicoccus*, while negatively correlated with *Enterococcus*. LEfSe was used to support the construction of POAH diagnostic model based on five characteristic genera. In addition, the prediction model based on the combination of five characteristics and three independent risk factors could predict the occurrence of POAH. Therefore, these finding revealed the close relationship between POAH and GM, and the characteristic GM could be used as a biomarker for early prediction of POAH.

However, several limitations of this study should be mentioned. First, this was a small sample observational study conducted in a single center. Meanwhile, we collected fecal sample of patients at a single time point, so we could not observe the dynamic changes of the interaction between GM and these parameters. In addition, the information on the concentration of microbial metabolites, such as SCFAs, was lacked, which was difficult to find out the causal relationship between GM and POAH. Despite these limitations, our study firstly described the Characteristic GM of POAH, which was helpful to understand the role of microbial biomarkers in predicting POAH.

In conclusion, these findings revealed the microbial characteristics of POAH, which were closely related to clinical parameters. The characteristic GM might facilitate the diagnosis of POAH, which highlighted the potential prediction of GM on POAH.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA894329.

Ethics statement

The protocol of the study was reviewed and approved by The Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (LCKY2020-207). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JL, JS and SC designed the experiments. JC, BC, JM, JZ, QG, HX, YK and SY performed the experiments and conducted the statistical analyses. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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The management of *Helicobacter pylori* infection and prevention and control of gastric cancer in China

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Helicobacter pylori (*H. pylori*) infection, a type-1 carcinogen, was closely associated with gastric cancer (GC). Successfully eradicating *H. pylori* infection could reduce the incidence of GC. China was a country with high incidence of GC and high prevalence of *H. pylori* infection. Nearly half of worldwide GC new cases and deaths attributed to *H. pylori* infection occurred in China. *H. pylori* prevalence varied over time with the improvement of socioeconomic status and sanitary conditions. The knowledge of antibiotic resistance rate in time was important to guide the clinical choice of antibiotics use in the regimens. With the publication of five Chinese consensus reports on the management of *H. pylori* infection and the effort of public preach of *H. pylori*-related knowledge, the standardization of *H. pylori* diagnosis and treatment by clinicians was improved. Bismuth-containing quadruple therapy was widely applied in clinical practice of *H. pylori* eradication because of high efficacy and safety. High-dose Proton Pump Inhibitor-amoxicillin dual therapy or vonoprazan-amoxicillin dual therapy showed comparable efficacy and lower side effects than bismuth-containing quadruple therapy, which were the alternative choice. The diagnosis rate of early GC was low and distinguishing Chinese GC risk population for the further endoscopy screening was important. Efforts have been done to establish prediction models to stratify GC risk in the Chinese GC risk population. We reviewed the current situation of the management of *H. pylori* infection and prevention and control of GC in China here.

KEYWORDS

***Helicobacter pylori*, prevalence, resistance, eradication, gastric cancer**

Introduction

In China, lung, colorectum and gastric cancer (GC) was the most common diagnosed cancer (Xia et al., 2022). The new case number and death number attributed to GC in China was 456, 124 and 390, 182 in 2018 (Bray et al., 2018). Meanwhile, the prevalence of *Helicobacter pylori* (*H. pylori*) infection was high in China, especially in area with high incidence of GC (Xie and Lu, 2015; Hooi et al., 2017). *H. pylori* infection was the main risk factor of GC (Malfertheiner et al., 2017), which was defined as type-1 carcinogen by the World Health Organization in 1994 and listed as the carcinogen by the 15th Report on carcinogens released by The U.S. Department of Health and Human Services in 2021. Nearly 90% of non-cardia GC were attributed to *H. pylori* infection, *H. pylori*-induced GC were estimated to be 810, 000 cases in 2018, which exceeded human papillomavirus-induced cervical cancer and hepatitis B virus-induced liver cancer, ranking the first among infection-attributable cancer (de Martel et al., 2020). China accounted for a third of worldwide cancer cases attributable to infection, especially *H. pylori* infection. We reviewed the current management of *H. pylori* infection and prevention and control of GC in China here.

Helicobacter pylori prevalence

H. pylori was a common infection worldwide, with different prevalence among different regions. *H. pylori* was mainly acquired in childhood. *H. pylori* gastritis was defined as an infectious disease by *Kyoto global consensus report on Helicobacter pylori gastritis* (Sugano et al., 2015), whose transmission route included oral-oral, fecal-oral and gastro-oral (Leja et al., 2016). As such, *H. pylori* was closely related with socioeconomic status and sanitary conditions (Goh et al., 2011). In 1990, China was a country with low human development index (value: 0.499), which gradually increased to 0.761 (defined as high human development index) in 2018. Hooi et al. (2017) included 26 studies (103, 128 subjects) conducted in China from 1983-2013 for pooled analysis of *H. pylori* prevalence, the results demonstrated that the overall infection rate of *H. pylori* was 55.82%, which indicated that estimated more than 700 million subjects in China were infected with *H. pylori*. However, the interval of included studies were 20 years, which might not reflect the current situation of *H. pylori* prevalence. Moreover, the studies published in Chinese might be missed in this analysis. A slightly decreased *H. pylori* infection rate was observed around the world (2000-2008: 46.8% vs. 2009-2016: 44.9%) (Zamani et al., 2018). Consistent with this trend, the prevalence of *H. pylori* infection was decreased over time in China from 1983 to 2013 (Nagy et al., 2016). The decreased prevalence of *H. pylori* infection was more obvious in urban areas in comparison with rural areas. The overall *H. pylori* prevalence was higher in rural areas (66%) than urban areas (47%), which might be attributed to the differences of socioeconomic status and sanitary conditions. The weighted mean prevalence of *H. pylori* infection increased from 48% among individuals aged 18-30 years to 59% among individuals aged 50-60 years.

A recent systematic review and meta-analysis focusing on *H. pylori* prevalence in China included 412 eligible studies (published in English or Chinese) with 1, 377, 349 subjects for pooled analysis of *H. pylori* prevalence (Ren et al., 2022). *H. pylori* infection rate was reported as 44.2% (range 35.8%-66.4%) in the mainland of China in this study, which indicated that estimated 589 million individuals were *H. pylori* positive. *H. pylori* prevalence were high (>60%) in developing regions (Xizang, Guizhou etc.) and low (<40%) in developed regions (Beijing, Chongqing, Tianjin etc.). The significantly decreased *H. pylori* prevalence was also observed (1983-1994: 58.3% vs. 2015-2019: 40%). The infection rate of *H. pylori* was 28.0% in children and adolescents and 46.1% in adults. The overall global infection rate of *H. pylori* in children was 32.3%. *H. pylori* prevalence was higher in older children (41.6% in 13-18 years old) than in younger children (33.9% in 7-12 years old and 26.0% in 0-6 years old). Lower economic status, more siblings or children, room sharing, no access to a sewage system, having a mother or a sibling or siblings infected with *H. pylori*, drinking unboiled or non-treated water and older age were significantly associated with paediatric *H. pylori* infection (Yuan et al., 2022). Considering the improvement of socioeconomic status and sanitary conditions, it was not surprised that *H. pylori* prevalence had been decreased during the past decades. However, high infection rate of *H. pylori* was reported in developing areas or regions with high incidence of GC. Eradicating *H. pylori* effectively is important for decreasing *H. pylori* prevalence. Moreover, forming good health habits are effective for prevention of *H. pylori* transmission. Zeng et al. (2015) reported that the oral recombinant *H. pylori* vaccine was effective, safe, and immunogenic for *H. pylori*-naive children, which could substantially reduce the incidence of *H. pylori* infection. Further *H. pylori* vaccine studies are still needed to explore the protection of the vaccine against *H. pylori* infection and *H. pylori*-associated diseases during a long period.

Helicobacter pylori recurrence

H. pylori recurrence remained a significant public health concern, which consisted of recrudescence or reinfection. Recrudescence was defined as reappearance of the original infection during short-term period (6 months), which was associated with the use of low efficient *H. pylori* regimen (Bell et al., 1993). Reinfection was defined as infection with a new strain of *H. pylori* occurred after 6 months, which required

reexposure and was therefore more likely occurred in countries with high *H. pylori* prevalence and poor sanitation (Niv and Hazazi, 2008). We included 132 studies (53, 934 patient-years) for analyzing global *H. pylori* recurrence rate and explored the influence factors (Hu et al., 2017c). The global annual *H. pylori* recurrence, reinfection and recrudescence rate were 4.3%, 3.1% and 2.2%, respectively. Only three researches (Mitchell et al., 1998; Zhou et al., 2003; Zhou et al., 2017) had been conducted in China to explore the *H. pylori* recurrence rate until the year 2017. The pooled annual *H. pylori* recurrence rate was 2.1%, which was relatively low. The included subjects from the three studies were in developed regions (Beijing, Guangzhou, Jinan etc.), which might not reflect the total *H. pylori* recurrence rate in the mainland of China, especially in regions with low socioeconomic status and sanitary conditions. We further analyzed the associations between *H. pylori* recurrence rate and human development index (reflecting the socioeconomic status), *H. pylori* prevalence (reflecting sanitary conditions) and different periods. *H. pylori* recurrence rate was inversely related to the human development index and directly related to *H. pylori* prevalence, which might be explained by that subjects living in a country with low socioeconomic status and high *H. pylori* prevalence had more possibility of exposure of *H. pylori*. *H. pylori* recurrence rate remained relatively stable during three periods (1990s, 2000s and 2010s).

Xue et al. (2019) prospectively enrolled patients with successful eradication in developed regions (Beijing and Jinan) from 2013 to 2014 to conduct ^{13}C-urea breath test at one year and 3 years after therapy. 743 patients finished the 1-year follow-up, 13 patients were *H. pylori*-positive, which indicated that the annual recurrence rate was 1.75%. 607 patients finished the 3-year follow-up, 28 patients recurred, which indicated that the 3-year recurrence rate was 4.61% and the annual recurrence rate was 1.5%. The invasive diagnoses or treatments, the level of income, and the hygiene standard of dining out place were the independent factors influencing *H. pylori* recurrence. A higher annual recurrence rate of *H. pylori* (4.75%) was reported in Jiangjin district, Chongqing (Zhou, 2020). Drinking and dining out were independent risk factors of *H. pylori* recurrence. Consistent with this study, Zhang et al. (2020a) also reported a high annual *H. pylori* recurrence rate (18.8%) among children. The follow-up of these two studies was short (1 year) and the enrolled patients were limited to one region. We conducted a large-scale multicenter, prospective open cohort, observational study to evaluate *H. pylori* recurrence rate during long-term follow-up (2012-2018, 7454.3 person-year) (Xie et al., 2020). 5, 193 subjects at 18 hospitals across 15 provinces were enrolled. The overall annual *H. pylori* recurrence, reinfection and recrudescence rate were 3.1%, 1.5% and 5.1%, respectively. Specific properties of ethnic groups, education level, family history or residence location was found to be at higher risk for *H. pylori* reinfection. *H. pylori* recurrence rate was relatively low in China, which could not be considered as the competing consideration to eradicate *H. pylori* and implement the "screen and treatment" strategy of *H. pylori*. Additionally, a recent systematic review and meta-analysis provided the sufficient evidences that whole family-based *H. pylori* treatment could increase eradication rate and reduce recurrence rate compared with single-infected patient treatment approach (Zhao et al., 2021). A family-based *H. pylori* prevention and eradication strategy would be an effective approach to prevent its intra-familial transmission (Ding et al., 2022).

Helicobacter pylori resistance

Antibiotic resistance of *H. pylori* was the important factor influencing the efficacy of *H. pylori* regimen (Hu et al., 2017a). For example, the efficacy of clarithromycin-containing regimens was shown to below 80% when the clarithromycin resistance rates were higher than 20%, which was then classified as unacceptable as the first-line treatment for *H. pylori* eradication (Graham et al., 2007). Clarithromycin resistance is due to mutations in the 23S rRNA gene. The 23S ribosomal RNA A2143G, A2142G, and A2142C mutations accounted for 80–90% of clarithromycin resistance. Similarly, levofloxacin resistance is mostly due to mutations at positions 87, 88, 91, 97 of gene *gyrA*. The occurrence of point mutations in the *pbp 1A* gene was the most common mechanism leading to moderate or low-level amoxicillin resistance. Resistance to tetracycline is mainly caused by point mutations in *tet*-1 in 16S rRNA genes. Metronidazole resistance is highly complex although *rdxA* mutations are highly predictive of metronidazole resistance (Peter et al., 2022). Furazolidone resistance is associated with Mutations in the *H. pylori porD* and *oorD* genes (Hu et al., 2017a). *H. pylori* resistance to common antibiotics (clarithromycin, metronidazole and levofloxacin) used in the regimen was quite serious (resistance rate >15%) in majority of World Health Organization Regions (Savoldi et al., 2018). In 2017, clarithromycin-resistant *H. pylori* was listed as antibiotic-resistant bacteria needing a high priority for antibiotic research and development of new antibiotics by World Health Organization (Tacconelli et al., 2018). In the Asia-Pacific region, the mean prevalence of primary *H. pylori* resistance was reported as 17% for clarithromycin, 44% for metronidazole, 18% for levofloxacin, 3% for amoxicillin, and 4% for tetracycline (Kuo et al., 2017). The resistance rate of clarithromycin and levofloxacin increased over time mainly after the year 2000. Primary *H. pylori* resistance to metronidazole remained stable but was above the alarming level from 1990 to 2016. Amoxicillin and tetracycline were considered to be susceptible antibiotics in this region. We also included 23 studies (published in English or Chinese) conducted in China focusing on primary antibiotic resistance of *H. pylori* to evaluate the overall pooled antibiotic resistance rate (Hu et al., 2017b). The results showed that the prevalence of *H. pylori* primary resistance to clarithromycin

(28.9%), metronidazole (63.8%), and levofloxacin (28.0%) was high and increased over time. However, the resistance rates to amoxicillin (3.1%), tetracycline (3.9%), and furazolidone (1.7%) were low and stable over time.

Considering the differences of antibiotic resistance existed among different regions, we conducted a multi-region prospective 7-year study (2010-2016) to explore the characteristics of *H. pylori* resistance in China (Liu et al., 2018). *H. pylori* were successfully cultured from 1, 117 patients in 13 provinces or cities. An E-test was used to determine the minimum inhibitory concentrations of amoxicillin, metronidazole, clarithromycin, levofloxacin and tetracycline. The Kirby-Bauer disc diffusion method was used to determine the inhibition zone for furazolidone. The resistance rate of metronidazole, clarithromycin and levofloxacin was above the alarming level (>15%), as well as the dual antibiotic resistance rate (metronidazole+clarithromycin or metronidazole+ levofloxacin). The resistance rate of amoxicillin, tetracycline and furazolidone resistance was relatively low (<4%). The resistance rate of antibiotics was higher in subjects with age ≥40 years than subjects<40 years old, which might be explained by the exposure time of antibiotics were longer in the elder subjects. Additionally, the resistance rate of metronidazole, clarithromycin and amoxicillin were significantly different among different regions of China. Recently, A retrospective cross-sectional observational study was conducted by the China Center for *H. pylori* Molecular Medicine to investigate resistance of *H. pylori* strains to antibiotics from 2018 to 2020 across different regions of China. The results included 4, 242 *H. pylori* strains. Kirby-Bauer disk diffusion method was used to determine the phenotypic resistance to antibiotics. Overall, the primary antibiotic resistance rates of *H. pylori* for clarithromycin, levofloxacin and metronidazole were above the alarming level (>15%). The primary antibiotic resistance rates of *H. pylori* for amoxicillin, furazolidone and tetracycline were relatively low (<4%) except in Northwest China (Zhong et al., 2021). It should be noted that the primary resistance rate of *H. pylori* for antibiotics in specific region of China (e.g., Northwest China) showed an increasing trend. The selection of antibiotics in the regimens should base on the antibiotic resistance rate in local area. The previously treated adult group exhibited higher resistance rate of antibiotics (99.2% for metronidazole, 58.3% for clarithromycin and 52.3% for levofloxacin) compared with treatment-naive adult (Liu et al., 2019). As such, elevating the efficacy of first-line treatment for *H. pylori* is important, which could avoid the occurrence of secondary resistance. The situation of *H. pylori* resistance was serious in China. The combinations of susceptible antibiotics (amoxicillin+furazolidone or amoxicillin+tetracycline or tetracycline+furazolidone) were recommended by the *Fifth Chinese National Consensus Report on the management of Helicobacter pylori infection* (Liu et al., 2018). Real-time monitoring of antibiotic resistance was also needed to further guide the selection of antibiotics used in the *H. pylori* regimens.

National survey regarding the management of *Helicobacter pylori* infection

In 2013, *Fourth Chinese National Consensus Report on the management of Helicobacter pylori infection* was published, including the indications, diagnosis and treatment of *H. pylori*. Proton Pump Inhibitor (PPI)-based triple therapy was not recommended as the first-line treatment of *H. pylori* infection because of its low efficacy (<80%). Bismuth-containing quadruple therapy (BQT) was recommended as the first-line and second-line regimen for eradicating *H. pylori*, the duration of *H. pylori* regimens increased from 7 days to 10 or 14 days (Liu et al., 2013). In 2018, *Fifth Chinese National Consensus Report on the management of Helicobacter pylori infection* was published, which contained 6 aspects including *H. pylori* indications for eradication, diagnosis, treatment, *H. pylori* and GC, *H. pylori* infection in special populations, *H. pylori* and gastrointestinal microbiota (Liu et al., 2018). *H. pylori* gastritis is defined as an infectious disease, which was consistent with the recommendation reported in the *Kyoto global consensus report on Helicobacter pylori gastritis*. *H. pylori* infection confirmed was included in the *H. pylori* indications. Considering the serious situation of antibiotic resistance, BQT (recommended 7 regimens) was still recommended as the main empirical therapy for *H. pylori* eradication because of its high efficacy especially for *H. pylori* resistant strains. Compared to *Fourth Chinese National Consensus Report*, two regimens of BQT (PPI+bismuth+amoxicillin +metronidazole, PPI+bismuth+amoxicillin+tetracycline) were extended. In order to investigate the management of *H. pylori* infection by clinicians in China, the Chinese Study Group on *H. pylori* conducted an authoritative survey at 14th (2014) and 17th (2017) Congress of Gastroenterology China (Song et al., 2019b). A total of 4, 182 valid samples were obtained in this study. Majority of the clinicians were aware of *H. pylori*-related diseases (chronic gastritis, peptic ulcer, GC and gastric MALT lymphoma). However, less than 50% of clinicians were familiar with the associations between *H. pylori* infection and extra-gastroduodenal diseases. Urea breath test were the preferred choice for detecting *H. pylori* infection by 80% of the clinicians. The demands for *H. pylori* detection by patients were increased mainly because of the increased awareness of *H. pylori* harmfulness by the publics and the preach of *H. pylori* consensus reports by the gastroenterologists. The proportion rate of quadruple therapy as the first-line treatment for *H. pylori* infection was higher (76%) in 2017 than that in 2014 (31%). The preferred antibiotic combinations were clarithromycin + amoxicillin, followed by amoxicillin + furazolidone. The combination of clarithromycin and metronidazole was used for the initial eradication by only 1% of the clinician because the dual resistance rate of clarithromycin and metronidazole was high

(>15%). From this survey, we found that the management of *H. pylori* infection has been improved. However, the proportion rate of BQT was only 57% in 2017 and the frequency of resistant antibiotics (clarithromycin, metronidazole and levofloxacin) used in the regimens were still high.

After 4 years, the Chinese Study Group on *H. pylori* conducted a nationwide, multicenter, cross-sectional questionnaire survey again to investigate the current state of knowledge and practice of *H. pylori* infection management (Song et al., 2022). The proportion of BQT was high (88%) in the first-line treatment of *H. pylori* infection. Amoxicillin was the most preferred antibiotic (93.1%) used in the regimen. The preferred antibiotic combinations were still clarithromycin + amoxicillin, followed by amoxicillin+furazolidone. The proportion of regimens used for 14 days or 10 days were 83.3% and 13.5%, respectively. Compared to the survey in 2017, the treatment of *H. pylori* infection was more standard in 2021. The awareness of *H. pylori* prevention and treatment in the general Chinese population was also important, an internet-based survey was carried out in 2019 by the National Clinical Research Center for Digestive Diseases, which enrolled 3, 211 people and 546 physicians (Wu et al., 2020). The majority of the surveyed population (87.0%) and physicians (82.2%) supported a national *H. pylori* screening plan to prevent GC. However, the proportion of subjects who answered correctly to all questions about *H. pylori*'s infectivity, harmfulness and preventive measures were only 16%, 35% and 43.6%, respectively. More works on health education are needed because the general population in China has insufficient awareness of *H. pylori*.

Helicobacter pylori treatment

Bismuth-containing quadruple therapy

Standard PPI-based triple therapy was not recommended for *H. pylori* eradication because of its low efficiency. The monotherapy of bismuth could directly eradicate 20% *H. pylori* successfully. The addition of bismuth in triple therapy might improve the cure rate, mainly in strains with antibiotic resistance (Dore et al., 2016). Numerous studies have evaluated the efficacy of different antibiotic combinations in BQT in China, including clarithromycin and amoxicillin (Sun et al., 2010), amoxicillin and furazolidone (Xie et al., 2014), amoxicillin and metronidazole (Zhang et al., 2015), amoxicillin and tetracycline (Xie et al., 2018b), tetracycline and metronidazole (Liang et al., 2013), tetracycline and furazolidone (Liang et al., 2013). The efficacy of different antibiotic combinations in BQTs was good or fair in most studies, with the intention-to-treat (ITT) analysis showing an efficacy above 85% and the per protocol (PP) analysis showing an efficacy above 90%. As such, BQT was recommended as the first-line treatment of *H. pylori* infection by the *Fifth Chinese National Consensus Report on the management of Helicobacter pylori infection* (Liu et al., 2018). The duration of regimens should be 14 days, except evidences showed that regimens for 10 days achieved high efficacy. Susceptible antibiotics (amoxicillin, tetracycline and furazolidone) were the preferred choices in the regimens. We performed a national, multicenter, open-label, randomized controlled trial to evaluate the efficacy of the combination of susceptible antibiotic (amoxicillin and furazolidone) in BQT for eradicating *H. pylori* (Xie et al., 2018a). A high efficacy (94.7%) and low side effect (9.7%) were achieved by furazolidone-containing BQT for 10 days. Additionally, an observational study of furazolidone-containing BQT for *H. pylori* infection in real-world settings was conducted from 2015 to 2018, the eradication rate of furazolidone-containing BQT for 10 days or 14 days was 93.7% and 98.2%, respectively. Meanwhile, low side effect (<20%) was also observed for this regimen (Song et al., 2019a). Another combination of susceptible antibiotic (amoxicillin and tetracycline) in BQT for 10 days was showed to achieve 91.9% eradication rate as the first-line treatment for *H. pylori* infection, which was confirmed by multicenter, randomized, parallel-controlled clinical trial (Xie et al., 2018b).

Public attentions were paid to the alterations of gastric or intestinal microbiota induced by BQT because antibiotics and PPI used in the regimes might influence the host microbiota to some extent. We included ten asymptomatic young adults with *H pylori*-related gastritis treated with BQT for 14 days, 7 age-matched adults with *H. pylori* negative were served as healthy controls. Both fecal and gastric mucosa samples were collected for 16S rRNA gene sequencing at the timepoint of before eradication, after eradication and 6 months after the therapy. The results demonstrated that BQT can restore the diversity of gastric microbiota with enrichment of beneficial bacteria. The composition of gut microbiota after *H. pylori* eradication trends toward healthy status (He et al., 2019). The main drawback of BQT was the complex of drug administration, which might influence the compliance of the subjects, leading to the decreased efficacy of regimen. Enhanced educational interventions were effective way to improve the adherence among infected patients (Zha et al., 2022). WeChat is a social media platform widely used in China. Luo et al. (2020) found that *H. pylori*-infected subjects intervened with WeChat as a reminder tool during the treatment exhibited better disease-related knowledge, medication adherence, and eradication rates than that in the control group. The following research with more *H. pylori*-infected subjects enrolled further confirmed this finding (Ma et al., 2021). Compared to the conventional patient education (verbal education and a specifically designed printout with detailed instructions), the addition of WeChat-based patient-doctor interaction did not yield better results of efficacy or compliance (Lin et al., 2022). Selecting efficient antibiotic combinations in BQT should depend on the local *H. pylori* resistance. Enhanced patient education (conventional education

or social media platform interactions) was also important for the success of *H. pylori* regimen.

High-dose PPI-amoxicillin dual therapy or vonoprazan-amoxicillin dual therapy

The role of PPI in *H. pylori* regimen was to maintain the gastric pH high enough for *H. pylori* to remain in the state of active replication where the organism becomes susceptible to amoxicillin (Graham and Fischbach, 2010) The eradication rate of *H. pylori* increased when the PPI dosage used in the regimen increased (Labenz, 2001). High-dose dual therapy (HDDT) was defined as the combination of amoxicillin and PPI in high dose for 14 days, which was evaluated for *H. pylori* eradication in recent years in China. Yang et al. (2019) firstly evaluated HDDT (amoxicillin 750mg q.i.d and esomeprazole 20mg q.i.d) as the first-line treatment of *H. pylori.* 91.1% eradication rate was achieved by HDDT in adherent infected subjects, which was non-inferior to BQT. The study conducted in Beijing with same regimen also reported the similar high efficacy for HDDT (Song et al., 2020). Changing the frequency of amoxicillin to 1000mg t.i.d in HDDT was also reported to achieve high efficacy (ITT: 89.4%, PP: 90.6%) (Guan et al., 2022). Increasing the dosage of esomeprazole to 40 mg t.i.d in HDDT was shown to achieve 91.7% eradication rate for ITT analysis and 95.7% in PP analysis (Tai et al., 2019). The addition of bismuth in HDDT only improved treatment efficacy among smoker (Yu et al., 2019). The eradication rate of HDDT was low when the duration was 10 days or the esomeprazole was given 20 mg t.i.d (Zhang et al., 2020b). The combination of amoxicillin and rabeprazole (10mg t.i.d) or Ilaprazole (5 mg bid) for 14 days was reported to be with high efficiency for eradicating *H. pylori* (Niu et al., 2022; Shao et al., 2022). Additionally, the combination of amoxicillin (1000mg t.i.d) and rabeprazole (10mg t.i.d) were also efficient for *H. pylori* eradication in elder infected subjects or those with multiple comorbidities (Gao et al., 2020). In China, the duration of HDDT should be 14 days. The optimized dosage of amoxicillin was 1000mg t.i.d or 750 mg q.i.d, the dosage of PPIs should be double (i.e. esomeprazole 20mg q.i.d or 40 mg b.i.d) to maintain the high gastric pH level. HDDT showed non-inferior efficacy and lowed side effects rate than BQT in adults or elder patients (Table 1), which was considered as an alternative first-line treatment for *H. pylori* infection in China. However, the influence of HDDT on the gut microbiota remained unclear and need to be explored in the future research.

Vonoprazan, a potassium-competitive acid blocker, was firstly introduced in Japan, which inhibited the gastric acid faster, longer and stronger in comparison with PPIs (Abadi and Ierardi, 2019). Its combination with amoxicillin (750mg b.i.d or 500mg t.i.d) for 7 days in adults (Furuta et al., 2020); Suzuki et al., 2020) or junior high school students (Gotoda et al., 2020) achieved acceptable eradication rate in Japan. We have conducted a systematic review and meta-analysis to evaluate the efficacy and safety of vonoprazan and amoxicillin (VA) dual therapy (Ouyang et al., 2022), the results showed that the crude eradication rate of VA dual therapy was 87.5% and 89.6% by ITT and PP analysis, respectively. Additionally, the side effect of VA dual therapy was 19.1%. Japan government restrictions limit the duration of regimen to 7 days. Chey et al. (2022) further explored the efficacy of VA dual therapy (amoxicillin 1g t.i.d and vonoprazan 20 mg b.i.d) for 14 days in the United States and

TABLE 1 Summary of HDDT or VA dual therapy as the first-line treatment of *H. pylori* infection in China.

Reference	Antibiotics	PPI or P-CAB	Days	n	ITT	PP	Adverse
Yang et al. (2019)	amoxicillin 750mg q.i.d	esomeprazole 20mg q.i.d	14	116	87.90%	91.10%	6.30%
Song et al. (2020)	amoxicillin 750mg q.i.d	esomeprazole 20mg q.i.d	14	380	87.10%	92.40%	17.60%
Guan et al. (2022)	amoxicillin 1000mg t.i.d	esomeprazole 20mg q.i.d	14	350	89.40%	90.60%	12.90%
Tai et al. (2019)	amoxicillin 750mg q.i.d	esomeprazole 40mg t.i.d	14	120	91.70%	95.70%	9.60%
Yu et al. (2019)	amoxicillin 1000mg t.i.d	esomeprazole 40mg b.i.d	14	80	92.50%	96.10%	7.50%
Zhang et al. (2020b)	amoxicillin 1000mg t.i.d	esomeprazole 20mg t.i.d	14	104	83.50%	86.40%	5.00%
	amoxicillin 750mg q.i.d	esomeprazole 20mg q.i.d	10	104	79.80%	81.30%	5.90%
Shao et al. (2022)	amoxicillin 1000mg t.i.d	rabeprazole 10mg t.i.d	14	120	85.80%	89.60%	13.00%
Niu et al. (2022)	amoxicillin 750mg q.i.d	Ilaprazole 5 mg bid	14	150	88.00%	93.00%	8.50%
Gao et al. (2020)	amoxicillin 1000mg t.i.d	rabeprazole 10mg t.i.d	14	198	–	90.90%	11.10%
Hu et al. (2022)	amoxicillin 1000mg b.i.d	vonoprazan 20mg b.i.d	10	37	89.20%	89.20%	29.70%
	amoxicillin 1000mg t.i.d	vonoprazan 20mg b.i.d	10	37	81.10%	81.10%	24.30%
	amoxicillin 1000mg b.i.d	vonoprazan 20mg b.i.d	7	24	66.70%	72.70%	16.70%
	amoxicillin 1000mg t.i.d	vonoprazan 20mg b.i.d	7	21	81.00%	81.00%	23.80%
Lin et al. (2022)	amoxicillin 750mg q.i.d	vonoprazan 20mg b.i.d	7	85	63.50%	65.10%	16.90%
	amoxicillin 500mg q.i.d	vonoprazan 20mg b.i.d	7	84	58.30%	66.20%	13.20%

PPI, proton-pump inhibitor; P-CAB, potassium-competitive acid blocker; N, number; ITT, intention-to-treat; PP, per protocol; HDDT, High-dose dual therapy; VA, vonoprazan and amoxicillin. b.i.d, two times daily; t.i.d, three times daily; q.i.d, four times daily.

Europe. 349 *H. pylori*-infected subjects received VA dual therapy. The eradication rate of VA dual therapy was 77.2% in all patients. The efficacy of regimen was unsatisfied although it showed superior to lansoprazole triple therapy. The different outcomes were achieved in Japan, United States and Europe, which might be explained by the differences of race and Body Mass Index. We conducted a prospective, randomized clinical pilot study to evaluate the efficacy of VA dual therapy in China (Hu et al., 2022b). 119 *H. pylori*-infected subjects were randomized to receive either low- or high-dose VA consisting of amoxicillin 1 g either b.i.d. or t.i.d plus VPZ 20 mg b.i.d for 7 or 10 days. Neither 7- or 10-day VA dual therapy with b.i.d. or t.i.d. amoxicillin provides satisfied efficacy (<90%). The following muti-centers study conducted in Lanzhou also showed that the efficacy of VA dual therapy for 7days was relatively low (Lin et al., 2022), which could be explained by the short duration of VA dual therapy and high antibiotic resistance of amoxicillin in this region (Zhong et al., 2021). VA dual therapy (VPZ 20mg bid with amoxicillin 1000mg b.i.d or t.i.d) for 14 days was shown to achieve high efficacy (eradication rate > 90%) as the first-line treatment for *H. pylori* infection in our center (unpublished data). For *H. pylori*-infected patients with history of treatment failure, the eradication rate of VA dual therapy (VPZ 20mg q.d or b.i.d with amoxicillin 1000mg t.i.d) for 14 days was 92.5%, which was effective and safe as rescue therapy for *H. pylori* infection (Gao et al., 2022). More importantly, our results showed that VA dual therapy showed minimal influence on the gut microbiota and short-chain fatty acids (Hu et al., 2022a). VA dual therapy for 14 days showed high efficacy and good safety for eradicating *H. pylori*. The included samples were limited and the application of VA dual therapy in different regions should be optimized. VA dual therapy is a promising *H. pylori* regimen in an era of increasing antibiotic resistance although more evidences are needed. Further prospective, multi-center, randomized control trail with more subjects included was needed to evaluate the efficacy and safety of low or high-dose VA dual therapy for 14 days in different regions of China.

The benefit of *Helicobacter pylori* eradication in the prevention of GC

China was a country with a high risk of GC (>20 per 100 000 person-years), especially in some regions (Changle, Linqu, Wuwei, Yangzhong, Zhuanghe etc.) (Liou et al., 2020). 2, 258 participants with *H. pylori* positive in Linqu county, Shandong province were randomized into *H. pylori* eradication, vitamin supplementation, garlic supplementation, or their placebos, who were followed up for 22 years (1995-2017). The results demonstrated that *H. pylori* treatment significantly reduce reduced risk of death due to GC (odds ratio 0.48, 95% confidence interval 0.32 to 0.71) (Li et al., 2019). In Changle, Fujian province, 1, 630 asymptomatic, *H. pylori*-infected subjects were randomly assigned to receive *H. pylori* eradication or placebo, who were then follow-up for 26.5 years (1994-2020). Subjects receiving *H. pylori* eradication had a lower incidence of GC than their placebo counterparts (hazard ratio, 0.57; 95% CI, 0.33-0.98), the benefit was more obvious in subjects without premalignant gastric lesions or dyspepsia symptoms at baseline (Yan et al., 2022). Mass eradication of *H. pylori* infection was launched in 2004 and continued until 2018 for a high-risk Taiwanese population aged 30 years or older dwelling on Matsu Islands with *H. pylori* infection. The effectiveness of *H. pylori* eradication in reducing GC incidence was 53% (95% CI 30% to 69%). Additionally, the decreased presence and severity of atrophic gastritis and intestinal metaplasia was also observed (Chiang et al., 2021). Acceptable cure rate of *H. pylori* and low rate of adverse effects were achieved during mass screening and eradication of *H. pylori* (Lei et al., 2022).

The benefit of *H. pylori* eradication in the prevention of GC was confirmed by randomized control trails with large subjects included and long-term follow-up in China, which supported the mass screening and eradication of *H. pylori*. The benefit was more obvious in subjects without the incidence of precancerous lesions. The subjects with precancerous lesions also benefit from *H. pylori* eradication because the successful treatment could prevent the progression of diseases. For example, *H. pylori* eradication could reduce the incidence of metachronous GC in subjects with early GC undergoing endoscopic mucosal resection (relative risk= 0.49; 95% CI 0.34 to 0.70) (Ford et al., 2020). Additionally, in an analysis of data from a public hospital database with 73, 237 *H. pylori* positive subjects included on Hong Kong, *H. pylori* infection eradication reduced the risk of GC in subjects 60 years or older compared to the matched general population (Leung et al., 2018). As such, the Chinese consensus (Du et al., 2020; Ding et al., 2022) have emphasized the importance of early detection, diagnosis, and treatment of *H. pylori* to reduce the occurrence of GC and a family-based *H. pylori* prevention and eradication strategy to prevent its intra-familial transmission and related diseases. Additionally, national, provincial and regional specialist clinic of *H. pylori* infection was established by the office of *H. pylori* infection and GC prevention and control, which could promote the standard of *H. pylori* diagnosis and treatment.

The stratification of risk for GC in the Chinese GC risk population

The 5-year survival rate was below 30% when advanced GC was diagnosed (Ajani et al., 2013). Early GC showed good prognosis (5-year survival rate >90%), which could be cured by the endoscopic submucosal dissection (Isobe et al., 2011).

However, the diagnosis rate of early GC was low (<10%) in China. An effective way to improve the current serious situation of diagnosis and treatment of GC was the endoscopy screening and treatment in patients with high-risk of GC. The current national screening guideline for GC in China recommended screening beginning at age 40 years for all in the high-risk population (those residing in high-incidence areas for more than 3 years or who have *H. pylori* infection, a positive family history of GC, or risk factors for GC etc.). Estimated exceed 300 million individuals were defined as high-risk of GC and needed to receive the screening gastroscopy, which is not likely feasible in the clinical practice. An applicable risk prediction rule to further stratify risk for GC in the Chinese GC risk population was needed.

Tu et al. (2017) conducted a cross-sectional study for identifying high-risk individuals and predicting risk of developing GC in Zhuanghe county, a rural county of northern China with high incidence and mortality of GC. The screening program included individuals with 35–70 years old or upper gastrointestinal symptoms or a positive family history of GC. Totally, 9, 002 participants underwent gastroscopy were recruited by the end of 2012. The five biomarkers (especially PGII, the PGI/II ratio, and *H. pylori* sero-positivity) (Area Under the Curve=0.803) were identified to be associated with the presence of precancerous gastric lesions or GC at enrollment. From 2015 to 2017, The Gastrointestinal Early Cancer Prevention & Treatment Alliance of China conducted a nationwide multi-center cross-sectional study to identify individuals with a high risk prior to gastroscopy, which recruited 14, 929 individuals aged 40–80 years who went to hospitals for a GC screening gastroscopy (Cai et al., 2019). Seven variables (age, sex, PG I/II ratio, G-17 level, *H. pylori* infection, pickled food and fried food) were comprised as the novel GC risk prediction rule, with scores ranging from 0 to 25. 70.8% of total GC cases and 70.3% of early GC cases were detected when individuals with medium risk (score: 12-16) and high risk (score: 17-25) received gastroscopy. Endoscopy requirements could be reduced by 66.7% according to the low-risk proportion. This nationwide study successfully developed and validated prediction rule identifying individuals at a higher risk in a Chinese high-risk population, which needed to receive further gastroscopy. Compared to ABC method (Miki, 2011), five markers-based method (Tu et al., 2017), the prediction model of seven variables (Cai et al., 2019) showed higher Area Under the Curve and Youden index values, which was further confirmed by a retrospective analysis of data from the Provincial Gastric Cancer Screening Program with 97, 541 individuals included (Hu et al., 2021). A future large population-based screening project should be launched to test and validate its efficacy of novel GC risk prediction rule.

Conclusions

China was a country with high incidence of GC and high infection rate of *H. pylori*. With the improvement of socioeconomic status and sanitary conditions and successful *H. pylori* eradication, the prevalence of *H. pylori* showed a decreased trend over time. Meanwhile, *H. pylori* recurrence rate remained low during the long-term follow-up. The situation of *H. pylori* resistant to antibiotics is serious worldwide, including in China. Suspectable antibiotics are recommended to be used in the regimens. With the publication of Chinese consensus report on the management of *H. pylori* infection and the effort of public preach of *H. pylori*-related knowledge, the standardization of diagnosis and treatment of *H. pylori* by clinicians was improved. Efforts were still needed to be done for education of public populations and the supervisor of *H. pylori* management in clinical practice. For example, the office for prevention and treatment of *H. pylori* infection and GC was established in 2021, which aimed to launch the national or provincial or county demonstration center of the standard outpatient clinic of *H. pylori* diagnosis and treatment. BQT was effective and safe, which was recommended as the first-line treatment for *H. pylori* infection. HDDT and VA dual therapy were the alternative choice. Successful *H. pylori* eradication reduce the incidence of GC and prevent the progression of *H. pylori*-related diseases. Prediction models had been developed and validated to stratify GC risk in the Chinese GC risk population.

"Healthy China 2030" proposed the improvement of public health with expectation of 5-year nationwide survival rate of cancer improved by 15%. The combination of primary (effective *H. pylori* eradication) and secondary prevention (increasing the diagnosis rate of early GC and treated them) was the effective way to decrease the incidence of GC and increase the survival rate of GC.

Author contributions

YH wrote the manuscript. YZ and N-HL designed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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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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Outer membrane vesicles from bacteria: Role and potential value in the pathogenesis of chronic respiratory diseases

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Infectious diseases are the leading cause of death in both adults and children, with respiratory infections being the leading cause of death. A growing body of evidence suggests that bacterially released extracellular membrane vesicles play an important role in bacterial pathogenicity by targeting and (de)regulating host cells through the delivery of nucleic acids, proteins, lipids, and carbohydrates. Among the many factors contributing to bacterial pathogenicity are the outer membrane vesicles produced by the bacteria themselves. Bacterial membrane vesicles are being studied in more detail because of their potential role as deleterious mediators in bacterial infections. This review provides an overview of the most current information on the emerging role of bacterial membrane vesicles in the pathophysiology of pneumonia and its complications and their adoption as promising targets for future preventive and therapeutic approaches.

KEYWORDS

bacteria, extracellular vesicles, outer membrane vesicles, mechanisms of disease, respiratory tract diseases

Introduction

Both Gram-positive and Gram-negative bacteria produce extracellular vesicles (EVs), which are referred to as membrane vesicles (MVs) and outer membrane vesicles (OMVs), respectively, based on their proposed mechanisms of release. For example, OMVs are thought to bleb from the outer membrane of Gram-negative bacteria and as a result, encapsulate periplasmatic content, whereas MVs are thought to bud from the cytoplasm

(Ñahui Palomino et al., 2021). OMVs resemble mammalian-derived EVs in size and are expected to promote bacterial-host communication by carrying a range of bioactive substances such as proteins, nucleic acids, lipids, and metabolites. OMVs are associated with bacterial-bacterial and bacterial-host interactions, promote health, or cause a variety of diseases. Vesicles are about 10 to 300 nm in diameter, originate from the outer membrane (OM), and are comprised of lipids, proteins, lipopolysaccharides (LPS), phospholipids, DNA, RNA, and the inner membrane (IM) (Figure 1) (Furuyama and Sircili, 2021). The passage components of OMVs into have cells are assorted and have not been totally explained due to the species, glue particles and their items. As a general rule, the OMVs are shipped into the cells through endocytosis. In endocytosis, film spaces invaginate, trailed by being squeezed off from the inward side of the cell layer and moved inside the cell. Likewise, some OMVs are recommended to meld with lipid pontoons in the film and the items are delivered into the cytoplasm.

OMVs have the potential to initiate inflammatory reactions in response to certain infections, such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *L. pneumophila* (Furuyama and Sircili, 2021). These bacteria are thought to be responsible for lung inflammation by triggering immunological responses in the respiratory epithelium. This has led some scientists to believe that OMVs can actually boost the immune system. When pathogenic bacteria break their outer membrane, they produce OMVs, which play a crucial role in the interaction between the host and the pathogen. These include establishing colonizing ecological niches, delivering virulence factors to host cells, forming bacterial communities (biofilms), and functioning as decoys for bacterial evasion of immunity and evasion of the host immune system (Cui et al., 2021).

Streptococcus pneumoniae

The bacterium known as *Streptococcus pneumoniae* is responsible for a considerable quantity of illness and death all across the globe (Codemo et al., 2018). Every year, pneumococcal infections claim the lives of around one million young children under the age of five all over the globe (Henriques-Normark and Tuomanen, 2013). Pneumococci are a key contributor to the development of community-acquired pneumonia, sepsis, and meningitis; nevertheless, they are also a substantial contributor to the development of less severe respiratory infections such as otitis media and sinusitis (Codemo et al., 2018; Xiong et al., 2019).

OMVs reduce pneumococcal-modulated phagocytosis by strongly binding components of the complement system

Streptococcus pneumoniae strain TIGR4 OMVs are high in lipoproteins and short-chain fatty acids, and they carry a wide range of surface proteins and toxins (hemolysins) (Yerneni et al., 2021). OMVs generated a rise in the production of pro-inflammatory cytokines in A549 lung epithelial cells as well as in human monocyte-derived dendritic cells, including interleukin 6 (IL-6), IL-8, IL-10, and tumor necrosis factor (TNF). OMVs induced high expression of the four cytokines,

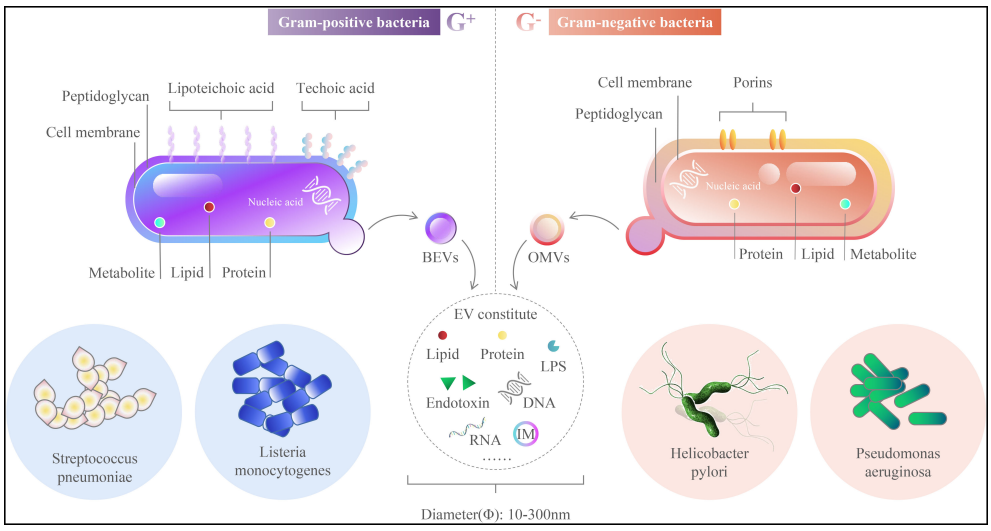

FIGURE 1
Outer Membrane Vesicles main components.

but they were unable to trigger the release of IL-1 or IL-12 into the supernatant (Codemo et al., 2018; Vitse and Devreese, 2020). The study also examined the ability of MV to regulate the release of interleukins from DCs and found increased TNF-α secretion by DC2.4 cells after exposure to streptococci MV, a slight increase in IL -10 production by pneumococcal OMVs treated DC cells, and no change in IL -12 secretion by OMVs treated DC2.4 cells, an observation consistent with previously reported results (Mehanny et al., 2020). On the other hand, pneumococcal OMVs readily binds factor H (FH) from human serum in a PspC-dependent manner; this factor is a negative regulator of the bypass route (Yuste et al., 2010; Codemo et al., 2018). First, FH increases the factor I-dependent cleavage of C3b that is attached to the surface of the bacterial cell, which results in the production of iC3b, which speeds up the degradation of C3b. Second, FH separates B factor from the bypass C3 convertase C3bBb, which decreases the amount of C3b that is deposited on bacteria (Yuste et al., 2010; Hyams et al., 2013). Third, FH inhibits the production of C3 convertase C3bBb on the surface of bacteria by preferentially binding C3b and preventing C3b from attaching to factor B (FB) (Quin et al., 2006; Quin et al., 2007; Li et al., 2007).

In conclusion, OMVs reduce pneumococcal conditioning phagocytosis by strongly binding components of the complement system. The capacity of OMVs to activate the alternative route (M2) shows that OMVs enhance pneumococcal chronicity. The OMVs stimulate significant NF-kB signaling in macrophages in a dose-dependent manner, and they differentiate human macrophages into the (M2) phenotype (Yerneni et al., 2021). OMVs are classified as pneumococcal immune regulators. They influence immune cell recruitment and cytokine production to influence the adaptive immune response (Yerneni et al., 2021). Our findings indicate that OMVs are incorporated into host cells after pneumococcal infection. OMVs help the host defend itself by inducing immune cell recruitment and cytokine responses. On the other hand, by creating an anti-inflammatory milieu for bacterial survival, these OMVs contribute to pneumococcal chronicity infected (Figure 2) (Yerneni et al., 2021). As a consequence, we hypothesize that OMVs are critical effectors of the nuanced interactions between bacteria and host that decide whether an infection is cleared, carried, or transmitted.

NET released by degraded human neutrophils

Pneumococcal virulence factors that facilitate host cell adhesion and/or invasion and/or antagonize the immune system have been found in studies. Several pneumococcal proteins, for example, have been linked to complement-mediated immunity evasion (Jhelum et al., 2018). The alveolar macrophage is the first kind of immune cell that engages in the fight against *Streptococcus pneumoniae* during the early stages of an infection (Jambo et al., 2010). As the lung infection progresses, pneumococci attract neutrophils, which are essential for bacterial clearance. Human bacterial pathogens such as Streptococcus pneumoniae are phagocytosed by neutrophils and killed by various oxidative and/or nonoxidative mechanisms. Neutrophils employ various strategies, such as the release of antimicrobial peptides and the production of reactive oxygen species, to combat these pathogens (Figure 2) (Winterbourn et al., 2016). NETs consist of chromatin adorned with antimicrobial peptides such as LL37, neutrophil elastase (NE), myeloperoxidase (MPO), defensins, etc (Cortjens et al., 2017). It has been reported that the formation of NET is mainly stimulated by various pathogens (von Köckritz-Blickwede et al., 2016). Examples include *Neisseria meningitidis* (Lappann et al., 2013), *Pseudomonas aeruginosa* (Rada et al., 2013), *Staphylococcus aureus* (Malachowa et al., 2013), *Mycobacterium tuberculosis* (Dang et al., 2018), *Streptococcus pyogenes* (Buchanan et al., 2006), and *Burkholderia pseudomallei* (de Jong et al., 2014), *Dengue virus* (Sung and Hsieh, 2021), and in mice that had pneumococcal infection, signs of *Aspergillus* were found in the lungs (Rafiq et al., 2022).

Secretion of OMVs-associated DNase by pneumococci was shown to degrade neutrophil- released NET and its absence reduced virulence of Streptococcus pneumoniae in a mouse model of sepsis, suggesting that OMVs-associated DNase is involved in bypassing the host innate immune system (Beiter et al., 2006). At least two DNases were found to be present in Streptococcus suis. Wild-type Vibrio cholerae rapidly degrades DNA components of NET through the combined activity of two DNases (Dns and Xds) (Jhelum et al., 2018). Secreted DNase contribute to the degradation of NET DNA and protects *P. aeruginosa* from NET-mediated killing (Seper et al., 2013).

Pseudomonas aeruginosa *(P. aeruginosa)*

P. aeruginosa is an opportunistic Gram-negative bacterium that attacks immunocompromised hosts. These immunocompromised hosts include cancer and AIDS patients, burn victims, and people on ventilators. *P. aeruginosa* releases OMVs, a method of interacting with hosts and microbes in their natural environment (Wilton et al., 2018). In addition to providing virulence factors and toxins to host cells, OMVs also triggers Pseudomonas quinolone signaling (PQS) (Figure 2). This process allows bacteria to develop a colonial ecological niche by selectively killing or promoting the growth of different types of bacteria (Kadurugamuwa and Beveridge, 1995; Kuehn and Kesty, 2005; Nakamura et al., 2008; Bomberger et al., 2009; Tashiro et al., 2012; Cooke et al., 2019; Cooke et al., 2020).

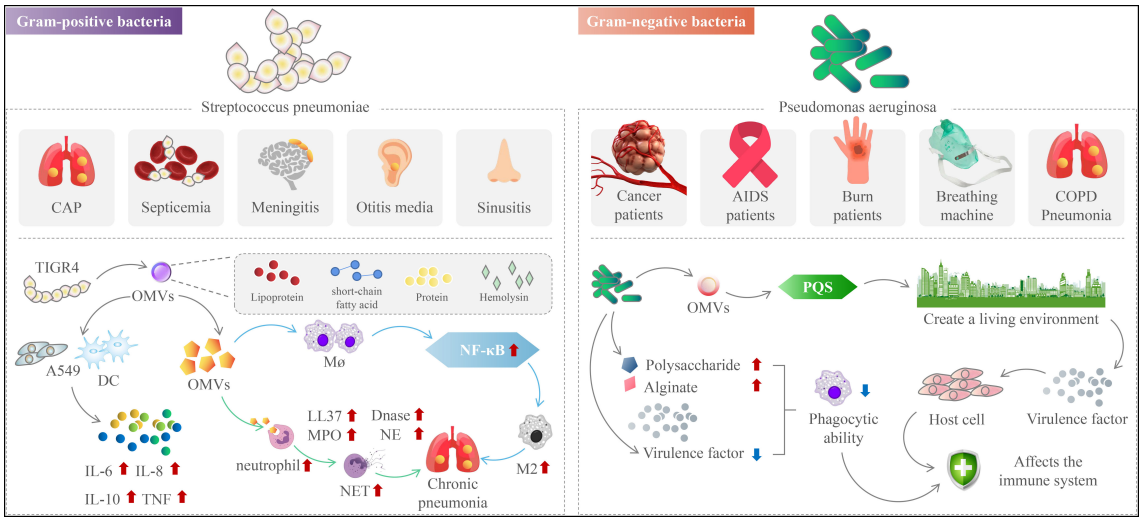

FIGURE 2
The mechanism of action of outer membrane vesicles in chronic respiratory diseases.

In recent years, it has been shown that the majority of pro-inflammatory host immune responses induced in the lung by pathogen-associated molecular patterns (PAMP) are mediated by OMVs. These nanoparticles, which are discharged in huge amounts into the bronchial lumen, include vital virulence components, among other things. PAMPs such as lipopolysaccharide (LPS), peptidoglycan (PG), flagellin (Flag), pore proteins (Por), and lipoproteins (Lip) bind to Toll-like receptors (TLR) in host cells, which signal *via* mitogen-activated protein kinase (MAPK), resulting in the release of pro-inflammatory cytokines and IL -8 in human airway epithelial cells (Saatian et al., 2006; Zhong and Kyriakis, 2007).

Cytokine release draws neutrophils and macrophages to infection sites, clearing bacteria. *P. aeruginosa* uses multiple ways to evade the human immune system to develop persistent infection. These techniques include upregulating polysaccharide and alginate synthesis, downregulating virulence factor expression, reducing phagocytic absorption of *P. aeruginosa* by immune cells, and eliminating flagellar mobility.

Regulation of virulence factor

Bacterial toxicity is the main obstacle to the use of bacteria or their derivatives. Mechanisms mediated by OMVs may also suppress the host immunological response to *P. aeruginosa*. For example, Cif (PA2934), a virulence factor in OMVs, reduces USP10-mediated deubiquitination of CFTR by inactivating host cell deubiquitinating enzyme (USP10) and increases CFTR degradation in lysosomes, which inhibits chloride secretion and thus reduces the ability to clear respiratory pathogens through mucus cilia, an important component of the pulmonary innate immune response (Figure 2) (Bomberger et al., 2011). Cif diminishes the adaptive immune response to viral infection, and thus reduces viral and bacterial clearance in the lung, primarily by decreasing TAP1, leading to a significant decrease in peptide transport to the ER, and inhibiting MHC class I-mediated viral and bacterial antigen presentation (Bomberger et al., 2014). Therefore, we hypothesize that the combined actions of the *P. aeruginosa* Cif virulence factors promote various microbial infections in the lungs of individuals who suffer from cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis. To address this escape mechanism, researchers found that filipin III is a compound that disrupts cholesterol-rich lipid rafts in the parietal membrane of airway epithelial cells, reduces OMVs fusion with CFBE cells, and prevents OMV-associated Cif delivery to human bronchial epithelial cells (Stanton, 2017). HPβCD and MβCD reduce OMVs inhibition by disrupting cholesterol-rich lipid rafts Phe508del CFTR Cl-secretion because cyclodextrins have many non-specific effects, including removing cholesterol from raft and non-raft domains of the plasma membrane and altering cholesterol distribution between the plasma membrane and intracellular membranes (Barnaby et al., 2019). They also observed that HPβCD and MβCD reduced the planktonic growth and biofilm formation of *P. aeruginosa*. These observations are consistent with previous studies indicating that cyclodextrins inhibit population induction of Gram-negative bacteria, which is a key factor in biofilm formation and bacterial resistance to antibiotics in CF lungs (Morohoshi et al., 2013).

P. aeruginosa was treated with an inhibitory concentration of tobramycin (1 μg/ml) or an untreated control PAO1 strain

and examined the effect of tobramycin on OMVs protein content using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. They discovered that tobramycin decreased the abundance of several virulence-associated proteins (including AprA) in OMV and attenuated the inhibitory effect of OMV on the secretion of Phe508del CFTR Cl - by VX-809-stimulated CF bronchial epithelial cells. In addition, they found that tobramycin inhibited the growth of CF bronchial epithelial cells. The host cells were killed by the alkaline protease AprA, which also suppressed the host's cellular and humoral immunological responses (Lee et al., 2018). They came to the conclusion that the elimination of AprA, AlpA, AlpD, and AlpE as well as other virulence determinants in tobramycin-induced OMV might enhance lung function and minimize lung damage, delivering a favorable therapeutic effect with only small reductions in bacterial burden (Koeppen et al., 2019).

Regulating vitronectin (VN)

In addition to utilizing modulators of the alternative and classical/agglutinin pathways, many microorganisms acquire human end metabolic pathway inhibitors, VN, to escape the immune system (Zipfel et al., 2013). Hic interacts with VN and FH, which may lead to *Streptococcus pneumoniae* serotype 3 colonization and invasive illness (Kohler et al., 2015). Recent studies have shown that endotoxin in *P. aeruginosa* OMVs induces the release of VN into the bronchoalveolar space, thereby protecting against complement-mediated killing. VN is a 75 kDa glycoprotein found in plasma and extracellular matrix and belongs to this class of complement regulators. The glycoprotein binds to C5b-7 at sub-stable membrane-bound sites, thereby inhibiting membrane insertion of the complex, but can also directly inhibit C9 polymerization (Podack et al., 1984). VN consists of several structural domains, including TGF-β structural domain, the integrin receptor binding motif Arg-Gly-Asp (RGD), the heme-binding protein-like structural domain, and three heparin-binding structural domains (HBD). VN is released into the blood by hepatocytes but may also be generated by respiratory epithelial cells (Salazar-Peláez et al., 2015). It prevents self-injury by blocking membrane attack complex formation (Sheehan et al., 1995). VN is also related with inflammatory processes, as shown by elevated glycoprotein levels in chronic lung disease patients (Carpagnano et al., 2003; Hallström et al., 2016). By binding Perlins to surface proteins, microorganisms suppress membrane attack complexes and achieve complement resistance. Surface-bound VN may promote bacterial adherence to the epithelium by boosting bacterial-host cell-cell interactions. *P. aeruginosa* from the bronchoalveolar area demonstrated improved boronin binding capability, indicating boronin-dependent pathogenicity in the lung (Paulsson et al., 2015). The researchers found that NTHi and *P. aeruginosa* cells were 3.9-fold and 2.6-fold more viable when compared to BALF preincubation with and without bollenin (Paulsson et al., 2018). These studies indicate that bacterial cells acquire BALF VN to avoid complement-mediated lysis and remain in mammalian hosts. This work uncovers a complicated host-pathogen interaction in which the innate immune system detects bacteria and their OMVs, reacts to them, and gives the bacteria tools to avoid the complement system's antimicrobial effects.

Secretion of small RNA (sRNA)

The fact that many bacterial regulatory small RNAs (sRNAs) have multiple mRNA targets places them at the center of regulatory networks that assist bacteria in adjusting to changes in their surrounding environment (Bossi and Figueroa-Bossi, 2016). OMVs and BEVs mediate the transfer of sRNA and tRNA fragments between bacterial and mammalian cells without direct contact. sRNA52320 was transferred from OMVs to host cells, resulting in a decrease in OMV-stimulated IL -8/KC production of human airway epithelial cells and mouse lungs and a decrease in neutrophil infiltration in an animal model exposed to OMVs. sRNA52320 mostly targets mRNAs in the TLR2/4-induced innate immune response pathway, whereas other receptors and pathways remain unaffected (Koeppen et al., 2016). Similarly, differently packaged sncRNAs were found in *H. pylori* OMVs, and results indicated that sncRNAs (sR-2509025 and sR-989262) were enriched for OMVs and inhibited LPS or OMV-induced IL-8 production from cultured AGS cells (Zhang et al., 2020). MicroRNA-sized RNA fragments identified in periodontopathogenic OMVs reduced IL-5, IL-13 and IL-15 secretion in lymphocyte Jurkat T cells (Choi et al., 2017). In addition, transfer of periodontal pathogen OMVs exRNA to the brain may contribute to neuroinflammatory diseases such as Alzheimer's disease (Han et al., 2019). The sRNA in OMVs secreted by *E. coli* is transferred to bladder epithelial cells and inhibits LPS-induced IL-1a secretion (Dauros-Singorenko et al., 2020). OMVs secreted by Listeria monocytogenes with sRNA rli32 promotes intracellular growth of the pathogen by relying on RIG-I (retinoic acid-inducible gene I) and stimulating the production of IFN-β by bone marrow-derived macrophages (Frantz et al., 2019). During Listeria monocytogenes infection, high levels of type I IFN antagonize IFN-γ signaling by downregulating interferon γ receptors (IFNGR) on antigen-presenting cells (APCs) (Rayamajhi et al., 2010), increase lymphocyte apoptosis (O'Connell et al., 2004), enhance macrophage cell death (Stockinger et al., 2002), reduce protective interleukin 12 (IL-12) and tumor necrosis factor α (TNF-α) production (Auerbuch et al., 2004), and inhibit neutrophil migration (Brzoza-Lewis et al., 2012), thereby contributing to bacterial growth. The microenvironment for bacterial growth was created by

increasing lymphocyte apoptosis, enhancing macrophage death, reducing protective interleukin 12 (IL-12) and tumor necrosis factor α (TNF-α) production, and inhibiting neutrophil migration (Osborne et al., 2017).

Inhibition of major histocompatibility complexes (MHC)

It has been postulated that OMVs directly or indirectly influence gene expression in target cells through host immune receptor signaling. The presentation of antigen is critical for the immune response (Armstrong et al., 2020). MHC molecules communicate peptides to other immune cells in order to activate adaptive immunological responses. Communication between antigen-presenting cells (APCs) and T lymphocytes is required for the host response to bacterial infection. This message is provided to helper T cells by MHC class II antigen presentation, which is followed by an adaptive T cell or B cell response. The interaction between T cells and MHC II, as well as their surroundings, is crucial to the phenotypic and effectiveness of the inflammatory response to infection.

Macrophages are important APCs in the lung because of their immunological flexibility and capacity to perceive and adapt to the local microenvironment (Xing et al., 2020). Pulmonary macrophages are important in the identification and clearance of germs, as well as in the polarization of innate and adaptive immunity. OMVs from the common Cystic Fibrosis (CF) bacteria *P. aeruginosa* were found for the first time to decrease the production of MHC-related markers in human lung macrophages (Armstrong et al., 2020). A study discovered that OMVs lowered the expression of nine distinct MHC II-associated genes, including HLA-DRA, -DRBs, -DMB, -DPs, CD74, CD9, and CTSS. HLA-DRA and -DRB are MHC II complex heterodimeric components that bind antigen fragments processed inside phagocytosed lysosomes. HLA-DMB is the enzyme responsible for removing CLIP from the HLA-DR cleft, allowing antigen fragments to bind. HLA-DP is a paralog of the HLA class II beta chain. CD74 is largely recognized as an invariant chain of MHC class II and plays a role in the molecular processing of MHC class II by the Golgi, as well as being a crucial component in the functional presentation of MHC class II restricted antigens (Roche and Furuta, 2015). CD9 controls MHC class II trafficking throughout the cell. CTSS is histone S, a cysteine protease found in the lysosome that degrades CD74 and pathogenic peptides. All of these components work together to process and deliver MHC class II antigens to $CD4^+$ T cells, acting as a link between innate and adaptive immunity during pathogen infection. This shows that *P. aeruginosa* OMVs, independent of chromosomal location, contain a variety of components that selectively target MHC molecular inhibition as an immune evasion strategy.

Altered methylation

P. aeruginosa OMVs causes dysregulation of the appropriate immune response to infection in macrophages by altering DNA methylation in human lung macrophages (Kyung Lee et al., 2021). Epigenetic modifications alter the pattern of gene expression and have been reported to be important during the innate immune response, regulating B-cell fate and function and controlling T-cell differentiation and memory responses (Lau et al., 2018; Zhang and Cao, 2019; Zhang and Good-Jacobson, 2019).

Gastric cancer is a disease that has been identified to be caused by epigenetic modifications following bacterial infection (Maekita et al., 2006). Alterations in the epigenome, including histone modifications and DNA methylation, are believed to be crucial activating or inhibitory factors, which raises the potential that fast alterations in DNA methylation play a role in the innate immune response (Marr et al., 2014; Morales-Nebreda et al., 2019; Qin et al., 2021). It is important to note that viruses have the potential to change the methylation of DNA and/or impact the expression and activity of DNA methylation modifiers like TET and DNMT. This may lead to changes in the expression of important host genes that are involved in immune response. These two groups of proteins are directly engaged in the mechanism of DNA methylation: DNMTs induce and maintain DNA methylation, whereas TETs catalyze demethylation *via* a series of stages. Infection of dendritic cells by Mycobacterium tuberculosis results in rapid loss of DNA methylation of distal enhancers of major immune transcription factors, including NF-kB and members of the interferon regulatory factor family, within 24 h of activation. This suggests that DNA methylation controls the innate immune response. DNA methylation actively blocks the binding of certain transcription factors (TFs) to the promoter, thereby impairing transcription. During development, activation, and tumor transformation, all three TETs contribute to dynamic demethylation, which is linked with significant transcriptional reprogramming in cells (Qin et al., 2021). During bacterial and viral infections, respectively, TET2 and TET3 have been demonstrated to decrease the expression of proinflammatory cytokines by bringing HDAC1/2 to the promoters of cytokine-encoding genes (Zhang et al., 2015; Xue et al., 2016; Fuster et al., 2017). TET2 also promotes the production of anti-inflammatory cytokines. TET2 also promotes the recruitment of the multicomb repressor complex 2 to the promoters of CpG dinucleotide-rich genes, which results in transcriptional repression (Wu et al., 2011). TET1 may bind to the SIN3A co-repression complex, resulting in a 5hmC-independent transcriptional effect (Williams et al., 2011), which might be a mechanism for TET1-mediated IL-1B transcriptional repression (Neves-Costa and Moita, 2013).

Aberrant DNA methylation in sepsis-related monocytes is linked to inflammatory cytokines and organ failure (Lorente-

Sorolla et al., 2019). Within twenty-four hours of the activation of distal enhancers of important immune transcription factors, such as NF-kB and members of the interferon regulatory factor family, Mycobacterium tuberculosis infection of dendritic cells causes a rapid loss of DNA methylation. This loss occurs as a direct result of the infection. According to this, DNA methylation is likely responsible for regulating the innate immune response (Pacis et al., 2015). DNA methylation may also affect the release of gingival cytokines, which in turn affects how the immune system reacts to *Porphyromonas gingivalis* (Drury and Chung, 2015; Jurdziński et al., 2020).

DNA methylation suppresses transcriptional processes in mature $CD4^+$ T cells in neonates with pneumonia, and these results imply that DNA methylation might serve as a therapeutic target for pediatric lung (McGrath-Morrow et al., 2018). For example, altered DNA methylation status of the Igf2 gene in mouse placental tissue has been associated with maternal infection with the peripheral pathogen Campylobacter. These results are consistent with immune evasion strategies employed by other microorganisms in host-pathogen interactions that may lead to altered innate immune responses.

Remodeling biofilm

Biofilm growth patterns are dominant in natural and disease environments, with over 65% of infections estimated to be biofilm related (Potera, 1999). Planktonic bacteria first attach to a surface, thus transforming into a biofilm lifestyle (Sauer et al., 2002). Afterwards, they produce an extracellular polymer (EPS) that envelops the bacteria and protects them from the environment. the EPS consists of polysaccharides, extracellular DNA, proteins and lipids, and OMVs (Hu et al., 2020). Recently, Schooling and Beveridge reported that bacteria with OMVs present in the biofilm matrix and biofilm of *P. aeruginosa* have higher OMVs synthesis rates than when cultured in planktonic environments (Schooling and Beveridge, 2006). The production of PaAP is controlled by a process known as population sensing (QS), and the protein is released through the Pseudomonas aeruginosa type II Xcp secretion pathway (Michel et al., 2007). Production of PaAP in *P. aeruginosa* leads to an increase in the activity of an endogenous protease that targets secreted OMVs. This in turn leads to OMV-induced cell separation and contributes to the remodeling of the overall biofilm structure. OMV-induced changes eventually lead to an increase in the Psl/biomass ratio in the early biofilm matrix, which helps to protect growing colonies from the deleterious effects of antimicrobial agents (Esoda and Kuehn, 2019). Based on these findings, there is a possibility that PaAP plays a role in fine-tuning pathogenesis, including biofilm production and infection. Proteomic investigation of *P. aeruginosa* biofilms confirmed that proteins associated with Membrane Vesicles make up more than 20% of the total matrix proteome. enzymes involved in the transport of small molecules, iron absorption, and antibiotic resistance were among the proteins shown to be related with OMVs (Couto et al., 2015). Additionally, vesicles isolated from late P. aeruginosa biofilms were richer in drug-binding proteins, which increased the antibiotic resistance of bacterial species inside these biofilms. It was further shown that MV secreted by *P. aeruginosa* is controlled by a population-sensing system and provides extracellular DNA (eDNA) and LPS to the forming biofilm (Nakamura et al., 2008). Furthermore, studies on *P. aeruginosa* biofilms have shown that OMVs secreted by one species is capable of lysing neighboring bacteria to release nutrients as a source of growth and to release eDNA for biofilm construction (Beveridge et al., 1997). However, subsequent studies have shown that OMVs itself is actually incorporated into the biofilm matrix (Schooling and Beveridge, 2006). In addition to this, 11 proteins linked with antimicrobial resistance were shown to be connected with OMVs. It was discovered that all of the proteins that are encoded by the vanA cluster of vancomycin-resistant strains are related with MV. This suggests that bacteria are able to employ Enterococcus faecalis OMVs to release proteins that increase virulence, pathogenicity, and antibiotic resistance (Schooling and Beveridge, 2006). OMVs are a factor in the creation and maintenance of biofilms. The discovery that enterococcal virulence factors AtlA, Esp, and SagA, all of which contribute to the production of enterococcal biofilm, are connected with OMVs may imply that E. coli may be able to produce OMVs. faecium OMVs have the potential to contribute to the production of biofilms (Kropec et al., 2011; Paganelli et al., 2015; Wang et al., 2015). The study investigated the production and functional activity of OMVs in surface-associated microbial communities or biofilms of the fungal pathogen Candida albicans. Biofilm vesicle cargoes include ESCRT subunits Hse1 and Vps27, and most ESCRT-deficient mutations result in reduced biofilm EVs production, reduced levels of matrix polysaccharides, and greatly increased susceptibility to the antifungal drug fluconazole. Exogenous administration of OMVs restores the biofilm resistance phenotype and matrix composition. The OMVs of the biofilm may deposit substances that directly contribute to the structure of the matrix, and they may also have catalytic activity involved in polysaccharide synthesis of the matrix (Zarnowski et al., 2018). Biofilm cells release EVs that promote extracellular matrix formation and resistance to antifungal drugs (Zarnowski et al., 2021).

It was also found that the release of OMVs, which contain two chromosomally encoded β-lactamases, increases when Stenotrophomonas maltophilia infection is treated with β-lactam antibiotics. They demonstrate the ability of these β-lactamase-packed OMVs to establish extracellular β-lactam

degradation. The investigation also reveals that the cohabitating species *P. aeruginosa* and *Burkholderia cenocepacia* have significantly higher apparent MICs for imipenem and ticarcillin.

Summary

Lung infection-associated bacteria pose a rising hazard to worldwide public health. OMVs are outer membrane vesicles that are secreted by Gram-negative bacteria. These OMVs have the ability to transfer infectious agent into the cytoplasm of the host cell, which induces a protective immune response in the body. This opens up a new avenue for the potential reduction of tissue damage caused by immune tolerance. Consequently, it is crucial to shed more insight on the techniques these bacteria use to improve their pathogenicity. The significance of OMVs in immune evasion is highlighted. By targeting OMV-related components implicated in the interaction of these vesicles with human lung cells or macrophages, new treatment approaches for these infections may become available. In addition, the immunomodulatory properties of OMVs might be used to develop vaccines that protect patients from bacterial infections.

Author contributions

Writing—Original Draft: CL and AS; Figure design: HZ; Data collection: MS and FH; Searching: YY and AS; Review & Editing: All authors. All authors read and approved the final manuscript.

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Conflict of interest

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

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Gut microbiota and fecal metabolic signatures in rat models of disuse-induced osteoporosis

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Background: Assessing the correlation between gut microbiota (GM) and bone homeostasis has increasingly attracted research interest. Meanwhile, GM dysbiosis has been found to be associated with abnormal bone metabolism. However, the function of GM in disuse-induced osteoporosis (DIO) remains poorly understood. In our research, we evaluated the characteristics of GM and fecal metabolomics to explore their potential correlations with DIO pathogenesis.

Methods: DIO rat models and controls (CON) underwent micro-CT, histological analyses, and three-point bending tests; subsequently, bone microstructures and strength were observed. ELISAs were applied for the measurement of the biochemical markers of bone turnover while GM abundance was observed using 16S rDNA sequencing. Metabolomic analyses were used to analyze alterations fecal metabolites. The potential correlations between GM, metabolites, and bone loss were then assessed.

Results: In the DIO group, the abundance of GM was significantly altered compared to that in the CON group. Moreover, DIO significantly altered fecal metabolites. More specifically, an abnormally active pathway associated with bile acid metabolism, as well as differential bacterial genera related to bone/tissue volume (BV/TV), were identified. Lithocholic acid, which is the main secondary bile acid produced by intestinal bacteria, was then found to have a relationship with multiple differential bacterial genera. Alterations in the intestinal flora and metabolites in feces, therefore, may be responsible for DIO-induced bone loss.

Conclusions: The results indicated that changes in the abundance of GM abundance and fecal metabolites were correlated with DIO-induced bone loss, which might provide new insights into the DIO pathogenesis. The detailed regulatory role of GM and metabolites in DIO-induced bone loss needs to be explored further.

KEYWORDS

osteoporosis, disuse, gut microbiota, 16s rDNA sequencing, metabolomics

Introduction

Osteoporosis is the most common bone disorder and is defined as a systemic skeletal disease characterized by low bone mass and the microarchitectural deterioration of bone tissue. It further leads to increased bone fragility and susceptibility to fractures (Locantore et al., 2020; Kim et al., 2021). The incidence of osteoporotic fractures is rapidly increasing in the aging population. Indeed, osteoporotic fractures seriously impact the quality of life and mortality of patients, as well as overall healthcare costs (Pisani et al., 2016).

Osteoporosis can be divided into primary and secondary osteoporosis depending on its etiology. Disuse-induced osteoporosis (DIO), which is a type of secondary osteoporosis with presently unsatisfactory treatment options, is a common complication caused by the lack, or disuse, of mechanical loading or systemic immobilization and constitutes a state of bone loss (Yang et al., 2020; Rolvien and Amling, 2022). Currently, drug therapy remains the primary treatment for osteoporosis (Ou et al., 2021). However, the long-term use of these drugs can lead to serious complications, including kidney damage, venous thrombosis, and an increased risk of developing tumors (Ou et al., 2021). Thus, more effective, safe, and novel treatment strategies are urgently required for DIO.

Gut microbiota (GM), referred to as the "second gene pool," comprise a collection of microorganisms that colonize the gastrointestinal tract (Qin et al., 2010; Tu et al., 2021). GM homeostasis is implicated in many aspects of human health, including neurological disorders, abnormal inflammatory responses, and metabolic diseases (Cani, 2018; Needham et al., 2020; Suganya and Koo, 2020; Yoo et al., 2020). Osteoporosis, which is a systemic metabolic bone disease, is closely related to the GM. In a study by Wen, a postmenopausal osteoporosis mouse model was constructed and revealed that *Ruminococcus flavefaciens* exhibited the greatest variation in abundance among the GM and was also associated with osteoclastic indicators and the estrobolome (Wen et al., 2020). Ma further carried out a metabolomics analysis of serum and fecal metabolites in animal models of postmenopausal osteoporosis and found that changes in the GM, as well as fecal and serum metabolites were responsible for the occurrence and development of postmenopausal osteoporosis (Ma et al., 2020b). Ma also observed an association between the composition and function of GM and senile osteoporosis in an aged rat model, demonstrating that variations in GM may contribute to senile osteoporosis through metabolic pathways (Ma et al., 2020a). In addition, glucocorticoid-, alcohol-, and high-fat-induced osteoporosis are all associated with dysbacteriosis in the GM (Schepper et al., 2020; Cheng et al., 2021; Lu et al., 2021).

To date, the GM, metabolites, and mechanisms by which the GM affect DIO remain poorly understood. In the present study, we constructed a DIO rat model and applied an integrated approach comprising 16S rDNA gene sequencing combined with fecal ultra-high-performance liquid chromatography-mass spectrometry to elucidate the association between GM and DIO. Understanding the characteristics of GM and GM-derived metabolites in DIO may provide insights that will contribute to the development, prevention, and treatment of osteoporosis.

Materials and methods

Animals

Twelve-week-old female Sprague-Dawley rats were purchased from the Ying Ze District Campus Animal Testing Center at Shanxi Medical University. The rats were housed in a non-specific microbial environment with a constant temperature of 23 ± 2°C, 12-h light/dark cycle, and allowed ad libitum access to sterile food and autoclaved water. After one week acclimatization, the rats were randomly divided into DIO and control (CON) groups (n = 6/group). Right leg sciatic neurotomies were performed on the rats to construct DIO models; the same amount of adipose tissue was taken from both groups (Monzem et al., 2021). The animals were sacrificed 10 weeks post-operation, and target samples were collected from each group. All animal experiments were performed in strict accordance with the National Institutes of

Health (NIH) Guidelines for the Care and Use of Experimental Animals and were approved by the Ethical Committee of Experimental Animal Care of Shanxi Medical University (permit number: 2021014).

Micro-CT scanning

Distal femurs were scanned at a high resolution using Micro-CT (vivaCT80, Scanco, Switzerland) to analyze differences in the volumes and structures of the trabecular bones between the two groups. We manually selected 100 contiguous cross-sectional slices above the limit of the femoral growth plate to analyze the bones. Histomorphometric parameters were computed using Scanco Medical software. The bone mineral density (BMD), bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), cortical bone thickness (Ct.Th), and cortical bone volume (Ct.V), were determined for each sample.

Histological analysis

The femoral samples were fixed with 4% paraformaldehyde for 24 h and decalcified in 20% ethylenediaminetetraacetic acid solution at 37°C for 6–7 weeks until the femurs had softened. The femurs were then dehydrated, embedded in paraffin, cut into 5-mm longitudinal sections, dried, and stained with hematoxylin and eosin. Their morphological characteristics were examined using light microscopy.

Three-point bending test

The femurs were subjected to a three-point bending test. The mechanical properties of the femurs were evaluated at the mid-diaphysis using an electronic universal testing machine (ElectroForce 3200 Series, TA Instruments, USA). The femoral samples were centered and fixed on a three-point bending test stent with two fixed loading points separated by a 20-mm gap. A bending load was applied at a constant displacement rate of 3 mm/min until fractures occurred. The internal and external major and minor axis lengths of the femurs at the fracture points were then measured. The following parameters of the samples were obtained: peak load, fracture load, maximum displacement, and stiffness.

ELISA

Blood samples were collected from the hearts of rats and centrifuged for 15 min at 3,000 rpm to separate the sera. The abundance of the N-terminal propeptide of type I procollagen (PINP) and C-terminal telopeptide of type I collagen (CTX-I) was measured using ELISA kits (Lunchang Shuo Biotechnology, Xiamen, China) according to the manufacturer's protocol.

Feces collection

Fecal samples were directly collected from the rats in both groups. At least two fecal pellets were collected from each rat: one was used for microbial analysis, while the other was subjected to metabolic analysis. The fecal samples were placed in sterile centrifuge tubes, frozen in liquid nitrogen immediately, and stored at −80°C for further sequencing.

16S rDNA sequencing

16S rDNA sequencing was carried out at Lc-Bio Technologies Co., Ltd. DNA from different samples were extracted using the cetyltrimethylammonium ammonium bromide (CTAB) method. We selected the V3–V4 region of the 16S rRNA gene using the custom barcode universal bacterial primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). The PCR products were confirmed with 2% agarose gel electrophoresis and purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, USA), and quantified by Qubit (Invitrogen, California, USA). The purified PCR products were assessed on an Agilent 2100 Bioanalyzer (Agilent, California, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, USA). The libraries were sequenced on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA).

16S rDNA microbial community analysis

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequence, and merged using FLASH (v1.2.8, http://ccb.jhu.edu/software/FLASH/). Quality filtering of the raw reads was performed to obtain high-quality clean tags using fqtrim (v0.94, http://ccb.jhu.edu/software/fqtrim/). Chimeric sequences were filtered using Vsearch (v2.3.4, https://github.com/torognes/vsearch). Subsequently, we performed DADA2 analysis using QIIME2 (v2019.7, https://qiime2.org/) to obtain Amplicon Sequence Variant (ASV) tables and sequences. Alpha diversity and beta diversity were analyzed based on the above ASV tables and sequences. Lastal+ (v2017.3, https://github.com/hallamlab/LAST-Plus/wiki) was used for sequence alignment, and the feature sequences were annotated with the NT-16s database for each representative sequence. Finally we conducted difference analysis and advanced analysis between two groups. The graphs were drawn using R (v3.5.2).

Extraction and UHPLC-MS/MS analysis of fecal metabolites

The collected samples were thawed on ice, and metabolites were extracted with 50% methanol buffer. Metabolite-containing supernatants were obtained after centrifugation at 4,000 g for 20 min and stored at -80°C prior to LC-MS analysis. In addition, pooled QC samples were prepared by combining 10 μL of each extraction mixture. We ensured that different groups were cross-sorted on the machine. All chromatographic separations were performed using a Thermo Scientific UltiMate 3000 HPLC system (Thermo Scientific, Waltham, USA). A high-resolution tandem mass spectrometer Q-Exactive (Thermo Scientific, Waltham, USA) was used to collect first and secondary order spectrum data of the metabolites eluted from the column, and operated in both the positive and negative ion modes. To evaluate the stability of the LC-MS during the data acquisition stage, a quality control sample was acquired after every 10 samples.

Bioinformatics of fecal metabolome data

Pretreatments of the acquired mass spectrum raw data were performed using Compound Discoverer 3.1.0 (Thermo Fisher Scientific, Waltham, USA). Each ion was identified by combining retention time (RT) and m/z data. We use the chemspider database as a plug-in for database search using the Compound Discoverer software. Using online Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Metabolome Database (HMDB), the metabolites were annotated by matching their molecular weights (mass difference less than 10 ppm), substance names and formulas. The intensity of peak data was further preprocessed using metaX (v1.4.16, https://www.ncbi.nlm.nih.gov/pubmed/28327092). The features that were detected in less than 50% of Quality Control (QC) samples or 80% of biological samples were removed, and the remaining peaks with missing values were imputed with the k-nearest neighbor algorithm to further improve data quality. Principal Component Analysis (PCA) was performed for outlier detection using the pre-processed dataset. Probabilistic Quotient Normalization (PQN) was used to normalize the data to obtain the normalized ion intensity data of each sample. In addition, the Coefficient of Variations of the metabolic features were calculated across all QC samples, and those > 30% were then removed. The P-values were adjusted for multiple tests using the False Discovery Rate and Benjamini-Hochberg method. Supervised Partial Least Squares-Discriminant Analysis (PLS-DA) was conducted using metaX (v1.4.16, https://www.ncbi.nlm.nih.gov/pubmed/28327092) to discriminate the different variables between two groups, and Variable Influence of Projection (VIP) values were calculated. A VIP cut-off value of 1.0 was used to select important features. KEGG enrichment analysis was performed on significantly different metabolites. The correlations between differential bacterial genera, bone phenotypes, and metabolites were analyzed using R (v3.5.2).

Statistical analysis

The statistical significance of bone mass and biochemical indices was assessed using Student's *t*-tests or a one-way analysis of variance in GraphPad Prism (v.9.0). Spearman's rank correlation was applied for the association analysis of the 16S microbiome, bone phenotypes and fecal metabolites. Differences were considered statistically significant at $P < 0.05$.

Results

DIO-induced bone loss in rats

Micro-CT was used to analyze distal femur trabecular bone microstructures. The associated 3D structures (BMD, BV/TV, Tb.N, Tb.Th, Ct.Th, and Ct.V) revealed that the distal femur trabecular bone microstructure was significantly destroyed in the DIO group compared with that in the CON group (Figure 1A). Moreover, distinct reductions in BMD, BV/TV, Tb.N, Ct.Th, and Ct.V were observed. (Figures 1B–D, F, G). However, there were no significant differences in the Tb.Th between the two groups (Figure 1E). These results indicate that DIO causes marked femoral bone loss.

Changes in bone morphology, reductions in bone mechanical properties, and inhibition of bone formation in DIO

To further determine the changes in bone morphology in rats with DIO, histopathological examinations were performed on bone tissues. Compared with that in the CON group, the trabecular bone in the DIO group became sparse, and its shape changed from plate- to rod-like (Figure 2A). We further observed a marked decrease in the area ratios of the trabecular bone (Figure 2B) and osteoblasts (Figure 2C) in the DIO-induced rats. Additionally, we tested the mechanical properties of the femurs using a three-point bending test. The peak load, fracture load, and stiffness of the disused femurs were significantly decreased compared to those in the CON group, and no significant difference was observed in the maximum displacement between the two groups (Figures 2D–G). Furthermore, we used ELISA to detect the serum bone transformation indicators. The results showed that DIO increased CTX-I (Figure 2H) and decreased PINP (Figure 2I)

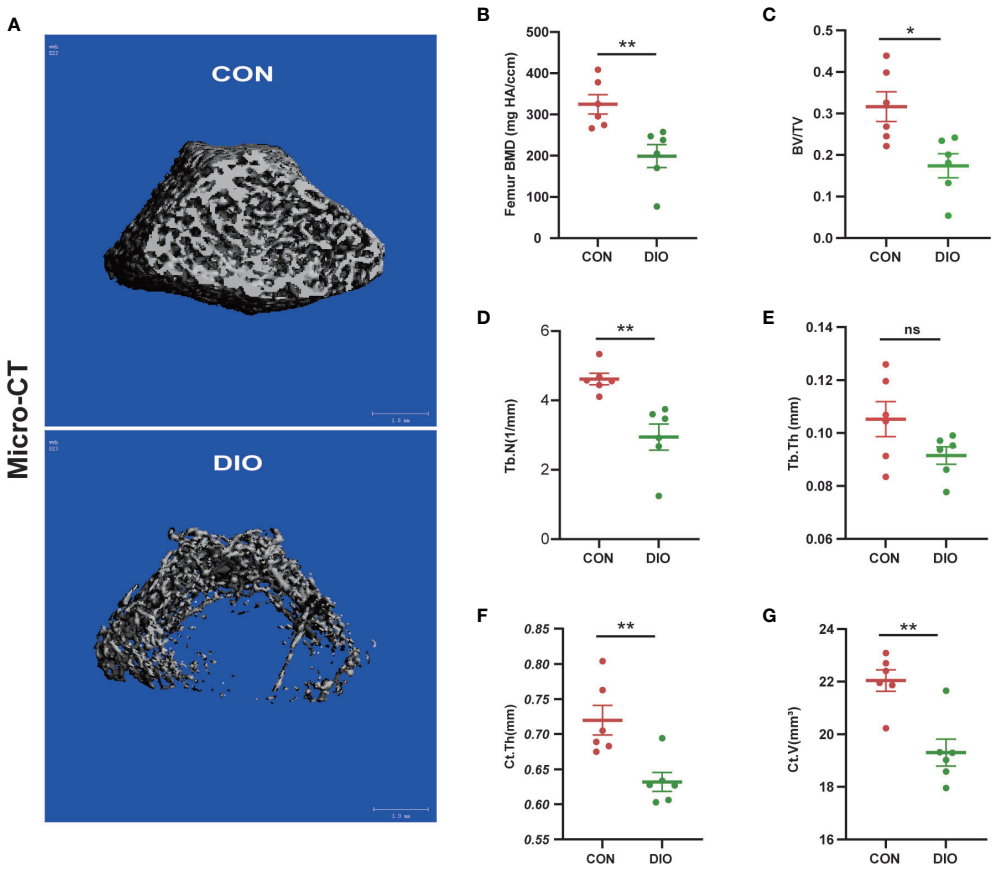

FIGURE 1
DIO-induced bone loss in rats. **(A)** Representative Micro-CT 3D reconstructions of the two groups. **(B–E)** Trabecular bone parameters at the distal femoral metaphysis after 10 weeks, including BMD, BV/TV, Tb.N, and Tb.Th. **(F, G)** Cortex bone parameters at the middle femur after 10 weeks, including Ct.Th and Ct.V. Data are expressed as the mean ± SEM. n = 6, *P < 0.05, **P < 0.01, ns, no significance.

abundance. In conclusion, DIO effectively altered bone morphology, attenuated the mechanical properties of the femurs, and inhibited bone formation.

DIO significantly changes the species abundance of gut microbiota

A Venn diagram of the ASV distribution revealed changes in the microbiota in the DIO group. We generated 7,020 ASVs from the CON and DIO groups, including 211 differential ASVs, out of which DIO had 3,342 unique ASVs, CON had 2,753 unique ASVs, while 925 ASVs were shared between both groups (Figure S1A; Table S1). We then performed an alpha diversity analysis of the Chao1 and Shannon indices to assess the sample species richness and sequencing depth (Figures S1B, C). The rarefaction curve can directly reflect the rationality of sequencing data and indirectly reflect species richness in samples. When the curve tends to be flat, it shows the sequencing depth is reasonable. (Figure S1D, E). We then assessed the beta diversity, which reflects species differentiation between the two groups. To that end, we performed a Principal Coordinate Analysis (PCoA) to observe differences in the microbiota between the CON and DIO groups. Each point in the resulting graph represents an independent sample; the closer two points are to each other, the more similar they are. Based on the 3D and 2D results of the PCoA, analyzed based on Jaccard distance matrices, the GM in the CON and DIO groups were divided into two distinct groups, indicating that the composition of the GM in the DIO group differed significantly from that in the CON group (P<0.05) (Figures 3A, B).

Next, we generated stacked bar charts and heat maps of the phylum, class, order, family, genus, and species levels to conduct a species analysis. The phylum-level analysis is shown in Figures 3C, D, while the results of the other levels are listed in Figure S2. Firmicutes and Bacteroidetes were identified as the main phyla of the GM, accounting for 80% of the total microbiome. In our study, the abundance of Firmicutes was

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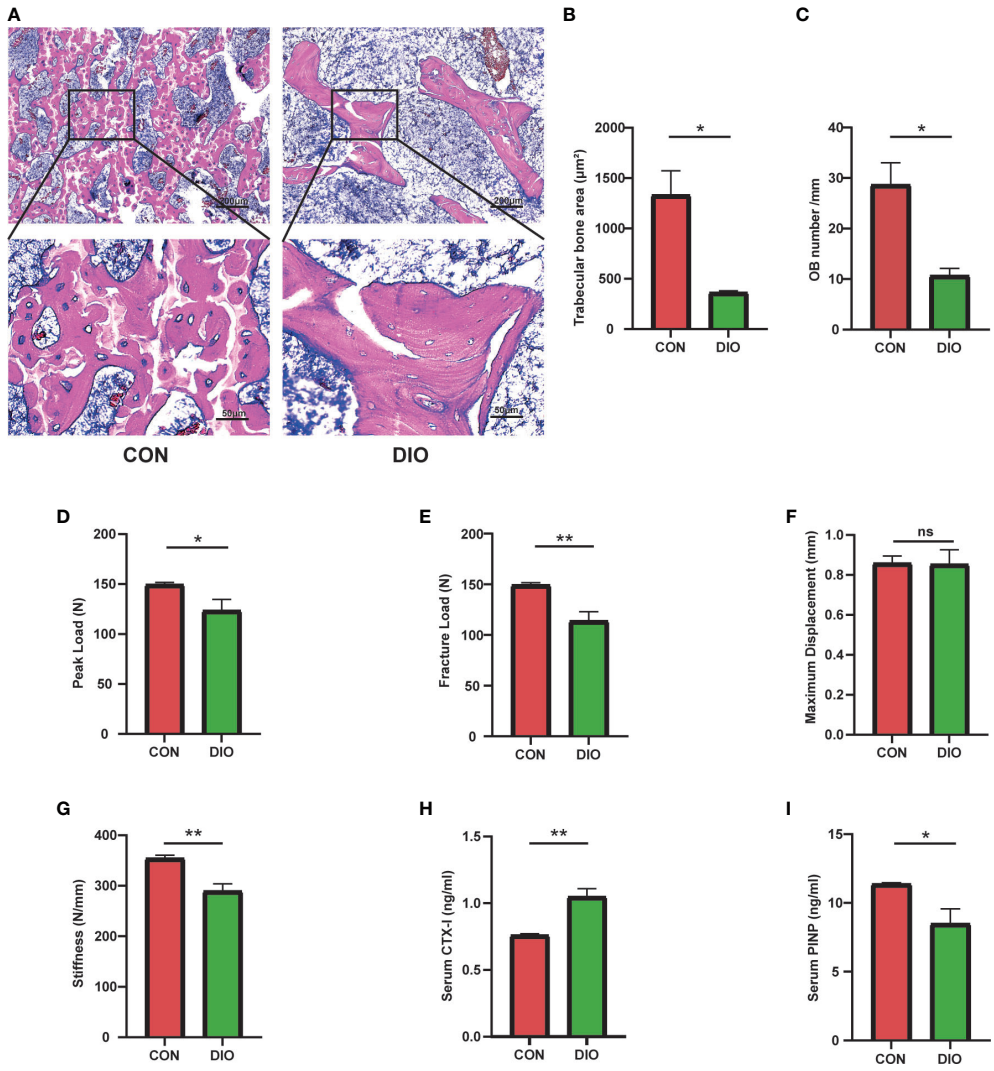

FIGURE 2
Changes in bone morphology, reduced bone mechanical properties, and inhibition of bone formation in DIO. **(A)** The bone tissue of distal femoral metaphysis was observed by H&E staining. **(B)** Trabecular bone area ratio. **(C)** Osteoblast numbers. **(D–G)** Parameters of the three-point bending test, including the peak load, fracture load, maximum displacement, and stiffness. **(H, I)** Serum levels of bone turnover biomarkers, including CTX-I and PINP. Data are expressed as the mean ± SEM. n = 6, *P < 0.05, **P < 0.01, ns, no significance.

higher in DIO, and the abundance of Bacteroidetes was lower (Figures 3C, D). To assess the significant differences in the abundance of the species between the two groups, we performed a linear discriminant analysis (LDA) effect size (LEfSe) analysis with an LDA fold of four. The relationships between the different microbiota from the phylum to species levels are shown in the cladogram in Figures 4A, B. We then performed a relative abundance analysis at each level and observed 34 species with significant differences at the genus level, out of which 13 were upregulated and 21 were downregulated (Figure 4C).

DIO markedly alters the fecal metabolome

Principal Component Analysis (PCA) was mainly used to observe the trend of separation between groups in experimental models and whether there were outliers, and to reflect the degree of variation between and within groups from the raw data. A PLS-DA was performed to identify the differences in the fecal metabolites between the two groups. Different from PCA, the PLS-DA is a supervised analysis that maximizes the differences between two groups using partial least squares regression to

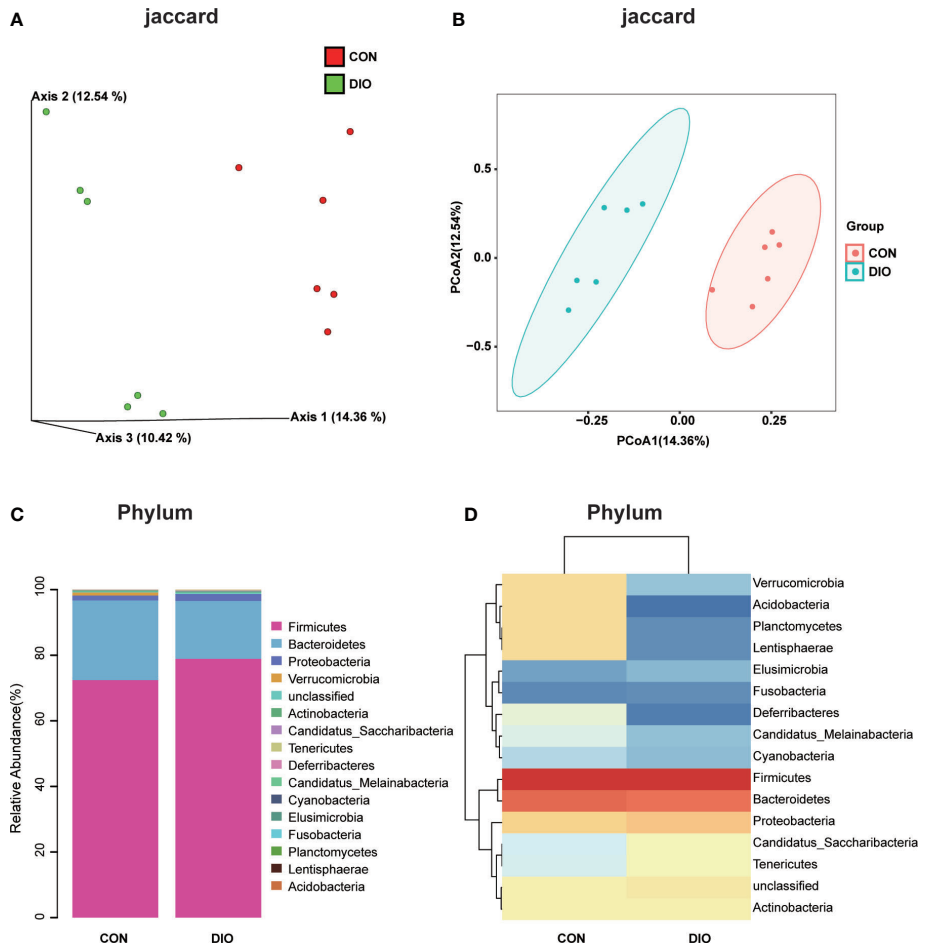

FIGURE 3
DIO significantly alters the abundance of gut microbiota. **(A, B)** 3D and 2D PCoA of GM analyzed with Jaccard distance matrices, n = 6. **(C)** Stacked bar chart at the phylum-level in the CON and DIO groups, n = 6. **(D)** Heat map at the phylum-level in the CON and DIO groups, n = 6.

model the relationship between metabolite expression and sample type. It can further be used to assess sample modeling and prediction. R2 is the interpretation rate of the model in the Y direction, and Q2 is the prediction rate of the model. We tested the R2 and Q2 model parameters 200 times, the values of which are shown in Figure S3. The PCA and PLS-DA results revealed that the metabolite profiles of the two groups were divided into two distinct zones, indicating that DIO can alter fecal metabolites (Figures 5A, B, S4). Compared with the positive ion mode in the CON group, we identified 4,579 metabolites with alterations in the DIO group (2,620 upregulated and 1,959 downregulated), and in negative ion mode, we identified 2,110 metabolites with alterations (1,111 upregulated and 999 downregulated) (Figures 5C, D). As shown in the differential metabolite heat map of the comparison group, there were significant cumulative differences in the metabolites between the two groups (Figure 5E), among which 857 were annotated based on the KEGG database. We performed a KEGG enrichment analysis of the differential fecal metabolites between the two groups, the top 20 of which have been listed in Figure 5F. Among the top ten metabolic pathways, we assessed primarily those related to bile, including primary and secondary bile acid biosynthesis and bile secretion (Figure 5F). Compared with the CON group, 16 differential metabolites were enriched in the above three metabolic pathways in the DIO group, namely, chenodeoxyglycocholate, bilirubin, cholic acid, glycochenodeoxycholic acid, glycocholic acid, deoxycholic acid, zalcitabine, lithocholic acid (LCA), cortisol, taurochenodeoxycholic acid, taurocholate, fexofenadine, fluvastatin, taurine, 5-beta-cyprinolsulfate, and beta-muricholic acid, of which 15 were upregulated and one was downregulated. LCA was particularly interesting to identify as it is the main

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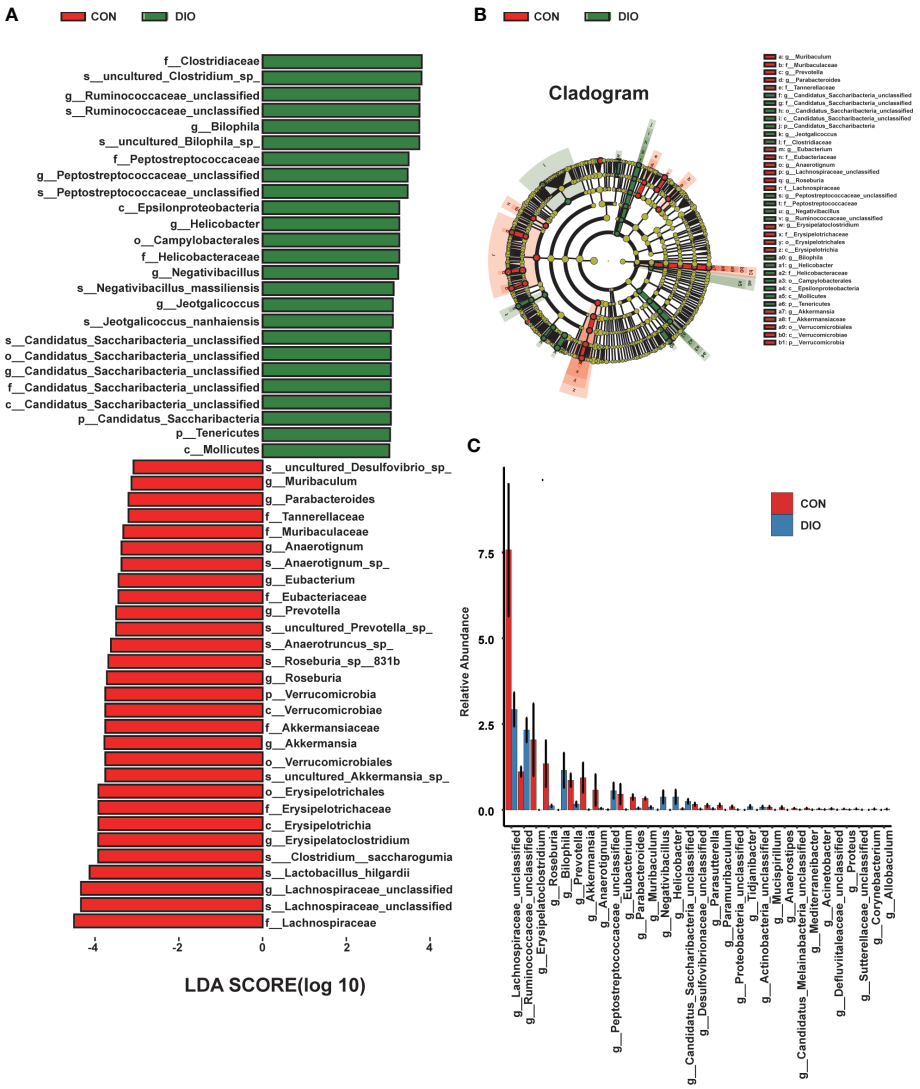

FIGURE 4
DIO changes the gut microbiota from the phylum to species levels. **(A)** LEfSe analysis of gut microbiota in the CON and DIO groups, n = 6, LDA score > 4.0. Red represents increased microbiota in the CON group; green represents increased microbiota in the DIO group. **(B)** The relationships among different microbiota from the phylum to species levels are shown in the cladogram, n = 6. **(C)** Significant difference analysis at genus level, n = 6, Wilcox test P-value < 0.05.

component of bile acids and closely related to bone metabolism (Ruiz-Gaspà et al., 2020B).

Correlations between differential gut microbial genera and bone phenotypes

The correlation heat map and network of differential bacterial genera and bone phenotypes are plotted in Figures 6A, B (|r| > 0.5). Among the differential bacterial genera that were significantly related to BV/TV (|r| > 0.5), 24 of 34 distinct genus were correlated with BV/TV, including 9 negatively correlated and 15 positively correlated. The nine bacterial genera that were negatively correlated with BV/TV were *Jeotgalicoccus* (r = –0.549, P = 0.064), *Negativibacillus* (r = –0.591, P = 0.042), *Bilophila* (r = –0.623, P = 0.030), *Actinobacteria* (r = –0.630, P = 0.027), *Staphylococcus* (r = –0.657, P = 0.020), *Helicobacter* (r = –0.717, P = 0.008), *Peptostreptococcaceae* (r = –0.751, P = 0.004), *Tidjanibacter* (r = –0.795, P = 0.002) and *Candidatus Saccharibacteria* (r = –0.818, P = 0.002). The 15 bacterial genera that were positively correlated with BV/TV were *Proteus* (r = 0.865, P = 0.001), *Anaerotignum* (r = 0.797, P = 0.003), *Erysipelatoclostridium* (r = 0.794, P = 0.002), *Paramuribaculum* (r = 0.775, P = 0.003),

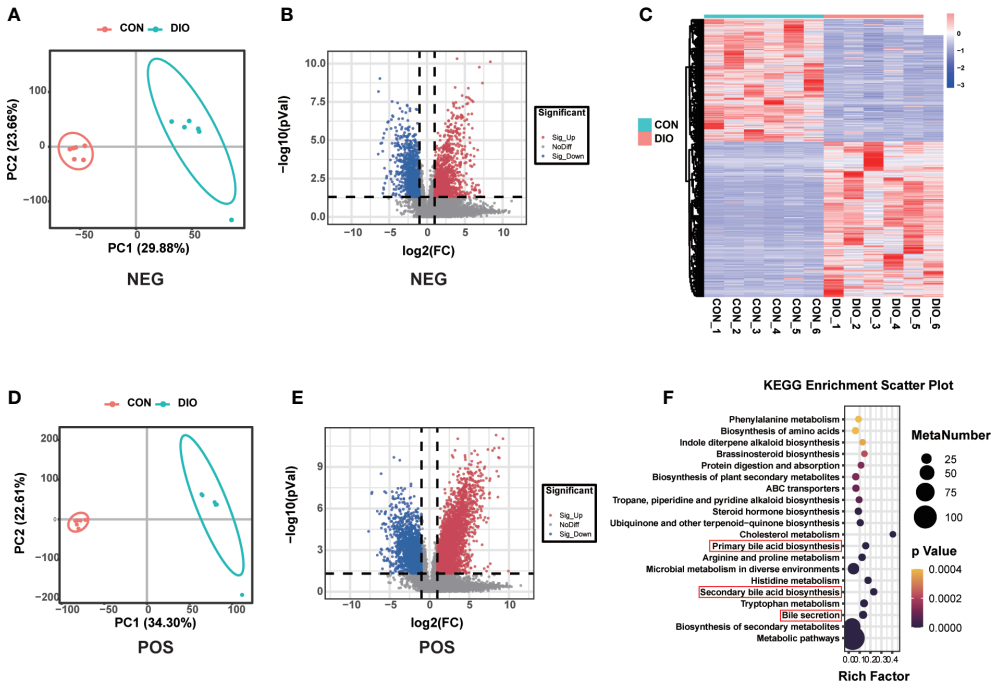

FIGURE 5
DIO markedly alters the fecal metabolome. **(A, B)** PLS-DA analysis of fecal metabolites between the two groups; variable importance in projection (VIP) > 1, R2 > 0.5, Q2 > 0.5. **(C, D)** Volcano maps displaying differential fecal metabolites. **(E)** Heat map of fecal metabolites in the two groups. **(F)** Bubble diagram of the top 20 enriched KEGG pathways.

Sutterellaceae (r = 0.761, P = 0.004), *Roseburia* (r = 0.741, P = 0.008), *Muribaculum* (r = 0.706, P = 0.013), *Parabacteroides* (r = 0.692, P = 0.016), *Defluviitaleaceae* (r = 0.690, P = 0.013), *Eubacterium* (r = 0.651, P = 0.021), *Acinetobacter* (r = 0.647, P = 0.023), *Desulfovibrionaceae* (r = 0.609, P = 0.035), *Proteobacteria* (r = 0.583, P = 0.046), *Akkermansia* (r = 0.576, P = 0.049), and *Prevotella* (r = 0.531, P = 0.079).

Association analysis of differential gut microbiota and fecal metabolome

The correlation heat map and network of differential bacterial genera and fecal metabolites related to bile acid metabolism are plotted in Figures 6C, D (|r| > 0.5). Multiple correlations between the differential bacterial genera and fecal metabolites were identified. For example, LCA was positively correlated with *Peptostreptococcaceae* (r = 0.854, P = 0.001), *Negativibacillus* (r = 0.781, P = 0.002), *Actinobacteria* (r = 0.780, P = 0.002), *Candidatus Saccharibacteria* (r = 0.769, P = 0.005), *Corynebacterium* (r = 0.750, P = 0.004), *Ruminococcaceae* (r = 0.692, P = 0.015), *Bilophila* (r = 0.639, P = 0.025), *Allobaculum* (r = 0.624, P = 0.030), and *Helicobacter* (r = 0.545, P = 0.066), and negatively correlated with *Erysipelatoclostridium* (r = –0.705, P = 0.010), *Defluviitaleaceae* (r = –0.661, P = 0.019), *Proteobacteria* (r = –0.633, P = 0.026), *Mediterraneibacter* (r = –0.626, P = 0.029), *Parabacteroides* (r = –0.615, P = 0.037), *Desulfovibrionaceae* (r = –0.602, P = 0.038), *Muribaculum* (r = –0.594, P = 0.045), *Candidatus Melainabacteria* (r = –0.588, P = 0.044), *Paramuribaculum* (r = –0.572, P = 0.051), *Mucispirillum* (r = –0.556, P = 0.060), *Proteus* (r = –0.542, P = 0.068), *Lachnospiraceae* (r = –0.538, P = 0.074), *Akkermansia* (r = –0.512, P = 0.088), *Eubacterium* (r = –0.507, P = 0.091), and *Roseburia* (r = –0.503, P = 0.098). Further, we analyzed the correlation between bone phenotypes and differential metabolites related to bile acid and found a negative correlation between LCA and bone phenotypes (Figure S5).

Discussion

GM play an indispensable role in host physiology, including nutrient absorption, immune system modulation, and homeostasis (Belkaid and Hand, 2014). GM composition is influenced by several internal and external factors, including diet, age, disease, and lifestyle (Sędzikowska and Szablewski, 2021). Imbalances in the GM, known as dysbiosis, often induce aberrant immune responses, which in turn disrupt the local and systemic homeostasis of the host leading to various disorders (Holmes et al., 2020). Increasing evidence has suggested that GM further play an important role in bone homeostasis (Ohlsson

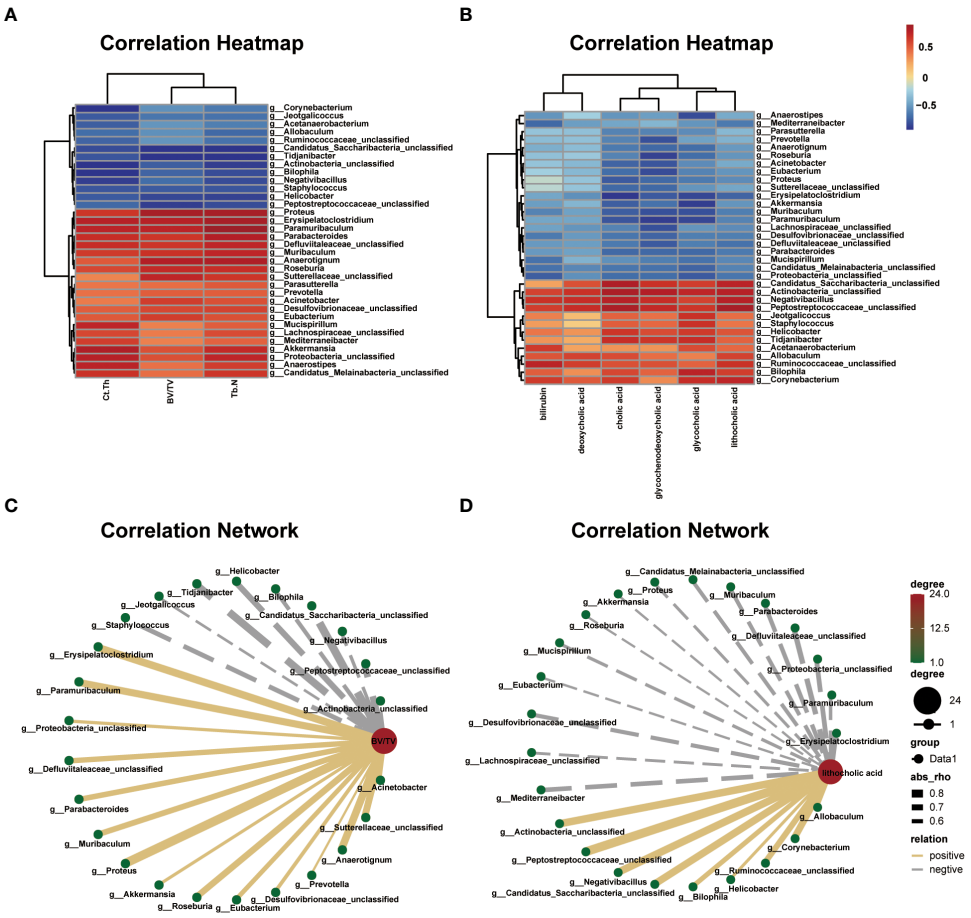

FIGURE 6
Correlations among differential gut microbial genera, bone phenotypes, and fecal metabolites related to bile acid metabolism. **(A)** Correlation heat between differential bacterial genera and bone phenotypes in the two groups, |r| > 0.5. Red represents positive correlations, and blue represents negative correlations. The darker the color, the stronger the correlation. **(B)** Correlation network map between BV/TV and differential bacterial genera, |r| > 0.5. **(C)** Correlation heat map between the differential bacterial genera and fecal metabolites related to bile acid metabolism in the two groups, |r| > 0.5. Red represents positive correlations, and blue represents negative correlations. The darker the color, the stronger the correlation. **(D)** Correlation network map between lithocholic acid (LCA) and differential bacterial genera, |r| > 0.5.

and Sjögren, 2015). The notion that the gut microbiome is a BMD regulator in health and disease is supported by an established correlation between microbiome diversity and osteoporosis (Wang et al., 2017). Extensive research has demonstrated that the effects of the GM and their metabolites in bones are secondary to immunomodulatory responses, and the perturbation of the GM can drive skeletal deterioration in pathophysiological states (Wu et al., 2010; Li et al., 2016; Li et al., 2019). However, the relationship between intestinal microorganisms and host metabolites in DIO has seldom been investigated. In this study, we analyzed the characteristics and relationships between the microbiome and fecal metabolome in DIO using a multi-omics correlation network approach.

In the 16S rDNA gene sequencing analysis, we observed that DIO was associated with GM dysbiosis. Although alpha diversity was similar between the two groups, the beta diversity was obviously different. Higher Firmicutes and lower Bacteroidetes abundance were observed in the DIO group, which is consistent with the changes reported in postmenopausal osteoporosis (Ma et al., 2020b; Wen et al., 2020) and high-fat diet-induced bone loss (Lu et al., 2021), and contrary to variations in glucocorticoid- (Li et al., 2020) and alcohol-induced osteoporosis (Cheng et al., 2021). Additionally, the abundance of *Verrucomicrobia* was decreased in DIO. Sun also observed this change in ovariectomized osteoporotic rats (Sun et al., 2021).

We further performed a correlation analysis between the bacterial genera and bone phenotypes and found that various bacterial genera were positively or negatively correlated with BV/TV (|r| > 0.5), including *Proteus*, *Erysipelatoclostridium*, *Akkermansia*, and *Roseburia*. *Proteus*, a gram-negative pathogenic bacterium, contributes to the inhibition of osteoclast formation and bone resorption by its outer-

membrane vesicles (Wang et al., 2022). *Erysipelatoclostridium*, which was found to be linked to BMD at multiple sites, has been reported to produce acetate, which is a short-chain fatty acid that inhibits inflammatory Th17 cell activation and promotes Treg cell differentiation (Zhang et al., 2016; Cao et al., 2021). Th17 cells are primarily responsible for initiating and stimulating bone resorption, while Treg cells are associated with bone resorption inhibition (Srivastava et al., 2018). *Akkermansia* of the phylum *Verrucomicrobia* is a member of the GM and plays a beneficial role in the prevention of metabolic disorders (Keshavarz Azizi Raftar et al., 2021). *Akkermansia* is also beneficial in the maintenance of bone mass and strength and is likely a critical mediator of child GM-induced anti-osteoporotic effects (Liu et al., 2021). *Akkermansia* can further induce adaptive intestinal immune responses under homeostatic conditions (Ansaldo et al., 2019). Benthe found that supplementation with *Akkermansia* could decrease the presence of B cells in the colon and that mature and immature B cell frequencies in the bone marrow were increased (van der Lugt et al., 2019). B cells are the main source of osteoprotegerin in the bone microenvironment and directly participate in the regulation of bone resorption. Activated B cells overexpress the receptor activator of nuclear factor kappa B ligand (RANKL), which promotes bone resorption (D'Amelio, 2013). Another bacterium, *Roseburia*, can decrease serum levels of proinflammatory cytokines and inhibit the activation of the nucleotide-binding oligomerization segment-like receptor family 3 (NLRP3) inflammasome in murine colitis (Wu et al., 2020). The NLRP3 inflammasome, which is a new target for the prevention and control of osteoporosis, can promote bone resorption and inhibit osteogenesis (Jiang et al., 2021). Overall, DIO can lead to dysbacteriosis in the GM, and it is speculated that osteoporosis induced by different factors may represent various dysbacteriosis. GM dysbiosis may further play a key role in contributing to bone loss in DIO.

Gut-derived bacterial metabolites regulate distant organs, thereby bridging the gap between the GM and skeletal system (Zaiss et al., 2019). Thus, we performed a metabolomic analysis to explore the metabolism in DIO and clarify the pathogenic mechanisms by which GM regulate bone metabolism. Metabolomics, which has traditionally been studied with the aim of identifying biomarkers for the diagnoses and prognoses of various diseases, has been redefined from a simple biomarker identification tool to a technology for the discovery of active drivers of biological processes (Rinschen et al., 2019). Yachida performed fecal metagenomic and metabolomic studies on samples from 616 patients who underwent colonoscopies and found that colorectal cancer progression may be influenced by the metabolic output of the entire microbiota (Yachida et al., 2019). Zhang further used metabolomics profiling and found that bile acid metabolism was impaired following the administration of a high-cholesterol diet. This promoted non-alcoholic fatty liver disease-associated hepatocellular carcinoma development (Zhang et al., 2021). In our study, fecal metabolites in DIO differed significantly from those in the CON group. We also found that bile acid metabolism was abnormally active. Based on these results, we hypothesize that DIO can activate bile acid metabolism and secretion, which are involved in the regulation of bone homeostasis.

Bile acids are important for GM and bone homeostasis. They are synthesized from cholesterol in the liver and form the major components of bile, including primary and secondary bile acids (Long et al., 2017). Bile acids are significantly modified in the gut by bacterial enzymes (Joyce and Gahan, 2016). A mechanistic link between GM composition and host physiology is thought to occur *via* microbiologically-produced secondary bile acids (Pushpass et al., 2021). The functional roles of bile acids as key pleiotropic signaling mediators in metabolism and inflammation have been identified through the discovery of the G protein-coupled bile acid receptor TGR5, farnesoid X receptor, vitamin D receptor (VDR), and pregnane X receptor (Thomas et al., 2008; de Aguiar Vallim et al., 2013). The degree of bile acid receptor activation is largely influenced by the GM (Jones et al., 2014). Vitamin D plays an important role in bone metabolism and the prevention of multifactorial pathologies, including osteoporosis (Marozik et al., 2021). Vitamin D and its active metabolites further participate in bone tissue mineralization, maintaining calcium homeostasis, and bone remodeling, which are mediated through the VDR (Banjabi et al., 2020). Recent research has shown that osteoporosis and vitamin D deficiencies are common complications in patients with chronic liver disease, especially end-stage disease and chronic cholestasis (Assis, 2018; Guañabens and Parés, 2018; Ishizawa et al., 2018). LCA, which is a secondary bile acid produced by intestinal bacteria, acts as an additional physiological VDR ligand (Ishizawa et al., 2018). VDR can be activated by LCA; the degree of activation is largely influenced by the GM (Jones et al., 2014). VDR activation by LCA decreases vitamin D signaling and induces vitamin D insufficiency or deficiency by inducing vitamin D catabolism (Ishizawa et al., 2018). In addition to catabolizing vitamin D, LCA reduces the expression of two other genes, including osteocalcin, which is closely associated with bone formation, and RANKL, which is expressed in osteoblasts and regulates osteoclast formation (Li et al., 2019). The principal physiological effect of vitamin D is in the enhancement of calcium absorption in the upper intestines (Holick, 2007). Vitamin D deficiencies and insufficiencies further cause rickets and osteomalacia and are associated with increased risks of osteoporosis, cancer, autoimmune disease, infection, cardiovascular disease, obesity, and diabetes (Rosen et al., 2012). Interestingly, in the present study, LCA was significantly increased in the DIO group compared to the CON group. Previous studies have further demonstrated the deleterious consequences of LCA and bilirubin on osteoblastic cells (Ruiz-Gaspà et al., 2020a; Ruiz-Gaspà et al., 2020b). Low bone formation due to diminished osteoblast activity is the main

cause of bone loss (Guañabens et al., 1990). Furthermore, we conducted a correlation analysis between metabolites and microbiota to better understand their crosstalk. We found that LCA was correlated with multiple differential bacterial genera including *Erysipelatoclostridium*, *Proteus*, *Akkermansia*, and *Roseburia*. These bacterial genera, which are significantly downregulated in DIO, are closely related to bone metabolism. Therefore, bile acid dysfunction may play a vital role in DIO pathogenesis. LCA may further be a key metabolite in DIO.

Despite our findings, our study had various limitations. Although a correlation was found between GM, fecal metabolites, and DIO, the causal and regulatory relationships among them were not clarified. Furthermore, we identified three metabolic pathways related to amino acids among the top ten pathways apart from those related to bile acid metabolism. This was noteworthy as amino acids are important energy sources in bone remodeling and affect bone resident cells through neuronal and hormonal mechanisms. These findings should therefore be further explored in future research. Our sample size was also small, and high-quality biomarkers require rigorous studies with adequate sample sizes. Therefore, a larger sample size should be used in future studies to identify potential novel biomarkers for DIO.

Conclusion

Our study demonstrated close correlations among GM, fecal metabolites, and DIO. DIO-induced alterations in the abundance and function of GM may represent key factors affecting bone homeostasis. Our findings provide new insights into the pathogenesis of DIO and may ultimately contribute to improving diagnostic and therapeutic options for patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by Shanxi Medical University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YF, ZL, ZT, and XQ contributed to the conception and design of the study. XQ, WW, KZ, RZ, and LY conducted experiments and maintained the animals. XQ, XL, YP, SY, WW, RZ, and LY participated in sample collection and data analysis. CX, PL, and XS gave technical support to the experiment. XQ and ZT uploaded raw data to the NCBI database. XQ drafted the manuscript. ZT, YF, and ZL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

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Gastric microbiota in gastric cancer: Different roles of *Helicobacter pylori* and other microbes

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Gastric cancer (GC) is one of the leading causes of cancer-related deaths worldwide. The gastric microbiota plays a critical role in the development of GC. First, *Helicobacter pylori* (*H. pylori*) infection is considered a major risk factor for GC. However, recent studies based on microbiota sequencing technology have found that non-*H. pylori* microbes also exert effects on gastric carcinogenesis. Following the infection of *H. pylori*, gastric microbiota dysbiosis could be observed; the stomach is dominated by *H. pylori* and the abundances of non-*H. pylori* microbes reduce substantially. Additionally, decreased microbial diversity, alterations in the microbial community structure, negative interactions between *H. pylori* and other microbes, etc. occur, as well. With the progression of gastric lesions, the number of *H. pylori* decreases and the number of non-*H. pylori* microbes increases correspondingly. Notably, *H. pylori* and non-*H. pylori* microbes show different roles in different stages of gastric carcinogenesis. In the present mini-review, we provide an overview of the recent findings regarding the role of the gastric microbiota, including the *H. pylori* and non-*H. pylori* microbes, in the development of GC.

KEYWORDS

gastric cancer, gastric microbiota, *Helicobacter pylori*, carcinogenesis, dysbiosis

Introduction

Gastric cancer (GC) is one of the leading causes of cancer-related deaths worldwide, ranking fifth in incidence and third in mortality of cancers (Bray et al., 2018). According to World Health Organization International Agency for Research on Cancer (WHO-IARC), the annual burden of GC will increase to approximately 1.8 million new cases and

1.3 million deaths by 2040. Compared with those in 2020, the numbers of new cases and deaths will increase by approximately 63% and 66%, respectively (Morgan et al., 2022). *Helicobacter pylori* (*H. pylori*) infection is a critical risk factor for GC (Amieva and Peek, 2016) and *H. pylori* was classified by the WHO-IARC as a type I carcinogen (WHO-IARC, 1994). In recent years, sequencing-based studies focusing on microbiota have shown that patients with GC have gastric microbiota dysbiosis, including reduced microbial diversity, altered microbial community structure, altered compositions, and abnormal bacterial interactions (Gantuya et al., 2020; Kadeerhan et al., 2021). Furthermore, non-*H. pylori* microbes might also promote gastric lesions and even GC (Coker et al., 2017; Yu et al., 2017; Ferreira et al., 2018; Kadeerhan et al., 2021). The interactions between *H. pylori* and other microbes may be also involved in gastric carcinogenesis.

In the present mini-review, we aim to discuss the recent findings regarding the role of gastric microbiota, including *H. pylori* and non-*H. pylori* microbes, in the development of GC.

H. pylori infection, eradication, and GC

H. pylori is a gram-negative, flagellated, microaerophilic bacterium belonging to the *Campylobacterota* phylum, which was first identified in 1982 (Warren and Marshall, 1983). *H. pylori* colonizes in the stomach and becomes the predominant microbe in stomach after infection (Schulz et al., 2018). In terms of the global epidemiology of *H. pylori* infection, according to a global meta-analysis (Hooi et al., 2017), there were about 4.4 billion *H. pylori*-positive cases worldwide in 2015. The prevalence rate of *H. pylori* infection varied by region, with the highest prevalence rate in Africa (70.1%, 95% CI: 62.6-77.7%) and the lowest prevalence rate in Oceania (24.4%, 95% CI: 18.5-30.4%). Furthermore, for the temporal trend of *H. pylori* infection, the prevalence in different regions is stable or decreasing, especially in the developed world and in children (Burucoa and Axon, 2017; Hooi et al., 2017).

H. pylori infection is considered a major risk factor for gastric carcinogenesis. Overall, a large-scale pooled analysis of case-control studies nested within prospective cohorts showed that *H. pylori* infection was associated with nearly six-fold increased risk of non-cardia cancer (Helicobacter and Cancer Collaborative Group, 2001). The mechanism that *H. pylori* induces GC has been explored (Ishaq and Nunn, 2015; Talebi Bezmin Abadi, 2016). First, *H. pylori* primarily triggers the transition from normal mucosa to non-atrophic gastritis and then initiates precancerous lesions (Díaz et al., 2018). The responses after infection are mainly mediated through the action of bacterial virulence factors, including cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), and other outer membrane proteins (Díaz et al., 2018; Alipour, 2021). CagA has multiple effects on epithelial cells, including stimulating cell proliferation, reducing epithelial cell apoptosis, etc. (Saadat et al., 2007; Nagy et al., 2009; Buti et al., 2011). Additionally, inflammatory cells can be recruited and oxygen species-induced damage can be induced after CagA and the type IV secretion system (T4SS) activate the inflammatory signaling (Viala et al., 2004; Chaturvedi et al., 2011). VacA can also cause alterations of cells, such as vacuolization and promoting immune regulation (Willhite et al., 2003; Yang et al., 2022). Further, the urease production by *H. pylori* and the glandular atrophy induced by *H. pylori* infection lead to reduced acid production and shifts in gastric pH value. As a result, the bacterial colonization environment in the stomach changes and gastric microbiota dysbiosis may occur (Schulz et al., 2015; Noto and Peek, 2017). The above-mentioned effects promote GC development.

For *H. pylori*-positive cases, eradication therapy could be given (Fallone et al., 2016; Malfertheiner et al., 2017; Liu et al., 2018). The effect of *H. pylori* eradication therapy on the GC risk has been evaluated. You et al. reported that, based on a randomized trial with a follow-up of 7.3 years, *H. pylori* treatment resulted in statistically significant decreases in the combined prevalence of severe chronic atrophic gastritis, intestinal metaplasia, dysplasia, or GC (OR = 0.77, 95% CI: 0.62-0.95) (You et al., 2006). With a follow-up of 22 years for this randomized trial, this team found that the protective effect of *H pylori* treatment on GC incidence (OR= 0.48, 95% CI: 0.32-0.71) and GC death (HR= 0.62, 95% CI: 0.39-0.99) persisted 22 years post-intervention (Li et al., 2019). Additionally, a recent well-designed meta-analysis enrolling randomized controlled trials (RCTs) with 10 or more years of follow-up found that the GC incidence decreased significantly with *H. pylori* eradication therapy (RR=0.54, 95% CI: 0.41-0.72); on the other hand, eradication of *H. pylori* showed significant reductions in GC mortality (RR=0.66, 95% CI: 0.46-0.95) (Ford et al., 2022).

H. pylori associated gastric microbiota dysbiosis

The gastrointestinal microbiota refers to microorganisms lived in the gastrointestinal tracts, which is critical to many aspects of human health (Clemente et al., 2012; Valdes et al., 2018). For human immune, the microbiota is key to the induction, training, and function of the host immune system (Belkaid and Hand, 2014; Ling et al., 2022). Regarding the gastric microbiota, due to the high acidity of the stomach, the human stomach was once assumed to be a sterile organ (Espinoza et al., 2018). However, *H. pylori* is able to colonize the human gastric mucosa and survive in the highly acidic environment of the stomach (Schulz et al., 2015). With the advent of novel techniques for analyzing the microbial community, the unique features of the gastric microbiota have been identified that the

major microbes in the healthy human stomach environment are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, and *Proteobacteria* (Guo et al., 2020; Guo et al., 2021).

For *H. pylori*-infected individuals, the stomach is dominated by *H. pylori* and accordingly, the abundances of non-*H. pylori* microbes reduce substantially (Brawner et al., 2017; Das et al., 2017). In addition to the changes of microbial composition, other phenomena of gastric microbiota dysbiosis have also been found. For the microbial alpha diversity, Gantuya et al. reported that individuals infected with *H. pylori* showed significant decreased microbial diversity compared with *H. pylori*-negative individuals (Gantuya et al., 2019). Another study found that there was a negative association between the gastric microbiome diversity and *Helicobacter* abundance (Das et al., 2017). In addition to microbial alpha diversity, infection with *H. pylori* results in alterations of the microbial community structure (beta diversity). According to a population-based study, the *H. pylori* positive group and negative group were clearly separated according to beta diversity (Llorca et al., 2017). Furthermore, studies focusing on the microbial ecological interactions found shifts of the interactions between *H. pylori* and other microbes in the stomach environment. In detail, according to an Indian study using16S rRNA gene sequencing, the network analyses showed that *Helicobacter* had negative interactions with other microbes of the gastric microbiome (Das et al., 2017); another Chinese study reported similar findings (Guo et al., 2020). Regarding the numbers of interactions, Coker et al. found that *H. pylori* infection reduces the number of gastric microbiome interactions (Coker et al., 2017). However, all the above-mentioned findings were based on statistical analyses of sequencing data. Thus, we need more clinical data supporting current presented concept (Rivas-Ortiz et al., 2017).

For *H. pylori*-positive individuals, the *H. pylori* eradication could reverse gastric microbiota dysbiosis and exert beneficial effects on the gastric microbiota (Guo et al., 2022). Firstly, for the reduced gastric microbial diversity among *H. pylori*-positive cases, the diversity could increase significantly after successful eradication of *H. pylori* (Guo et al., 2020; Mao et al., 2021). Also, significant differences were observed for the microbial community structure (the beta diversity) following eradication (Guo et al., 2020; Sung et al., 2020b; Mao et al., 2021; Watanabe et al., 2021; Yuan et al., 2021). For the gastric microbiota composition, after removing *H. pylori* in the stomach environment, the gastric commonly dominant commensals are enriched (Guo et al., 2020; Shin et al., 2020). Different changes of specific microbes were reported, which may be resulted from different population, sequence methods, and sampling details. The common reported commensals included *Firmicutes*, *Streptococcus, Prevotella.*, etc. (He et al., 2019; Guo et al., 2020; Mao et al., 2021; Watanabe et al., 2021; Yuan et al., 2021). In terms of interactions between gastric commensal bacteria, a reduction in these interactions was reported after eradication of *H. pylori* (Sung et al., 2020b; Yuan et al., 2021), which were also based on statistical analyses of sequencing data and required further validation. Moreover, due to the development of bioinformatics, microbiota function could be predicted and analyzed. According to the bioinformatic analysis of functional capacity, the bacteria reproduction-related pathways are down-regulated and pathways of gastric acid secretion, etc. are up-regulated (He et al., 2019; Guo et al., 2020), indicating beneficial effect of eradication on the recovery of gastric microbiota. In combination with the prevention effect of *H. pylori* eradication on GC, the alterations in gastric microbiota after eradication may contribute to the reduction in GC risk; further studies with long-term follow-up are needed (Guo et al., 2022).

The overall features of the gastric microbiota associated with GC

In recent years, the characterization of the gastric microbiota associated with GC has been identified, indicating that gastric microbiota dysbiosis occur in gastric carcinogenesis (Yang et al., 2021). In the year of 2009, the team of Prof. Engstrand compared the gastric microbiota of patients with GC and controls using the terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA gene cloning and sequencing. They found that diversity indices of GC microbiota were not significantly different from that in controls according to the T-RFLP. In terms of gastric microbiota composition of GC, the abundance of *H. pylori* was low and the GC microbiota was dominated by the following genera: *Streptococcus*, *Lactobacillus*, *Veillonella* and *Prevotella* (Dicksved et al., 2009). However, the sample size of this study was small (only ten patients and five controls); additionally, 16S rRNA sequencing technology and related procedures are not yet developed and extensively used, therefore this work is an initial investigation of this field.

In following decade, other findings have been reported. Firstly, the gastric microbial diversity alteration in GC has been the most focused topic. Several studies reported that compared with the gastritis status, gastric microbial diversity is significantly reduced; analyses showed that the microbial community structure (beta diversity) is significantly altered in GC patients (Coker et al., 2017; Ferreira et al., 2018). Similarly, according to studies based on comparison between GC tissues and non-cancerous tissues, GC tissues also have reduced diversity and shifted microbiota structure (Chen et al., 2019). However, the conclusions are inconsistent across studies. For instants, two studies showed that the alpha diversity of GC gastric microbiota was increased (Eun et al., 2014; Linz et al., 2017). The difference of results may be caused by different populations, sampling sites and stage of gastric disease.

In addition to microbial diversity analysis, with the development of bioinformatics, more in-depth analysis methods have been developed and used. The function prediction analyses have been applied to explore potential

mechanisms of gastric carcinogenesis. The most studies did function prediction analyses using PICRUSt (Langille et al., 2013). Ferreira et al. identified the presence of a nitrosating microbial community in GC cases, indicating that nitrate-reducing bacteria may contribute to gastric carcinogenesis (Ferreira et al., 2018). Meanwhile, a switch towards purine metabolism, D-alanine metabolism, drug metabolism, etc. in GC were reported in another study (Coker et al., 2017). These findings suggested that the microorganisms in the stomach may contribute to the development of GC through specific functional effects. Similarly, these findings need further validation of mechanisms.

The non-*H. pylori* microbes associated with GC

In addition to *H. pylori*, more and more studies have been focusing on other non-*H. pylori* gastric microorganisms. Similar to the bacterial driver-passenger model in the development of colorectal cancer (Tjalsma et al., 2012), the hypothesis of GC has been proposed that: *H. pylori*, as the "driver", causes pathological changes of gastric mucosa and dysbiosis of gastric microbiota; with the progression of gastric lesions, the number of *H. pylori* decreases and the number of other microorganisms in the stomach, i.e. non-*H. pylori* microbes as the "passengers", increases correspondingly. These non-*H. pylori* microbes play an important role in the pathogenesis of GC.

The above hypothesis has been confirmed in animal research. An animal study using hypergastrinemic insulin-gastrin (INS-GAS) transgenic mice found that compared with the specific pathogen free (SPF) INS-GAS mice, the duration of gastric lesions development was longer for germ-free INS-GAS mice; compared with INS-GAS mice infected with *H. pylori* only, INS-GAS mice with complex gastric microbiota had more severe gastric lesions and an earlier onset of gastrointestinal intraepithelial neoplasia (Lofgren et al., 2011). Another INS-GAS mice-based study reported that INS-GAS mice coinfected with *H. pylori* and other intestinal bacteria had a higher rate of development of gastrointestinal intraepithelial neoplasia than those infected with *H. pylori* alone (Lertpiriyapong et al., 2014). These findings indicate the potential role of non-*H. pylori* microbes and the interactions between *H. pylori* and non-*H. pylori* microbes in gastric carcinogenesis.

More researchers are paying attention to human studies as the hypothesis is supported in animal studies. In a population-based study using the 16S rRNA gene sequencing method, compared with individuals with gastritis, GC showed gastric microbiota dysbiosis and a lower abundance of *Helicobacter* and the over-representation of intestinal commensals was seen in GC gastric microbiota. In detail, 16 enriched taxa and 13 depleted taxa in GC according to the LEfSe analysis (Ferreira et al., 2018). Another study comparing gastric microbiota of GC patients and superficial gastritis reported that 21 bacterial taxa were enriched in GC and 10 bacterial taxa were depleted in GC. Specifically, enrichment of oral microbes was observed in the stomach of GC (Coker et al., 2017). In addition to above two cross-sectional studies, a cohort study with a 4-year follow-up reported that *Helicobacter* abundance was lower in the subjects with progression of gastric lesions compared with non-progression group. Specifically, the remarkable decline in *Helicobacter* was observed after the progression to stage of dysplasia/GC compared with non-progression controls (Kadeerhan et al., 2021). The key non-*H. pylori* microbes associated with GC are summarized in Table 1. However, inconsistent results were found, necessitating additional validations.

Furthermore, based on the current findings, a panel of differential gastric bacteria can be developed to distinguish GC and the progression of GC with outstanding performance. A recently published meta-analysis, which enrolled six independent studies, reported that eight bacterial taxa could serve as a panel of biomarkers to discriminate GC from superficial gastritis with an area under the curve (AUC) of 0.850 (Liu et al., 2022). Regarding the progression of GC, Kadeerhan et al. reported a combination of four genera (*Bacillus*, *Capnocytophaga*, *Helicobater*, *Prevotella*) with age and sex to distinguish subjects after lesion progression from non-progression controls (AUC = 0.927) (Kadeerhan et al., 2021). In addition to a panel of bacteria, a new single index called Microbial Dysbiosis Index (MDI) has been presented. MDI is calculated by log (total abundance of genera increased in GC/total abundance of genera decreased in GC); a higher value of MDI means a higher risk of GC. The application of MDI has been applied in the evaluation of GC: the GC gastric microbiota had a higher MDI and the findings were confirmed in the validation cohorts (Ferreira et al., 2018).

The different roles of *H. pylori* and non-*H. pylori* microbes in gastric carcinogenesis

The progression of gastric carcinogenesis is detailed in Figure 1. Like bacterial driver-passenger model of colorectal cancer, the development of GC showed similar change pattern of gastric microbiota. Thus, *H. pylori* and non-*H. pylori* microbes show different roles in different stages of gastric carcinogenesis. First of all, the load of *H. pylori* in the stomach increases after the initial infection, especially in the active gastritis stage (Stewart et al., 2020). Interestingly, the *H. pylori* load decreases with the progression of gastric lesions. A population-based study showed that a lower *Helicobacter* abundance was observed in subjects with the progression of gastric lesions (Kadeerhan et al., 2021); another study reported that the abundance of *Helicobacter* was

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TABLE 1 Key non-*H. pylori* microbes associated with gastric cancer.

PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcacaeae*	*Lactococcus*: potential harmful microbes for gastric mucosa (Coker et al., 2017; Ferreira et al., 2018; Hsieh et al., 2018)	*Lactococcus lactis*: potential beneficial microbes for gastric mucosa (Chen et al., 2019)
				Streptococcus: potential harmful microbes for gastric mucosa (Chen et al., 2019; Liu et al., 2019); also reported as potential beneficial microbes for gastric mucosa (Ferreira et al., 2018)	*Streptococcus anginosus*: potential harmful microbes for gastric mucosa (Coker et al., 2017; Liu et al., 2019) *Streptococcus infantis*: potential harmful microbes for gastric mucosa (Coker et al., 2017)
		Bacillales	*Bacillaceae*	*Bacillus*: potential harmful microbes for gastric mucosa (Kadeerhan et al., 2021)	
		Lactobacillales	*Lactobacillaceae*	*Lactobacillus*: potential harmful microbes for gastric mucosa (Ferreira et al., 2018; Hsieh et al., 2018)	*Lactobacillus brevis*: potential beneficial microbes for gastric mucosa (Chen et al., 2019) *Lactobacillus salivarius*: potential harmful microbes for gastric mucosa (Coker et al., 2017) *Lactobacillus fermentum*: potential harmful microbes for gastric mucosa (Coker et al., 2017)
	Clostridia	*Clostridiales*	*Clostridiaceae*	*Clostridium*: potential harmful microbes for gastric mucosa (Ferreira et al., 2018; Hsieh et al., 2018)	
Bacteroidetes	*Bacteroidetes*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*: potential harmful microbes for gastric mucosa (Chen et al., 2019; Sung et al., 2020a; Kadeerhan et al., 2021); also reported as potential beneficial microbes for gastric mucosa (Ferreira et al., 2018; Gantuya et al., 2020)	*Prevotella melaninogenica*: potential harmful microbes for gastric mucosa (Liu et al., 2019) *Prevotella oris*: potential harmful microbes for gastric mucosa (Coker et al., 2017) *Prevotella intermedia*: potential harmful microbes for gastric mucosa (Coker et al., 2017)
Proteobacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*	*Neisseria*: potential beneficial microbes for gastric mucosa (Ferreira et al., 2018)	
Fusobacteria	*Fusobacteria*	*Fusobacterales*	*Fusobacteriaceae*	*Fusobacterium:* potential harmful microbes for gastric mucosa (Coker et al., 2017; Hsieh et al., 2018; Chen et al., 2019)	*Fusobacterium nucleatum*: potential harmful microbes for gastric mucosa (Coker et al., 2017)

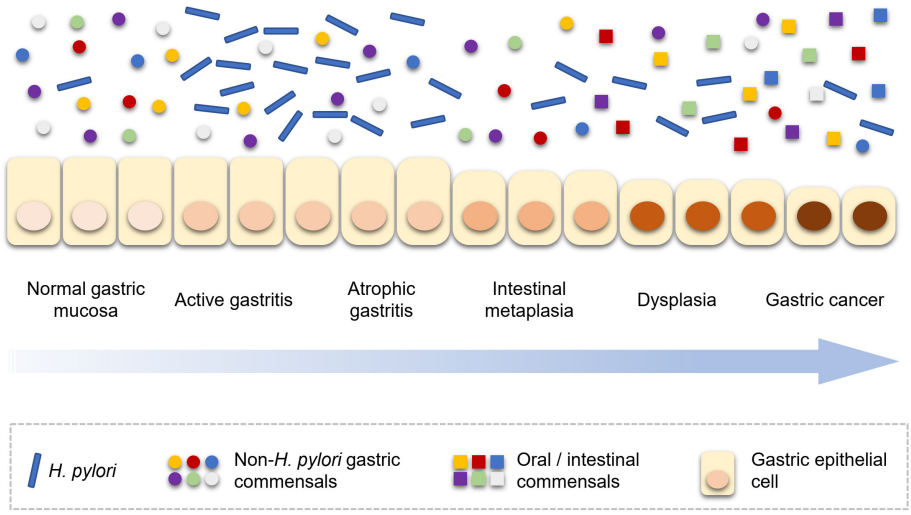

FIGURE 1
H. pylori and non-*H. pylori* microbes in the development of gastric carcinogenesis. *H. pylori*, *Helicobacter pylori*.

substantially lower in GC patients than gastritis (Ferreira et al., 2018). This phenomenon could be explained that, following *H. pylori* infection, due to the persistence of inflammation and the loss of acid-secreting parietal cells, the gastric environment becomes more favorable for the colonization of other bacteria and progression of lesions are accelerated (Polk and Peek, 2010). In detail, with the development of gastric lesions, oral or intestinal commensal microbes are enriched (Coker et al., 2017; Ferreira et al., 2018; Stewart et al., 2020). However, by the late stage of gastric precancerous lesions, the stomach environment is no longer suitable for *H. pylori* and the abundance *H. pylori* of decreases. This phenomenon has been confirmed in human studies (Ferreira et al., 2018; Kadeerhan et al., 2021). The key roles of *H. pylori* in different stages of gastric carcinogenesis were shown in the Table 2. In addition to the overall description of the progression of gastric carcinogenesis, the roles of certain bacteria remain to be clarified and further mechanism investigation is needed for a deeper understanding of this issue.

Future perspectives

Non-*H. pylori* microbes and their interactions may also play a critical role in the development of GC. However, inconsistent findings were reported for non-*H. pylori* microbes associated with GC. Accordingly, further mechanism investigation is needed to validate these potential GC-associated non-*H. pylori* microbes, such as animal studies. Additionally, most human studies are case-control studies, which compared gastric microbiota of gastric mucosa between GC patients and control population. Due to this study design, we cannot infer a causal relationship between gastric microbiota dysbiosis and development and progression of GC. In other words, it is

TABLE 2 Key roles of *H. pylori* in gastric carcinogenesis.

Stages in the development of GC	Descriptions
Uninfected stage	• The major microbes in the healthy human stomach environment are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, and *Proteobacteria* (Guo et al., 2020; Guo et al., 2021) • The abundance of *H. pylori* in the gastric microbiota of uninfected status is low (Guo et al., 2020; Guo et al., 2021).
H. pylori-dependent stage	• *H. pylori*, as the "driver", causes pathological changes of gastric mucosa and dysbiosis of gastric microbiota. • After *H. pylori* infection, the stomach is dominated by *H. pylori* and accordingly, the abundances of non-*H.pylori* gastric commensals reduce substantially (Brawner et al., 2017; Das et al., 2017). • *H. pylori* associated gastric microbiota dysbiosis includes: decreased microbial diversity, alterations in the microbial community structure, negative interactions between *H. pylori* and other microbes, etc. (Das et al., 2017; Llorca et al., 2017; Gantuya et al., 2019).
H. pylori-independent stage	• With the progression of gastric lesions, the number of *H. pylori* decreases and the number of non-*H. pylori* microbes, as the "passengers", increases correspondingly. • The "passengers" are considered oral or intestinal commensal microbes (Coker et al., 2017; Ferreira et al., 2018; Stewart et al., 2020).

unclear whether gastric microbiota dysbiosis causes GC or whether GC causes gastric microbiota dysbiosis. Therefore, cohort studies with long-term follow-up are needed to confirm the major findings.

Author contributions

YG drafted the manuscript, conceptualized the idea, and revised the manuscript. X-SC and M-GZ performed the literature search and revised the manuscript. YG and M-GZ contributed to drawing the figure. BY critically revised the manuscript and supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Lactobacillus for the treatment and prevention of atopic dermatitis: Clinical and experimental evidence

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Atopic dermatitis (AD) is a chronic inflammatory skin disease, accompanied by itching and swelling. The main pathological mechanism of AD is related to the imbalance between Type 2 helper cells (Th2 cells) and Type 1 helper cells (Th1 cells). Currently, no safe and effective means to treat and prevent AD are available; moreover, some treatments have side effects. Probiotics, such as some strains of *Lactobacillus*, can address these concerns *via* various pathways: i) facilitating high patient compliance; ii) regulating Th1/Th2 balance, increasing IL-10 secretion, and reducing inflammatory cytokines; iii) accelerating the maturation of the immune system, maintaining intestinal homeostasis, and improving gut microbiota; and iv) improving the symptoms of AD. This review describes the treatment and prevention of AD using 13 species of *Lactobacillus*. AD is commonly observed in children. Therefore, the review includes a higher proportion of studies on AD in children and fewer in adolescents and adults. However, there are also some strains that do not improve the symptoms of AD and even worsen allergies in children. In addition, a subset of the genus *Lactobacillus* that can prevent and relieve AD has been identified *in vitro*. Therefore, future studies should include more *in vivo* studies and randomized controlled clinical trials. Given the advantages and disadvantages mentioned above, further research in this area is urgently required.

KEYWORDS

atopic dermatitis, *Lactobacillus*, type 2 helper cells, gut microbiota, immunomodulation

1 Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease, and patients frequently experience complications from concurrent allergic conditions. The annual incidence of AD has increased in the recent years, particularly in children. Based on the Finnish national database, the prevalence of AD varies by age group, with the highest prevalence in the age group 0-14 years (47.46%), followed by that in 15-60 year olds (43.74%) (Salava et al., 2022).

The actual prevalence of AD between 6 months and 12 years has been reported at 11% in Israel (Weil et al., 2022). One report showed that the incidence of AD in infants aged 3 months in China was 40.81% (Guo et al., 2019). Patient quality of life can be severely affected, as AD causes scratching, itching, and a rash. The financial burden on households increases with disease severity (Weil et al., 2022). The incidence of AD is strongly correlated with heredity and environment (Figure 1). In other words, people with AD often have a family history of AD, and the incidence of AD may increase when the father or mother has a particular allergy. Although the exact mechanisms of AD have not yet been elucidated, impaired immunological function and dysregulation of the skin barrier are considered to be the primary pathogenic mechanisms (Yang et al., 2020). Concurrently, environmental factors such as poor eating habits, sudden lifestyle changes, and certain allergenic stimuli are also associated with AD. A climatic conditions closely associated with an elevated risk of AD is low vapor pressure (Yokomichi et al., 2022). In Chongqing, China, infants exposed to polluted air are at an increased risk of developing AD (Luo et al., 2022). Psychological factors also play an important role in AD development; subsequently, AD leads to fluctuating depressive symptoms (Chatrath et al., 2022). As a result, individuals with AD can be caught in a vicious cycle.

Although the pathogenesis of AD is not clear, decades of research indicate that the mechanisms of AD can potentially be attributed to genetic factors, environmental exposures, impaired skin barrier, abnormal immune function, and microbial imbalances. This suggests that AD is a systemic organ-related allergic disease. One study demonstrated an increased probability of early AD in maternal offspring due to dysregulated interferon signaling cascade (Schedel et al., 2023). Patients with high-risk genes tend to develop AD earlier (Hikino et al., 2022). A notable manifestation of AD is pruritus, which causes patients to scratch vigorously (Yosipovitch et al., 2020; Steinhoff et al., 2022). After skin rupture, the epidermal barrier is damaged, and antigens penetrate the skin, producing chemokines and inflammatory mediators (IL-25, IL-33, and thymic stromal lymphopoietin). Th cells polarize to Th2 and produce IL-4, IL-13, and IL-5 (Cosmi et al., 2019; Renert-Yuval and Guttman-Yassky, 2020). Th1, Th17, Th22, and other pathways are activated; a variety of cytokines and growth factors involved in inflammatory immune responses through the Janus kinase (JAK) pathway are produced and enhance Th2 cell differentiation (Bao et al., 2013; Rerknimitr et al., 2017). Scratching coupled with endogenous and other exogenous triggers, such as histamine, proteases, substance P, various interleukins, and environmental allergens leads to keratinocyte activation, and intensified skin inflammation. Inflammatory mediators and multi-pathway inflammation cause intensive scratching and further damage to the skin barrier. Moreover, bacteria take advantage of the situation and the vicious

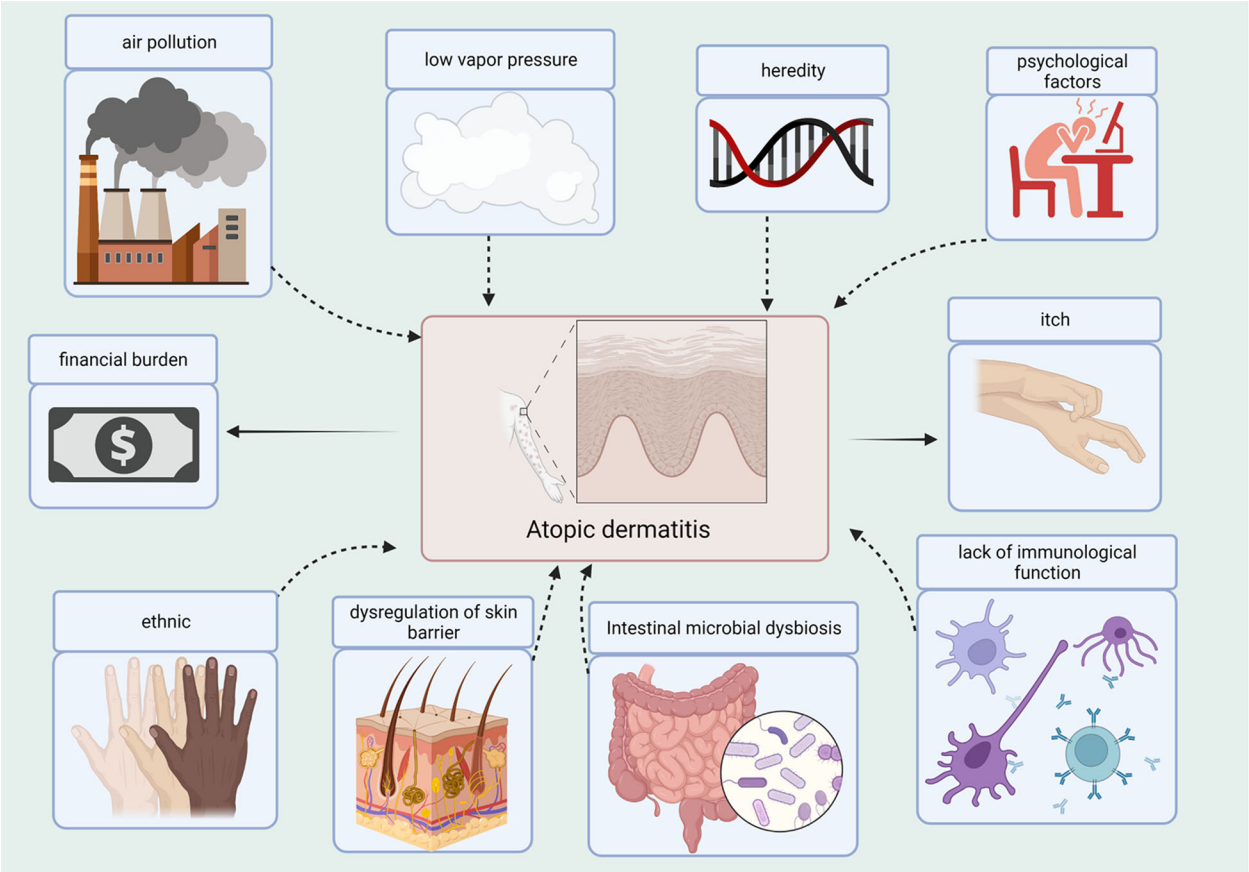

FIGURE 1
Causes and Consequences of the AD. Several factors contribute to the development of AD, such as air pollution, low vapor pressure, heredity, psychological factors, ethnicity, dysregulation of the skin barrier, dysbiosis of gut microbes, and lack of immune function. AD contributes to the itchiness and financial burden.

circle continues. Under physiological conditions, the skin microenvironment maintains immune homeostasis and reduces skin colonization by pathogenic bacteria. Diversity of the gut microbiome is significantly lower in infants with AD (Abrahamsson et al., 2012; Penders et al., 2013). The presence of *Staphylococcus aureus* was detected on the skin of 90% of patients with AD, and this pathogen can lead to disease progression (Powers et al., 2015; Blicharz et al., 2019). *Staphylococcus aureus* secretes staphylococcal enterotoxins A, B, and C and toxic shock syndrome toxin 1 (TSST-1), which activate lymphocytes and macrophages (Nakatsuji and Gallo, 2019). In addition, staphylococcal enterotoxin B promotes the expression of IL-31. IL-31 inhibits the expression of polyfilament proteins and antimicrobial peptides, which favor *Staphylococcus aureus*. Importantly, IL-31 is a key factor in itching (Meng et al., 2018). Previous studies showed that mediators produced by *Staphylococcus aureus* promotes adhesion, colonization, and spread to the skin. These mechanisms are complex and interact. In future, scientists may also identify new mechanisms that are yet to be discovered.

The gut plays an important role in the immune response. At the same time, Natural killer (NK) T cells, innate lymphoid cells, and intestinal flora regulate each other to maintain intestinal homeostasis and normal immune function (Cairo and Webb, 2022). In addition, healthy gut flora has a protective effect against food allergies (Méndez et al., 2021). Disturbances of the intestinal microbiome in early infancy worsen immune dysfunction in children with AD (Lee et al., 2022). In addition, a study showed that transplanting fecal microbiota to restore gut ecology provides a new method for treating AD (Kim et al., 2021). Lower microbial diversity has been associated with a higher incidence of AD (Galazzo et al., 2020). Moreover, greater severity of clinical manifestations in patients with AD has been associated with a lower number of *Bifidobacteria* in the intestine (Watanabe et al., 2003). Conversely, higher amounts of pathogenic *Clostridium difficile* have been detected in the stool of patients with AD (Penders et al., 2007). In one region of Brazil, children with AD have a higher prevalence of *Clostridium difficile* and a lower abundance of *Lactobacillus* (Melli et al., 2020). *Clostridium difficile* causes a decrease in beneficial bacteria, loss of immune function, and increased intestinal permeability. Studies have shown that colonization of the gut flora precedes AD changes (Galazzo et al., 2020); therefore, a timely intervention in the gut microbiota could be a promising preventive approach. Diet has an important impact on the colonization of gut microbes in early infancy (Galazzo et al., 2020). In early infancy, microbes are primarily affected by type of delivery; however, food becomes an important factor starting at 13 weeks (Galazzo et al., 2020). Food allergies and AD are closely related, and approximately one-third of children have both AD and food allergies (Hui-Beckman et al., 2023). Food-induced AD most likely occurs in children with severe AD (Robison and Singh, 2019). Food allergies increase the permeability of the intestine, making it easier for allergens to trigger the submucosal immune system through the intestinal barrier (Lee et al., 2013; Gao et al., 2021). Cytokines and inflammatory mediators produced after the activation of the immune system further increase intestinal permeability. The interaction between the gut microbiota and skin has been called the gut-skin axis by some authors (Mahmud et al., 2022; Varela-Trinidad et al., 2022). Healthy gut flora is beneficial for healthy skin.

Various treatments have been used in AD, including topical glucocorticoids and immunosuppressive agents, phototherapy, and narrow-spectrum ultraviolet radiation B. AD is a chronic inflammatory disease that affects patients with impaired skin barrier function. The long-term administration of topical corticosteroids carries a high risk. Topical corticosteroids are the mainstream treatment for moderate to severe AD; however, they have side effects, such as hormonal dermatitis, when used in large, long-term doses in combination with potent hormonal creams. In addition, prolonged use of topical corticosteroids may cause serious side effects, such as adrenal insufficiency (Böckle et al., 2014). Additionally, patients hesitate to use glucocorticoids (Maghen et al., 2019). Immunosuppressant treatment for AD may cause conjunctivitis (Wollenberg et al., 2018) or lymphopenia (Bakker et al., 2018) in some patients. Narrow-spectrum ultraviolet radiation B has a relieving effect on AD (Ben Mordehai et al., 2022); however, long-term use may cause abnormal skin reactions. Moreover, some biological drugs are expensive and have side effects (Puar et al., 2021). Patients receiving dupilumab treatment were reported to suffer from eye discomfort (Miniotti et al., 2022). Owing to the above reasons, safe and effective treatments for AD remain limited. Recently, treatment with probiotics has been proposed to regulate the gut microbiome in AD. The gut microbiome plays an essential role in the maintenance of host homeostasis and immunomodulation. Imbalances in microbial flora can contribute to many diseases. Intestinal microbial dysbiosis is the leading cause of AD-like symptoms (Kim et al., 2020a). Oral administration of *L. sakei* proBio65 (Rather et al., 2021) and *L. salivarius* LS01 can improve the quality of life of children (Niccoli et al., 2014) and adults (Drago et al., 2011) with AD. In an experimental model, maternal mice and their offspring orally supplemented with *L. reuteri* Fn041 maintained the balance of the immune response to prevent AD (Qi et al., 2022; Zhao et al., 2022; Zhou et al., 2022).

Lactobacillus is a naturally occurring rod-shaped bacterium that is a part of the normal flora in some organs of humans, animals, and plants. The storage conditions for *Lactobacillus* are simple. *L. sakei* proBio65 live and inactivated bacteria can improve AD symptoms and enhance the function of skin barrier (Jeong et al., 2020a; Rather et al., 2021). Administration *L. paracasei* KBL382 alleviated AD by modulating immune responses (Kim et al., 2020a). *L. paracasei* KBL382 reduced serum levels of immunoglobulin E (IgE) and immune cell infiltration (Kim et al., 2020a). Moreover, supplementation with *L. rhamnosus* HN001 substantially reduced the cumulative prevalence of AD (Wickens et al., 2008). These findings suggest that *Lactobacillus* may provide an alternative strategy for the treatment and prevention of AD. In this review, we focus on the role of *Lactobacillus* as a novel therapeutic agent for AD.

2 Classification of *Lactobacillus* and its mechanism of prevention and treatment of AD

Probiotics are living microorganisms such as *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bacillus cereus* spp., and *Saccharomyces*

boulardii that benefit the host. *Lactobacillus* spp. is the most widely used probiotic microorganism. The genus *Lactobacillus* includes more than 200 species (Sun et al., 2015), and can be subdivided into at least 24 phylogenetic groups (Zheng et al., 2015). Several *Lactobacillus* species have been studied for AD prevention and treatment (Figure 2), including *L. rhamnosus*, *L. plantarum*, *L. acidophilus*, *L. sakei*, *L. reuteri*, *L. salivarius*, *L. paracasei*, *L. casei*, *L. delbrueckii*, *L. fermentum*, *L. johnsonii*, *L. pentosus*, and *L. brevis*. These *Lactobacillus* species have been reported to produce a variety of substances, such as organic acids, hydrogen peroxide, low-molecular-weight antimicrobials, bacteriocins, and adhesion inhibitors. *Lactobacillus* products stimulate innate immunity and promote balanced microbial communities through the competitive rejection and antimicrobial activity against pathogenic bacteria (Goldstein et al., 2015). Administration of *Lactobacillus* decreased the serum levels of IgE (Sunada et al., 2008; Wakabayashi et al., 2008; Tanaka et al., 2009; Prakoeswa et al., 2017), and achieved a balance of Th1/Th2 (Wickens et al., 2008; Kwon et al., 2018; Kim et al., 2019a; Zhao et al., 2022). Moreover, the intestinal barrier (Wickens et al., 2008; Kim et al., 2020a; Zhao et al., 2022), immune function (Wickens et al., 2008; Kim et al., 2019a; Kim et al., 2020a) and skin barrier (Mariman et al., 2016) have been improved after the administration of *Lactobacillus*. The mechanisms of the 13 kinds of *Lactobacillus* are listed in Table 1. *Lactobacillus* shows certain effects on both animals and humans with AD (Tables 2, 3). *Lactobacillus* has high economic value in biotechnology, food production, and therapeutic applications.

3 Monostrain *Lactobacillus* in the treatment and prevention of AD

3.1 *Lactobacillus rhamnosus* -effective prevention and treatment of AD in both animal and clinical experiments

L. rhamnosus is the most studied species of Lactobacillus (Petrova et al., 2021). The peptidoglycan of *L. rhamnosus* CRL1505 can regulate the immune function of human intestinal epithelial and dendritic cells (Salva et al., 2021). Early exposure to LGG in dogs with AD had long-term immune effects and significantly reduced allergen-specific IgE despite the lack of a clear clinical effect (Marsella, 2009; Marsella et al., 2012). In clinical trials, infants aged 0–2 years had a reduced incidence of atopic eczema when their mothers have been administered LGG during pregnancy (Kalliomäki et al., 2003). The preventive effect of LGG extended to 4 years (Kalliomäki et al., 2003). LGG may enhance the gut barrier function and promote immune response development in infants with AD (Nermes et al., 2011). In

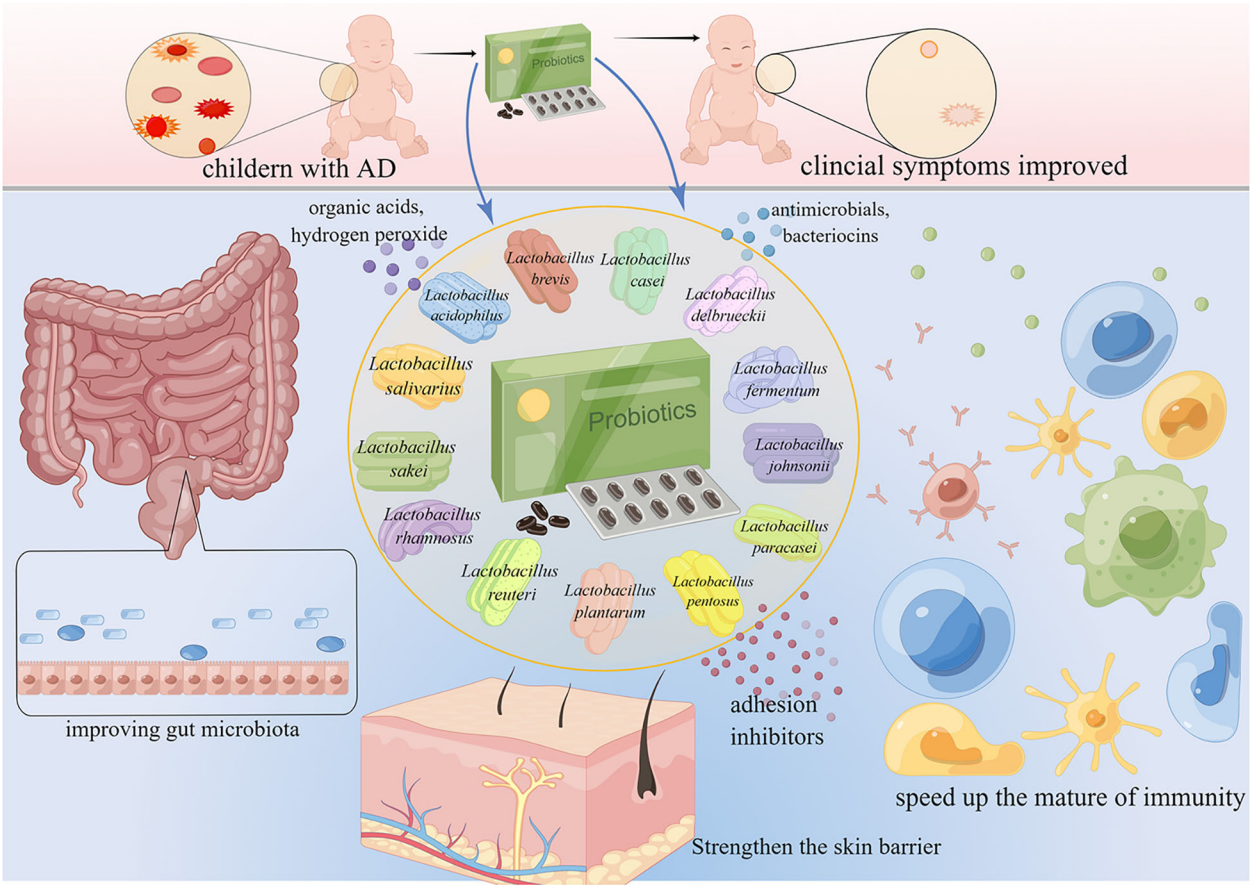

FIGURE 2
Lactobacillus for the treatment and prevention of AD. AD is common in children. *Lactobacillus* accelerates the maturation of the immune system, maintains intestinal homeostasis, improves the gut microbiome, and ultimately improves the symptoms of AD.

TABLE 1 Mechanism of action of *Lactobacillus* in the treatment of AD.

Probiotic	strain	Mechanism of action	Reference
L. rhamnosus	RHT3201	decrease of eosinophil cationic protein, eosinophil count, IL-31, and serum IgE concentration	(Lee et al., 2016; Jeong et al., 2020a)
	HN001	enhanced gut barrier function, influence on the immune system and a more balanced Th1/Th2 immune profile	(Wickens et al., 2008)
	LGG	decreased IL-10 protection against pathogenic macromolecules in the gut and accelerated immunological maturation, regulation of the immune response, stimulation of peripheral blood cells to secrete IL-10 and transforming growth factor β	(Kalliomäki et al., 2003; Sawada et al., 2007; Boyle et al., 2008; Marsella, 2009; Nermes et al., 2011; Marsella et al., 2012; Carucci et al., 2022)
	CGMCC 1.3724 (LPR)	reduction of plasma total IgE, upregulation of IFN-γ production at the skin level	(Tanaka et al., 2009)
L. acidophilus	L-92	activation of regulatory T cells and Th1 cells, decrease of eosinophil count and increase of change ratio for serum TGF-β, prevention of IgE-mediated hypersensitivity, modulation of Th1/Th2 balance	(Shah et al., 2010; Torii et al., 2011; Inoue et al., 2014; Yamamoto et al., 2016)
	L-55	decrease of serum total IgE level	(Sunada et al., 2008)
	LAVRI-A1	increase of allergen sensitization in infants	(Taylor et al., 2007)
L. plantarum	MG4221	inhibition of inflammatory and allergic reactions and regulation of the NF-κB/MAPK pathways in keratinocytes	(Hong et al., 2021a)
	LM1004	decrease of mRNA levels of Th2 and Th17 cell transcription factors, increase of transcription factors of Th1 and Treg cells, galactin-9, filaggrin, enhanced immunomodulation	(Kim et al., 2019a)
	CJLP133	upregulation of total IgE levels, increased TGF-β expression, increased proportion of regulatory T cells at baseline	(Kim et al., 2017)
	IS-10506	decreased levels of serum IgE, IL-4, and IL-17, increased the levels of Foxp3+ to IL-10 ratio, downregulate Th2 adaptive immune response	(Prakoeswa et al., 2017)
	NCIMB8826	amelioration of skin pathology, improvement of skin barrier integrity, skin thickening, and diminished excoriations	(Mariman et al., 2016)
L. sakei	ProBio65	increased expression of Foxp3+ transcription factor, Modulation of the expression levels of inflammatory cytokines IL-10 and IL-12, inhibition of mast cell activation, improvement of allergen-induced skin inflammation, downregulation of IgE and IL-4 production	(Park et al., 2008; Kim et al., 2013; Rather et al., 2021)
	KCTC 10755BP	decreased chemokine levels	(Woo et al., 2010)
	WIKIM30	modulation of allergic Th2 responses, enhanced Treg generation and increased relative abundance of intestinal bacteria that are positively related to Treg generation	(Kwon et al., 2018)
L. reuteri	Fn041	regulation of systemic Th1 and Th2 cytokine ratios and promotion of CD4+CD25+Foxp3+ regulatory T cell proliferation in mesenteric lymph nodes, regulation of intestinal microbiota	(Zhao et al., 2022)
	JCM 1112	dependent on the presence of Toll-like receptor 2 and the induction of TNF-α-induced protein 3 and cylindromatosis in HaCaT cells	(Kawahara et al., 2018)
	ATCC55730	modulation of the *in vivo* the cytokine pattern at an extra-intestinal site	(Miniello et al., 2010)
L. salivarius	LS01	Initiation of intestinal immunity, rebalancing of the changed intestinal microbiota, modulation of Thl/Th2 cytokine profiles	(Drago et al., 2011; Drago et al., 2012; Niccoli et al., 2014)
	PM-A0006	immune-modulating effects	(Wu et al., 2012)
L. paracasei	CBAL74	steroid sparing effect	(D'Auria et al., 2021)
	KBL382	increased immunosuppressive response and modified metabolic functions of gut microbiota	(Kim et al., 2020a)
	WK3001	diminished mast cell infiltration and plasma IgE levels, suppression of immediate hypersensitivity reactions and IL-4 mRNA expression in the auricles	(Wakabayashi et al., 2008)
L. casei	KCTC 12398BP	isolation of P14 protein decreased the levels of IL-4 in RAW264.7 and Balb/c splenocytes *in vitro* and ex vivo	(Kim et al., 2015b)
	DN-114001	effect on gut microbiota	(Klewicka et al., 2011)

(Continued)

TABLE 1 Continued

Probiotic	strain	Mechanism of action	Reference
	JCM 1134^{T}	modulation of the serum levels of IgE and cytokines and eosinophil count	(Ogawa et al., 2006)
L. delbrueckii	subsp. Bulgaricus LB-2	influence on both Th1 and Th2 through induction of Treg cells and secretion of IL-10	(Sheikhi et al., 2017)
	OLL1073R-1	attenuation of IL-6 secretion from lymph node cells and reduced IL-6 levels in inflamed tissues, such as auricles	(Kano et al., 2013)
	R-037	induce IL-12 and Th1	(Watanabe et al., 2009)
L. fermentum	VRI 003 PCC^{TM}	increased TNF-α responses to both heat-killed *Staphylococcus aureus* and heat-killed *Lactobacillus*	(Prescott et al., 2005)
L. johnsonii	NCC533	reduction of the gene expression of proinflammatory cytokines (IL-8, IL-12 and IL-23) and CD86	(Inoue et al., 2007)
L. brevis	NS1401	restoration of the Th1/Th2 balance through enhancing Th1-mediated immunity	(Choi et al., 2017)
	SBC8803	increased IgE production, increased production of immunosuppressive cytokines such as IL-10 and TGF-b1	(Segawa et al., 2008a)

TABLE 2 Effects of *Lactobacillus* on the clinical manifestations of AD.

Probiotic	strain	Participants	Interventions	Outcome	Reference
L. rhamnosus	RHT3201	100 children (aged 1–12 years) with moderate AD	1.0 × 10^{10} CFU (no details were provided)	decreased SCORAD total score and levels of eosinophil cationic protein	(Jeong et al., 2020a)
	HN001	mothers from 14–16 weeks gestation	6×10^{9} CFU/d, from 14-16 weeks gestation until 6 months post-partum if breastfeeding	no significant differences	(Wickens et al., 2018)
		High risk infants	6×10^{9} CFU/d, 9×10^{9} CFU/d, indirectly from 35 weeks gestation until 6 months after birth if breastfeeding, and directly from birth until 2 years	decreased cumulative prevalence of eczema, SCORAD score, and skin prick tests sensitization	(Wickens et al., 2013)
		425 children	6 ×10^{9} CFU, 9 ×10^{9} CFU/d, from 35 weeks gestation until birth, continued to 6 months after birth in mothers if breastfeeding, and from birth until 2 years in all infants	reduced cumulative prevalence of eczema at 4 years	(Wickens et al., 2012)
		Pregnant women, 474 infants at risk of allergic disease	6×10^{9} CFU/d, pregnant women from 35 weeks gestation until 6 months if breastfeeding, infants receive the same treatment from birth to 2 years	reduced risk of eczema	(Wickens et al., 2008)
	LGG	250 pregnant women carrying infants at high risk of allergic disease	1.8×10^{10} CFU/d, from 36 weeks gestation until delivery	no significant differences	(Boyle et al., 2011)
		100 AD patients (aged 6– 36 months)	1×10^{10} CFU/d, for 12 weeks	reduced SCORAD scores	(Carucci et al., 2022)
		159 mothers, 132 children at high risk	1×10^{10} CFU/d, for 4 weeks	improved AD	(Kalliomäki et al., 2003)
		39 infants with AD	3.4×10^{9} CFU/d	increased proportions of $CD19^{+}$ $CD27^{+}$ B cells and fewer infection	(Nermes et al., 2011)
		131 children (6–24 months old)	10^{10} CFU, 6 months	no significant differences	(Rose et al., 2010)

(Continued)

TABLE 2 Continued

Probiotic	strain	Participants	Interventions	Outcome	Reference
		35 infants (aged less than 1 year) with atopic eczema	4 weeks	no significant differences	(Salmi et al., 2010)
		11 adults and 73 infants	1.8×10^{10} CFU/d, for 7 days. 1.8×10^{10} CFU/d, from 36 weeks gestation until delivery	no significant differences	(Boyle et al., 2008)
		105 pregnant women	5×10^{9} CFU, twice daily	no significant differences	(Kopp et al., 2008)
		infants (aged 3–12 months) with mild-to-moderate AD	5×10^{9} CFU, for 12 weeks	no significant differences	(Grüber et al., 2007)
		54 infants (aged 1–55 months) with moderate to severe AD	10×10^{9} CFU/d, for 8 weeks	no significant differences	(Fölster-Holst et al., 2006)
L. acidophilus	L-92	57patients with AD(≥ 16 years)	20.7 mg/d, for 24 weeks	decreased investigator global assessment, eczema area and severity index, and AD score	(Yamamoto et al., 2016)
		49 AD patients (aged ≥ 16 years)	20.7 mg/d, for 8 weeks	decreased SCORAD scores and eosinophil count	(Inoue et al., 2014)
		20 children (age 4–15 years)	3×10^{10} CFU/d, for 8 consecutive weeks	ameliorated symptoms of AD in Japanese children and effect on serum concentrations of thymus	(Torii et al., 2011)
	LAVRI-A1	153 children	3×10^{9}/d, from birth to 6 months.	no significant differences	(Prescott et al., 2008)
		231 pregnant atopic women and babies	3×10^{9}/d, for the first 6 months of life	no significant differences	(Taylor et al., 2007)
		178 children	3×10^{9} CFU/d, for 6 months	no significant differences	(Jensen et al., 2012)
L. plantarum	CJLP133	76 children (median age of 7.1 years) with moderate-to-severe AD	1×10^{10} CFU/d, for 12 weeks	increased proportion of Treg cells with concurrent decrease in TGF-β mRNA expression	(Kim et al., 2017)
		children (aged 12 months to 13 years)	0.5×10^{10} CFU, twice a day for 12 weeks	lower SCORAD score at week 14 in the probiotic group than that in the placebo group; higher SCORAD score in the probiotic group from weeks 2 to 14 than that in the placebo group; *L. plantarum* CJLP133 significantly decreased total eosinophil count, logarithmic IFN-γ and IL-4	(Han et al., 2012)
	IS-10506	22 children with mild and moderate AD	10^{10} CFU, twice daily for 12 weeks	decreased SCORAD and levels of IL-4, IFN-γ, and IL-17; upregulation of Foxp3+ to IL-10 ratio	(Prakoeswa et al., 2017)
L. sakei	ProBio65	Children (aged 3–9 years) and adolescents (aged 10 to 18) with AD	1×10^{10} cells/d per sachet (400 mg)	decreased SCORAD total score when compared with baseline and potential improvement of skin barrier functions	(Rather et al., 2021)
	KCTC 10755BP	88 children (aged 2–10 years) with AEDS	5×10^{9} CFU, twice daily for 12 weeks	decreased total SCORAD scores	(Woo et al., 2010)
L. reuteri	ATCC55730	patients (aged 4–10 years)	10^{8} CFU/d, for 8 weeks were prescribed as 1 tablet once per day (2 hours before meals)	no significant differences	(Miniello et al., 2010)
		mothers and their babies	1×10^{8} CFU/d, from gestational week 36 until delivery	diminished IgE-associated eczema and skin prick test reactivity	(Abrahamsson et al., 2007)

(Continued)

TABLE 2 Continued

Probiotic	strain	Participants	Interventions	Outcome	Reference
L. salivarius	LS01	patients (aged 25–63 years)	5×10^9 CFU/d, for a month	decreased SCORAD index and the count of Staphylococcus Aureus.	(Drago et al., 2014)
		43 patients (aged 0–11 years) with AD	1×10^9 CFU/sachet, 2 sachets/d, for 8 weeks, and 1 sachet/day for the following 8 weeks	improved SCORAD score and itching	(Niccoli et al., 2014)
		38 patients (aged 18–46 years) with moderate/ severe AD	1×10^9 CFU/g, twice daily, for 16 weeks	decreased SCORAD score	(Drago et al., 2012)
		38 patients (aged from 18 to 46 years) with moderate/severe AD	1×10^9 CFU/g, twice daily, for 16 weeks	improved SCORAD score and dermatology life quality index	(Drago et al., 2011)
	PM-A0006	60 children (aged 2–14 years) with moderate to severe AD	2×10^9 CFU, twice daily, for 8 weeks	decreased SCORAD scores range 8 weeks and 10 weeks. *L. salivarius* PM-A0006 significantly reduced medication use frequency and eosinophil cationic protein levels at 8 weeks. *Lactobacillus salivarius* PM-A0006 reduced AD intensity	(Wu et al., 2012)
L. paracasei	CBAL74	58 infants and young children with moderate to severe AD (aged 6–36 months)	8 g/d for 12 weeks	decreased SCORAD index	(D'Auria et al., 2021)
	GM-080	infants with AD (aged 4–30 months)	1× 1,010 equivalent CFU, for 16 weeks	decreased CCL17 levels and TEWL in lesions and unaffected skin	(Yan et al., 2019)
	K71	34 adults with AD	100 mg/d (~2×10^{11} bacteria), over 12 weeks	decreased skin severity scores compared with baseline	(Moroi et al., 2011)
L. casei	DN-114001	40 children (aged 6–18 months) with AD	10^9 cells/d, for 3 months	decreased SCORE index	(Klewicka et al., 2011)
L. delbrueckii	subsp. Bulgaricus LB-2	20 children (age 1–12 years) with AD	co-cultured with different concentrations of UV killed bacteria in RPMI-1640 plus 10% FCS for 48/ 72 h	upregulated the secretion of IL-10, IL-12, and IFN-γ, and decreased secretion of IL-4.	(Sheikhi et al., 2017)
L. fermentum	VRI 003 PCC™	53 children with moderate or severe AD	1×10^9 twice daily for 8 weeks	increased Th1-type cytokine IFN-γ responses to PHA and SEB	(Prescott et al., 2005)

2016, researchers observed that *L. rhamnosus* IDCC 3201 tyndallizate (RHT3201) had the potential to treat AD. The mast cell count and serum IgE concentration in axillary lymph node cells were decreased in RHT3201-fed NC/Nga mice compared with those in the control group (Lee et al., 2016). Another animal experiment concluded that heat-treated LGG could improve the symptoms of NC/Nga mice with AD (Sawada et al., 2007). In 2020, Jeong and colleagues (Jeong et al., 2020a) found that RHT3201 had a therapeutic effect on AD in children. *L. rhamnosus* GG (LGG) is a strain of *L. rhamnosus* that regulates gut flora and reduces conditional pathogenic bacteria (Chen et al., 2020b). When children with AD were supplemented with LGG, the clinical severity and quality of life improved (Carucci et al., 2022). Simultaneously, the use of topical steroids was reduced (Carucci et al., 2022).

L. rhamnosus decreases the concentrations of eosinophilic cationic protein, eosinophil count, IL-31 (Jeong et al., 2020a), and serum IgE (Tanaka et al., 2009; Lee et al., 2016); supports a better Th1/ Th2 balance (Wickens et al., 2008); prevents pathogenesis caused by large molecules in the intestine; accelerates immune maturation (Nermes et al., 2011); stimulates peripheral blood cells to secrete IL-10 (Kalliomäki et al., 2003; Sawada et al., 2007); converts growth factor β (Kalliomäki et al., 2003), and upregulates the production of IFN-γ at the skin level (Tanaka et al., 2009). However, not all *L. rhamnosus* strains are equally effective. Wickens et al. (2012) studied the efficacy of *L. rhamnosus* HN001 and HN019 in atopic diseases and demonstrated that *L. rhamnosus* HN001 was more effective in improving AD than *L. rhamnosus* HN019 (Wickens et al., 2012). Moreover, the protective effect on eczema can persist two years when *L. rhamnosus* HN001 is administered to children with AD (Wickens et al., 2008). In addition, *L. rhamnosus* HN001 can prevent atopic sensitization in the long term (Wickens et al., 2013). Moreover, several studies have shown that the protective effect of LGG against AD requires further investigation. Two randomized controlled trials have shown that prenatal LGG treatment was not associated with a reduced risk of eczema (Boyle et al., 2008; Boyle et al., 2011). No clear causal relationship between the positive effects of LGG and infantile

TABLE 3 Effects of *Lactobacillus* on the experimental AD.

Probiotic	strain	Experimental animal	Interventions	Outcome	Reference
L. rhamnosus	RHT3201	six-week-old female NC/Nga mice	1×10^8, 1×10^9, or 1×10^{10} cells/d, for 8 weeks.	improved dermatitis scores and frequency of scratching	(Lee et al., 2016)
	LGG	two litters of Beagles (same sire and dam) with AD	200×10^9 CFU/d, ten Culturelle® capsule; offspring from the second pregnancy, LGG, 100×10^9 CFU/d	decreased allergen-specific IgE and partially prevented AD	(Marsella et al., 2012)
		2 adult beagles with severe AD and 16 pups	One capsule containing a minimum of LGG 20×10^9 CFUs; first litter female dogs did not receive LGG. During the second pregnancy, a dosage of 10 capsules/d LGG from week 3 of gestation and continued throughout lactation. During the third pregnancy, 5 capsules/d from 3 weeks to 6 months of age	decreased serum titer of allergen-specific IgE and moderated reaction to intradermal testing	(Marsella, 2009)
		specific pathogen-free NC/Nga mice	30–50 mg/d	increased plasma IL-10 levels and enhanced IL-10 mRNA expression in both Peyer's patches and mesenteric lymph nodes	(Sawada et al., 2007)
	CGMCC 1.3724 (LPR)	specific-pathogen free pregnant NC / NgaTnd mice, pups until 12 weeks of age	5×10^8 CFU/ ml	decreased clinical symptoms of dermatitis, reduced scratching frequency	(Tanaka et al., 2009)
L. acidophilus	L-92	ICR mice BALB/c mice BALB/c and NC/Nga mice	3 and 30 mg/kg	inhibited vascular permeability in both passive cutaneous anaphylaxis	(Shah et al., 2010)
	L-55	female NC/Nga mice (5 weeks old) with AD-like skin lesions	1 and 10 mg/d, for 75 days	inhibited dermatitis score, ear swelling, scratching behavior	(Sunada et al., 2008)
L. plantarum	MG4221	NC/Nga mice (male, 4 weeks old)	a single dose ($7\ \mu g\cdot cm^{-2}$) of 200 µL of PM2.5 ($500\ \mu g\cdot mL^{-1}$) with 2% dinitrochlorobenzene another single dose ($7\ \mu g\cdot cm^{-2}$) of 200 µL of PM2.5 ($500\ \mu g\cdot mL^{-1}$) with 0.2% dinitrochlorobenzene	decreased transepidermal water loss and erythema; decreased scratching behavior	(Hong et al., 2021a)
	LM1004	AD-induced rat (histamine-induced vasodilation) and mouse (pruritus and contact dermatitis)	2×10^{12} cells, for 28 days	reduced vasodilation, pruritus, edema, and serum histamine	(Kim et al., 2019a)
	NCIMB8826	APOC1+/+ mice	3×10^8 CFU, three times a week, for 8 weeks	ameliorated skin pathology, improved skin barrier integrity, eliminated of skin thickening, and fewer excoriations	(Mariman et al., 2016)
L. sakei	ProBio65	dogs with CAD	2×10^9 CFU/g, for 2 months	reduced disease severity index, CASESI score values	(Kim et al., 2015a)
		25 male 6-week-old NC/Nga mice	5×10^9 CFU/ml, 200 µL/d, for 2 weeks	improved condition of skin and reduced scratching frequency	(Kim et al., 2013)
		mice triggered by allergen	1×10^8 CFU/mL, 200 µL/d, for 2 weeks	faster recovery of AD	(Park et al., 2008)
	WIKIM30	wild-type male BALB/c mice	2×10^9 CFU bacteria, 200 µL/d	reduced AD-like skin lesions	(Kwon et al., 2018)
L. reuteri	Fn041	seven-week-old male and female BALB/C mice	1×10^9 CFU/d, once a day, 100 µL each time, each time	suppressed AD symptoms such as skin swelling, mast cell and eosinophil infiltration	(Zhao et al., 2022)
	Japan Collection of Microorganisms 1112	specific pathogen-free male NC/Nga mice (aged 10 weeks)	0.1% (w/v) *Lactobacillus* water extract (LW)-treated, and 1.0% (w/v) LW-treated; 0.1% LW in 80% ethanol, or 1.0%	suppressed the development of house dust mite-induced atopic skin lesions and thymus and activation-regulated chemokine expression	(Kawahara et al., 2018)

(Continued)

TABLE 3 Continued

Probiotic	strain	Experimental animal	Interventions	Outcome	Reference
			LW in 80% ethanol, twice weekly for one week		
L. paracasei	KBL382	mice with Dermatophagoides farinae extract -induced AD	1×10^9 CFU/d, for 4 weeks	reduced AD-associated skin lesions and epidermal thickening	(Kim et al., 2020a)
	K71	41 dogs with mild to moderate cAD	5 mg/kg, once daily, for 12 weeks	decreased CADESI, and pruritus scores; the reduced medication scores	(Ohshima-Terada et al., 2015)
	WK3001	five-week-old male NC/Nga mice	basic diet at concentrations of 0.03% (low dose) or 0.3% (high dose)	reduced development of AD-like skin lesions	(Wakabayashi et al., 2008)
L. casei	KCTC 12398BP	male NC/Nga mice	1, 10, and 100 μg/mL of P14 0.1, 0.2, 1, 5, and 10 μg/mL of P14	downregulated AD index and scratching score in AD-like NC/Nga mice	(Kim et al., 2015b)
	JCM 1134^T	six-week-old male NC/Nga mice	NC/Nga mice were divided into four groups of six each and administered CD, DD (500 mg of dextran per day) LD (1×10^7 CFU of lyophilized *L.* casei subsp. casei per day) LDD (1×10^7 CFU of lyophilized *L.* casei subsp. casei and 500 mg of dextran per day) (control diet; CD) (dextran diet; DD) (*L.* casei subsp. casei diet; LD) (*L. casei* subsp. casei and dextran diet; LDD)	decreased clinical skin severity scores and total IgE levels	(Ogawa et al., 2006)
L. delbrueckii	OLL1073R-1	specific-pathogen-free female NC/Nga mice and BALB/c mice, (4- or 5-wk-old).	bacterial, 1 mg/d	inhibited development of dermatitis and elevation of an acute inflammation marker, serum amyloid A	(Kano et al., 2013)
	R-037	female BALB/c mice (5-weeks-old) and male NC/Nga mice (7-weeks-old)	5 g/d/mouse, from day 0 to day 55	reduced inflammatory auricular thickness and alleviated the AD clinical score	(Watanabe et al., 2009)
L. johnsonii	NCC533	NC/NgaTnd mice	4 weeks	suppressed exacerbation of the clinical severity of dermatitis and suppressed epidermal hyperplasia and infiltration of inflammatory cells in skin	(Tanaka et al., 2008)
		pups of 4 pregnant NC/Nga mice.	10^{10} cells, from 20 to 22 days of age *via* oral administration	enhanced gene expression of the proinflammatory cytokines [interleukin-8 (IL-8), IL-12 and IL-23] and decreased gene expression of CD86	(Inoue et al., 2007)
L. brevis	NS1401	female NC/Nga mice (24 Six-weeks old)	5×10^8 CFU/d per mouse, for 8 weeks	reduced skin thickness and infiltration of mast cells and eosinophils in skin lesions and the size and number of immune cells in draining lymph nodes	(Choi et al., 2017)
	SBC8803	male 5-week-old NC/Nga mice	0%, 0.05% or 0.5%, once a week for 9 weeks.	inhibited ear swelling, and suppressed the development of dermatitis.	(Segawa et al., 2008a)

eczema has been reported (Fölster-Holst et al., 2006; Grüber et al., 2007; Kopp et al., 2008; Rose et al., 2010).

Some probiotics affect multiple immune pathways through different mechanisms and protect against the pathogenesis of eczema (Wickens et al., 2008). However, supplementation mothers with *L. rhamnosus* HN019, was not effective in preventing eczema in infants, indicating that *L. rhamnosus* HN019 is less likely to be passed on to infants through breast milk (indirect supplementation route) (Wickens et al., 2018). One of the reasons that prenatal LGG was not shown to prevent eczema in infants may be that the impact of prenatal probiotic therapy on fetal B cell development was not excluded (Boyle et al., 2008). Prenatal LGG intake may not directly contribute to the effects of postpartum breast milk regulation. It is possible that postpartum intake by nursing mothers can alter immunity and/or microbiota to benefit breast milk composition (Boyle et al., 2011). Probiotics are commonly added to dairy products (Grüber et al., 2007), which can also affect the efficacy of *L. rhamnosus* in AD. It is important to note that the number of participants who completed the study and the subgroups analyzed were insufficient. In addition, the complexity of human life makes it challenging to achieve homogeneity. In addition, eczema in early infancy can naturally improve, and more than 40% of patients with AD recover around

the age of 3 years (Illi et al., 2004). Moreover, it is more difficult for clinical trials to recruit sufficient human participants than for animal studies to include a sufficient number of animals.

L. rhamnosus has been shown to be effective in the prevention and treatment of AD in both animal and clinical experiments. However, future research needs to continue to explore different strains of *L. rhamnosus* and use strains with excellent laboratory efficacy in clinical trials. Effective strains of *L. rhamnosus* can be passed on to the offspring from the mother, and subsequently, infants may potentially not develop AD.

3.2 Some strains of *Lactobacillus acidophilus* alleviate symptoms of AD and show good safety

L. acidophilus is a commercially significant probiotic isolated from the human gastrointestinal tract (Bull et al., 2013). Moreover, it is an important class of bioprotective agents (Anjum et al., 2014). Supplementation with *L. acidophilus* L-92 significantly reduced vascular permeability in diseased mice and attenuated the clinical symptoms of AD (Shah et al., 2010). The administration of *L. acidophilus* L-92 not only significantly attenuated AD symptoms in children (Torii et al., 2011), but also improved the symptoms in adults (Inoue et al., 2014). *L. acidophilus* triggers an anti-inflammatory response (Goh et al., 2021). *L. acidophilus* L-92 inhibited the inflammatory response dominated by Th2 cells by activating regulatory T (Treg) and Th1 cells (Yamamoto et al., 2016). *L. acidophilus* L-55 decreased the occurrence of anaphylactic dermatitis-like skin lesions in NC/Nga mice by decreasing serum total IgE levels (Sunada et al., 2008). However, the findings on several strains of *L. acidophilus* have been inconsistent. For example, there is no evidence that *L. acidophilus* NCFM can improve AD (Larsen et al., 2011). Supplementation with *L. acidophilus* LAVRI-A1 in children with allergies did not reduce the risk of dermatitis (Taylor et al., 2007; Prescott et al., 2008; Jensen et al., 2012), and the sensitization rate in the *L. acidophilus* LAVRI-A1 group was significantly higher than that in the control group (Taylor et al., 2007).

In early studies, *L. acidophilus* demonstrated good safety and efficacy in children with AD, and future studies in mothers are ongoing or are expected to begin soon. In addition, the time required to evaluate clinical outcomes is insufficient. The detailed interplay between the early microbial environment and the developing immune system remains largely unknown and requires further exploration. Overall, the findings suggest that not every strain of *L. acidophilus* has the potential to prevent or treat AD. The effect of *L. acidophilus* on the prevention of AD requires further study.

3.3 *Lactobacillus plantarum* regulates the host immune system to improve AD symptoms

L. plantarum is a rod-shaped lactic acid-producing bacterium that is used in probiotics and silage production. *L. plantarum* has the potential to be a highly effective immunomodulatory probiotic in the human gut microbiome. In recent years, an increasing number of studies have shown the health benefits of *L. plantarum*. (Seddik et al., 2017). The extract of fermented blueberry black rice containing *L. plantarum* MG4221 had an effect similar to that of dexamethasone, but with fewer side effects; oral administration of FBBBR in NC/Nga mice reduced skin dryness, erythema, and scratch behavior (Hong et al., 2021a). *L. plantarum* NCIMB8826 can soothe the skin of mice with AD, strengthen the skin barrier, and alleviate scratching (Mariman et al., 2016). Supplementation with *L. plantarum* CJLP133 and IS-10506 has been shown to be beneficial for treating AD in children (Han et al., 2012; Kim et al., 2017; Prakoeswa et al., 2017). In addition, *L. plantarum* IS-10506 has an immunomodulatory effect and can effectively relieve AD symptoms in adults (Prakoeswa et al., 2022).

L. plantarum BF_15 can successfully colonize murine intestines by rebalancing intestinal microbiota (Zhang et al., 2020). The extract of fermented blueberry black rice containing *L. plantarum* MG4221 inhibited the production of serum IgE and Th2 cell-related cytokines, suggesting that fermented blueberry black rice may be an essential functional food in AD (Hong et al., 2021a). Additionally, oral administration of *L. plantarum* NCIMB8826 reduced the number of mast cells in the colon. Finally, *Staphylococcus aureus* infection is the main reason for the exacerbation of AD-like symptoms, but *L. plantarum* can alleviate AD-like symptoms by inhibiting *Staphylococcus aureus* (Kim et al., 2020b). Additionally, lipoteichoic acids isolated from *L. plantarum* and *Staphylococcus aureus* have shown anti-AD effects. Lipoteichoic acid combination therapy can alleviate AD by reducing the formation of membrane attack complexes and inhibiting Th1 reactions (Kim et al., 2019c). Notably, *L. plantarum* LM1004 not only regulates the host immune system and gut microbiota, but is also promising for the treatment of AD and obesity in humans (Kim et al., 2019a). Collectively, the preliminary evidence suggests *L. plantarum* is a potential therapeutic strategy. Moreover, it has an acceptable safety profile in adults. *L. plantarum* improves the symptoms of AD, although it is ineffective in preventing AD. Future research should focus on the preventive effects of *L. plantarum* on AD. *L. plantarum* is a promising *Lactobacillus* strain that can be further explored to improve treatment options and efficacy.

3.4 *Lactobacillus sakei* has potential as a supplement for the treatment of AD due to its anti-inflammatory and skin barrier protective properties

L. sakei was isolated from fermented meat, fish, and kimchi. *L. sakei* KDP is a potent antioxidant and antibacterial agent (Ghoneum and Abdulmalek, 2021). *L. sakei* 07, combined with *Bifidobacterium bifidum* B10, regulates immunity and the gut microbiota (Wang et al., 2019). Oral administration of live and inactivate (Kim et al., 2013; Rather et al., 2021) *L. sakei* probio65 can increase skin sebum content and improve the function of the skin barrier (Rather et al., 2021). *L. sakei* probio65 inhibits AD-like skin lesions and may serve as an influential novel anti-inflammatory medication that resolves AD symptoms in mice (Kim et al., 2013). In experimental dogs (Kim et al., 2015a) and mice (Park et al., 2008) with AD, orally administered *L. sakei* probio65 significantly reduced the disease severity index

without clear side effects. Supplemental treatment with *L. sakei* KCTC 10755BP has the potential to alleviate the clinical severity of AD syndrome in children (Woo et al., 2010).

L. sakei WIKIM30 was isolated from kimchi and can significantly reduce AD-like skin lesions, regulate allergic Th2 responses, increase the relative abundance of intestinal bacteria positively correlated with Treg production, and has potential in AD treatment (Kwon et al., 2018). Current research shows that *L. sakei* can be anti-inflammatory and can protect the skin barrier. *L. sakei* has the potential to be used as a therapeutic supplement in AD. *L. sakei* originates from fermented foods, and direct intake of fermented foods may have the same effect. As mentioned above, both live and inactivated *L. sakei* have been shown to be effective. Fermented foods such as kimchi are popular and readily available, and patients can effortlessly benefit and achieve improvement of AD. Future research can further apply *L. sakei* in clinical practice and investigate its preventive effect on AD. In addition, the therapeutic effects of other strains of *L. sakei* can also be explored.

3.5 *Lactobacillus reuteri* supplementation is effective in preventing AD

L. reuteri has been reported to occur naturally in the intestines of all vertebrates and mammals. *L. reuteri* induces neonatal IgA production (Mu et al., 2021). *L. reuteri* survives in the gastrointestinal tract of mammals and benefits host health (Engevik et al., 2021). *L. reuteri* NK33 can be used to improve gut dysbiosis (Han et al., 2020). *L. reuteri* strain, the Japan Collection of Microbiology 1112, significantly inhibited the expression of allergic lesions and thymus and activation-regulated chemokines at the site of lesions in NC/Nga mice (Kawahara et al., 2018). Prenatal and postnatal supplementation with *L. reuteri* Fn041 effectively prevented the fetus from developing AD, remodeled the intestinal ecology, and improved the immune function of Peyer's patches (Qi et al., 2022; Zhou et al., 2022). *L. reuteri* Fn041 regulated the intestinal flora and significantly inhibited AD symptoms by regulating the systemic ratio of Th1 and Th2 cytokines in mice (Zhao et al., 2022). *L. reuteri* DYNDL22M62 attenuated AD symptoms by modulating gut bacteria in mice (Fang et al., 2022). Mothers and their babies supplied with *L. reuteri* ATCC 55730 had a lower prevalence of IgE-associated eczema at 2 years of age (Abrahamsson et al., 2007). However, in another clinical trial, *L. reuteri* ATCC 55730 did not improve clinical symptoms (Miniello et al., 2010). This may be because the small sample size. Alternatively, the duration of the experiment may have been too short. *L. reuteri* has been extracted from the gastrointestinal tract of all mammals, and future studies could explore the reasons for an absence of this *L.* and whether higher abundance of *L. reuteri* is beneficial. *L. reuteri* appears to have preventive and therapeutic effects in animals. Further clinical research on *L. reuteri* is required to explore the treatment and prevention of AD.

3.6 *Lactobacillus salivarius* actively improves the quality of life in children and adult patients with AD

L. salivarius is a Lactobacillus species that occurs in the human gastrointestinal tract and oral mucosa. It produces bacteriocins, and is used as a probiotic. It modifies the gastrointestinal system to alleviate intestinal diseases and promote host health (Neville and O'Toole, 2010). *L. salivarius* is valuable for both animals and humans. *L. salivarius* can reduce pathogen colonization of the gastrointestinal tract of animals (Hong et al., 2021b). Administration of *L. salivarius* can prevent and treat a variety of chronic diseases in humans (Chaves et al., 2017), including AD, asthma, cancer, and bad breath (Drago et al., 2014). The combined administration of *L. salivarius* PM-A0006 and fructooligosaccharides exhibited a notable anti-AD effect compared with either therapy alone in the treatment of children with moderate-to-severe AD (Wu et al., 2012). *L. salivarius* LS01 can help manage AD in children and improve their quality of life; moreover, partial effect remains after termination of medication (Niccoli et al., 2014). *L. salivarius* LS01 actively improves the quality of life in adult patients with AD by regulating the balance of Th1/Th2 (Drago et al., 2011; Drago et al., 2012; Kim et al., 2014). Additionally, *L. salivarius* has shown efficacy in improving AD in existing studies. As the name suggests, *L. salivarius* is present in the oral cavity. The study of the oral environment is of great significance for increasing *L. salivarius*. The preventive effect of *L. salivarius* on AD requires further study. However, the long-term safety and persistence of *L. salivarius* remains to be studied. More potent subspecies of *L. salivarius* are yet to be discovered.

3.7 *Lactobacillus paracasei* has anti-inflammatory properties that can reduce the development of AD-like skin lesions

L. paracasei originates from the healthy human gastrointestinal tract and is widely distributed in food. *L. paracasei* has anti-inflammatory properties that can reduce antigenic pro-inflammatory responses. *L. paracasei* improves immune function by enhancing NK cell function and IFN-γ concentrations (Lee et al., 2017). In addition to a preventive effect on AD-like skin changes in mice *L. paracasei* KW3110 demonstrated inhibitory effects even when supplementation was started after symptoms have appeared (Wakabayashi et al., 2008). Supplementation with *L. paracasei* KW3110 can significantly reduce the development of AD-like skin lesions in mice, while regulating immunity (Wakabayashi et al., 2008). Oral supplementation with *L. paracasei* K71 can be used to treat dogs with AD (Ohshima-Terada et al., 2015). A diet with added K71 can be used as a complementary therapy for adult AD patients (Moroi et al., 2011). Moreover, *L. paracasei* NL41 reduced inflammation by improving the intestinal environment and maintaining intestinal integrity in rats (Zeng et al., 2021). Daily oral administration of *L. plantarum* HEAL9 and *L. paracasei* 8700 has been shown to regulate the peripheral immune response in children with celiac disease autoimmunity (Håkansson et al., 2019). *L. paracasei* KBL382 can significantly reduce AD-related lesions and epidermal thickening in mice by modulating the immune response and changing intestinal microbiota composition(Kim et al., 2020a).

Additionally, heat-killed *L. paracasei* CBA L74 has a minimal effect on steroid use, but its effect on reducing the severity of AD needs to be further studied (D'Auria et al., 2021). However, there is no evidence that *L. paracasei* GM-080 has a retention effect equivalent to that of glucocorticoids (Yan et al., 2019). This may be due to the

inappropriate selection of *L. paracasei* strains, timing of administration, timing of exposure, and failure to achieve appropriate dosing levels. In conclusion, *L. paracasei* enhances NK cell function and IFN-γ concentrations to regulate immune mechanisms and achieve anti-inflammatory effects. Concurrently, *L. paracasei* can improve the intestinal environment and maintain intestinal homeostasis to achieve anti-inflammatory effects. The duration of *L. paracasei* administration does not require special emphasis, and intervention in patients with AD before and after the appearance of symptoms can achieve the desired effect. Hence, these studies might help clarify whether *L. paracasei* can improve intestinal barrier function and maintain immune system balance. Oral administration of *L. paracasei* can prevent and treat AD. However, more clinical experiments are needed to explore prevention of AD with the administration of *L. paracasei*.

3.8 *Lactobacillus casei* treats AD by balancing the gut microbiota and immune responses

L. casei is found in many fermented foods and coexists with gut microbiota. It is involved in housekeeping functions, metabolism, cell wall biogenesis, and environmental adaptation (Licandro-Seraut et al., 2014). *L. casei* CCFM1074 can balance gut microbiota and immune responses (Fan et al., 2021). *L. casei* regulates the host immune response (Aktas et al., 2016) and can be used to treat AD. During the Japanese cedar pollen season, NC/Nga mice orally administered *L. casei* Japan Collection of Microorganisms (JCM) 1134T combined with dextran experienced a possible effect on the prevention and treatment of allergic reactions (Ogawa et al., 2006). Furthermore, researchers screened an active ingredient, protein P14, from the *L. casei* extract, which specifically lowered IgE and IL-4 levels in AD-like NC/Nga mice, suggesting potential therapeutic effects in AD (Kim et al., 2015b). Similarly, the administration of *L. casei* DN–114001 to the diet of children with AD was beneficial to the increase in the count of intestinal flora and its maintenance for five months after the cessation of probiotics (Klewicka et al., 2011). *L. casei* DN—114001 can improve clinical symptoms in children with AD long term (Klewicka et al., 2011). Overall, these studies provide a good foundation for developing future therapeutic or preventive approaches using *L. casei* in individuals with AD. *L. casei* has an extended effect after stopping supplementation; therefore, the effect of permanent colonization of the intestine after regular supplementation should be studied. Few studies have been conducted on *L. casei* for the treatment of AD. More subspecies of *L. casei* are yet to be identified.

3.9 *Lactobacillus delbrueckii* alleviates AD by maintaining and improving intestinal barrier function by stimulating immune cells to reduce inflammatory responses

L. delbrueckii is one of the most economically valuable fermented Lactobacillus species. *L. delbrueckii* maintains and improves the intestinal barrier function by stimulating immune cells (Kobayashi et al., 2019). *L. delbrueckii* also improved intestinal integrity and immune responses in piglets (Chen et al., 2020a). *L. delbrueckii* subsp. bulgaricus may be involved in regulation of immune factor secretion in patients with AD (Sheikhi et al., 2017). IL-6 is a leading cause of dermatitis; whereas oral administration with *L. delbrueckii* subspecies bulgaricus OLL1073R-1 attenuated dermatitis by inhibiting the IL-6 response and restoring the elevation of serum amyloid levels in the NC/Nga mouse model of AD (Kano et al., 2013). In addition, oral supplementation with heat-treated *L. delbrueckii* R-037 can inhibit the rise of serum total IgE in allergic model mice, decrease inflammation, and alleviate AD; however, its effect on serum total IgE levels needs to be further studied (Watanabe et al., 2009). Studies in animal (mouse) models and have shown that *L. delbrueckii* plays a role in the management of AD. Experimental samples are easier to obtain in animal experiments than in clinical studies. However, only results of clinical studies that show the efficacy of *L. delbrueckii* will enable its widespread use in the management of patients with AD. In future, it will be necessary to further investigate the use of *L. delbrueckii* in patients with AD, including factors such as the time of supplementation, dosage, and strain activity. Moreover, the understanding of the preventive function of *L. delbrueckii* requires further experiments in both animals and humans.

3.10 *Lactobacillus fermentum* regulates the immune response and benefits children with AD

L. fermentum is a gram-positive bacterium. It can improve the functionality and nutritional value of foods (Naghmouchi et al., 2020). *L. fermentum* can restore homeostasis of the intestinal microflora and regulate the immune response in mice (Rodríguez-Nogales et al., 2017). Mice were immunized with *L. fermentum* NWS29 and exposed to ovalbumin. *L. fermentum* NWS29 inhibited the expression of certain inflammatory factors to achieve an anti-inflammatory effect (Nawaz et al., 2015). Mice were inoculated with the Salmonella vaccine and *L. fermentum* PC2. When mice were challenged with live *Salmonella typhimurium*, *L. fermentum* enhanced mucosal and immune responses and played a protective role (Esvaran and Conway, 2012). *L. fermentum* KBL374 and KBL375 can modulate the innate immune response by improving intestinal barrier function and reducing leukocyte infiltration in mice (Jang et al., 2019). *L. fermentum* CJL-112 protected mice from the deadly influenza virus infection by stimulating macrophages, activating Th1 cells, and increasing immunoglobulin A production (Yeo et al., 2014). In a clinical trial, *L. fermentum* PCCTM strengthened Th1 IFN-γ responses and achieved clinical benefits in children with AD (Prescott et al., 2005).

L. fermentum MS15 inhibited exogenous IL-10 induced human β-defensin-2 and regulated the response to the inflammatory stimulus (Habil et al., 2014). In future, *L. fermentum* can be combined with other vaccines to enhance their protective effect (Esvaran and Conway, 2012). These different strains of *L. fermentum* have been shown to regulate immunity and may also have some effect in the prevention of AD. In future, more research is required to explore the potential of *L. fermentum* in the treatment of AD.

3.11 *Lactobacillus johnsonii* improves intestinal inflammation and alleviates the severity of AD

L. johnsonii is a probiotic that can be isolated from dairy products. Notably, *L. johnsonii* BS15 can regulate intestinal inflammation (Charlet et al., 2020; Xin et al., 2020a; Xin et al., 2020b; Wang et al., 2021). Oral administration of *L. johnsonii* NC553 can relieve the severity of AD and inhibit epidermal hyperplasia and infiltration of inflammatory cells into the skin (Inoue et al., 2007). Additionally, it relieves skin damage by inhibiting pro-inflammatory cytokines and CD86 (Inoue et al., 2007). Therefore, early administration of *L. johnsonii* NC553 in mice with allergies may help reduce AD exacerbations (Tanaka et al., 2008). *L. johnsonii* has been shown to improve intestinal inflammation in animal models. *L. johnsonii* is present in fermented dairy products, and it is worth investigating whether an effective dose can be achieved through daily yogurt intake in children. *L. johnsonii* can also ameliorate skin damage in mice. However, there have been few experiments related to the treatment of AD with *L. johnsonii*, and further research is needed to determine its effectiveness in the prevention and treatment of AD.

3.12 *Lactobacillus pentosus* regulates the host immune system and improves systemic inflammatory response

L. pentosus regulates the host immune system and plays an integral role in intestinal health (Ma et al., 2020). *L. pentosus* KF340 regulates systemic immunity and improves systemic inflammatory response (Kim et al., 2019b). *L. pentosus* S-PT84 may be involved in the modulation of immune mechanisms to alleviate clinical allergy symptoms (Majumder et al., 2020). Although administration of *L. pentosus* and placebo can improve symptoms, *L. pentosus* significantly improved the average subjective ratings evaluated using the SCORAD index for allergen-sensitizing AD (Ahn et al., 2020). *L. pentosus* KF340 reduced cell infiltration and serum IgE levels at the site of lesions in mice by inducing type 1 regulatory T cells (Tr1 cells) that produce IL-10 (Kim et al., 2019b). *L. pentosus* S-PT84 reduced the concentrations of histamine in the serum, mouse mast cell protease, total IgE, and IgG (Majumder et al., 2020). The detailed function of *L. pentosus* remains largely unknown and requires further investigation. *L. pentosus* has been shown to exert anti-inflammatory effects and benefit intestinal health. However, there is limited evidence on the efficacy of *L. pentosus* in the treatment and prevention of AD, and further research is needed.

3.13 *Lactobacillus brevis* alleviates symptoms of AD by regulating the immune response

L. brevis is a gram-positive, rod-shaped *Lactobacillus* that is frequently used as a starter culture in silage fermentation, sourdough, and lactic acid-fermented beer and wine. *L. brevis* is widely used in the fermentation industry. Orally administered *L. brevis* SBC8803 can significantly inhibit the production of IgE and severity of AD symptoms, and long-term use can inhibit AD development. However, it did not affect the production of cytokines produced by Th1 and Th2 (Segawa et al., 2008a). *L. brevis* NS1401, isolated from kimchi, stimulated immune cells to secrete Th1 or Th2 cytokines, balance Th1/Th2, and alleviate AD symptoms (Choi et al., 2017). *L. brevis* stabilizes the gut microbiota, prevents the growth of pathogenic bacteria, and reduces intestinal inflammation (Han et al., 2021). *L. brevis* can not only improve disease but also regulate immunity. In nine-week-old female BALB/c mice managed with *L. brevis* KB290 (3 × 109 CFU/g), cytotoxicity mediated by mouse splenocytes increased (Fukui et al., 2013). Similarly, the spleen cells of mice treated with *L. brevis* KCTC12777BP also expressed high levels of TNF-α. *L. brevis* 12777BP improves immunity in mice and prevents organisms from being invaded by pathogens (Jeong et al., 2020b). The supernatant of *L. brevis* BGZLS10-17 can be divided into two components: a GABA-containing and a GABA-free component (Bajić et al., 2020). The supernatant containing GABA relies on ATG5 autophagy to stimulate Foxp3+, IL-10, and transforming growth factor-β, CTLA4 and Sirp-α isoimmunoregulatory molecule expression (Bajić et al., 2020). The GABA-free supernatant can also regulate the immune response through other mechanisms (Bajić et al., 2020). Heated *L. brevis* KB290 accelerated the secretion of IL-8, induced ERK1/2 phosphorylation, increase p38MAPK phosphorylation, and enhanced the expression of IL-8 mRNA (Yakabe et al., 2013). Heated *L. fermentans* SBC8803 inhibited IgE production and histamine secretion (Segawa et al., 2008b). *L. brevis* regulates immunity through various mechanisms and has anti-inflammatory effects. Unfortunately, the prevention and treatment of AD by *L. brevis* remain unclear. *L. brevis* may be an innate probiotic to inhibit AD development, and is a promising *Lactobacillus* strain that requires further research.

4 Multi-strain *Lactobacillus* for the treatment and prevention of AD

By critically reviewing the current literature, we also address the advantages and major disadvantages of the simultaneous use of two or more strains of probiotics. *Lactobacillus* supplementation can improve AD by regulating the intestinal microbiome. Intestinal flora has the potential to improve AD. In two experiments, administration of a *Lactobacillus* mixture had a preventive effect on AD in hairless mice (Holowacz et al., 2018a; Holowacz et al., 2018b). *L. plantarum* CJLP55, CJLP133, and CJLP136 isolated from kimchi inhibited AD-like skin lesions, reduced serum IgE levels, and restored the condition of the skin (Won et al., 2011). Moreover, multi-strain probiotics have been shown to have immunomodulatory effects and prevent AD in high-risk infants (Kukkonen et al., 2007). A mixture of heat-inactivated *L. casei* LOCK 0900, *L. casei* LOCK 0908, and *L. paracasei* LOCK 0919 modulated *in vitro* cytokine profiles of allergic children to produce anti-allergic Th1 reactions (Cukrowska et al., 2010). More specifically, prenatal and postnatal supplementation with a mixture of *Bifidobacterium* BGN4, *L.* aD011, and *Acidophilus* A031

can substantially reduce the probability of developing AD before the age of one year (Kim et al., 2010). Compared to a single strain, mixed lactic acid bacteria significantly enhanced the ability of Th1 cells to respond. Multi-strain probiotics help maintain good skin function, are beneficial for the treatment of AD (Rosenfeldt et al., 2003; Wang and Wang, 2015), and maintain intestinal barrier function in children with AD (Rosenfeldt et al., 2004). In addition, a mixture of *Lactobacillus* spp. improves the clinical symptoms of adults with AD (Iemoli et al., 2012).

However, benefits of the use of mixed probiotics should be interpreted with caution. Consumption of formula containing probiotics (*Bifidobacterium longum* BL999 and *L. rhamnosus* LPR) before the age of one year did not effectively prevent eczema in infants at high risk of allergies in Asia (Soh et al., 2009). Eczema symptoms in infants did not change when *L. paracasei* CNCM I-2116 or *Bifidobacterium lactate* CNCM I3446 were used as an adjunct to basic topical therapy (Gore et al., 2012). Moreover, there is no evidence that *L.* NFCM and *L.* Bi-07 affect the intestinal flora of children with AD (Larsen et al., 2011). The combination of probiotics did not have a positive effect on AD treatment, which may be due to several reasons. First, there were differences between strains, different combinations of strains, and individual differences in the study subjects. Second, although the researchers prudently selected the infants, irreversible immune-related events occurred. Therefore, probiotic supplementation had no effect on AD. Moreover, of the reason for the probiotic mixture not achieving the desired effect may be because the mother did not supplement with probiotics before antenatal administration. Asian populations may differ, as this is the first randomized controlled trial to be conducted in Asia (Soh et al., 2009). In addition, the lack of effect may be due to a smaller bacterial population and a smaller number of experimental subjects (Larsen et al., 2011). *Lactobacillus* species are complex, the strains are diverse, and their combinations are random and varied. Countless combinations of different strains of the same or different species occur. Perhaps, we can find an effective way to explore the therapeutic effects of these different combinations of strains on AD. Inappropriate strains can have negative effects. Perhaps strains with side effects negate the efficacy of beneficial strains, which needs to be explored further. Moreover, research in Asian populations is limited. The efficacy of mixed strains in AD treatment and prevention should be investigated in Asia, a region with a large population.

5 Conclusion

Based on reviews and meta-analyses, probiotics can prevent or treat AD, and perinatal administration of probiotics can prevent AD (Kuitunen, 2013; Mansfield et al., 2014; Panduru et al., 2015). Children (Tan-Lim et al., 2021) and adults with moderate to severe AD can also be administered probiotics to treat AD (Kim et al., 2014). In addition, the preventive effect of probiotics on pediatric AD is better than that of treatment (Lee et al., 2008). A mixture of *Lactobacillus* and *Bifidobacteria* can effectively reduce the incidence of eczema in infants and young children during the first three years of life (Sun et al., 2021). The preventive effects of probiotics on eczema appear to last until age of two (Dang et al., 2013) and extend to the age four years (Kuitunen, 2013). The variety and specificity of probiotic strains enriches therapeutic options. Nevertheless, there is substantial evidence that LGG supplementation does not reduce the prevalence of eczema (Kim et al., 2014). Not all *Lactobacillus* strains can be used to treat AD, and further experiments are needed to screen for the most effective *Lactobacillus* strains. Additionally, probiotics require repeated experiments to determine the mechanism of their efficacy against AD, effective dose, optimal administration time, and other characteristics.

In conclusion, since the discovery of *Lactobacillus*, numerous studies have demonstrated that this bacterium has a positive effect on the host (Tables 2, 3). In addition, previous research demonstrated the robust anti-inflammatory and homeostatic effects of *Lactobacillus*. The various health properties of *Lactobacillus* have been confirmed by different research groups. The mechanisms and beneficial effects of *Lactobacillus* are diverse (e.g., inflammation, immunity, gut health, brain function). Importantly, various types, species, and strains of *Lactobacillus exist*. Different strains from the same species have different functions. Therefore, research findings must be considered with utmost caution. The effect of some *Lactobacillius*, such as *L. rhamnosus* HN001 and *L. casei*, is retained for longer after supplementation; therefore, extending their function and permanent colonization of the intestine after regular supplementation can be studied. A portion of Lactobacillus is isolated from human organs. The absence of normal *Lactobacillus* colonization in some patients and options for restoration of these *Lactobacillus* strains are worth examining. In addition, different subtypes of the same *Lactobacillus* strain may have opposite effects; therefore, the positive and negative effects of the subtypes remain to be studied. A more potent subtype may suppress the effects of the effective subtype. However, most of the *Lactobacillus* strains are commensal or are present in food. Thus, ruling out an external interference in experiments is challenging. Finally, as a promising step towards precision and personalized medicine, *Lactobacillus* may become a food supplement to improve future AD treatments. Additional research is needed to explore other varieties of probiotic strains to enrich treatment options. Moreover, effective methods to preserve the activity and effects of probiotics should be explored. Importantly, additional human studies are needed to support the growing evidence of the beneficial effects observed in animal models of various diseases such as cancer, depression, and obesity.

Author contributions

AX is responsible for the collection of data and writing of the original manuscript. AC and YC are responsible for the organization of the original manuscript. ZL and SJ are responsible for editing. DC and RY are responsible for the concept development, review of the manuscript and revision. RY is responsible for funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Uncovering the specificity and predictability of tryptophan metabolism in lactic acid bacteria with genomics and metabolomics

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Tryptophan is metabolized by microorganisms into various indole derivatives that have been proven to alleviate diseases and promote human health. Lactic acid bacteria (LAB) are a broad microbial concept, some of which have been developed as probiotics. However, the capacity of most LAB to metabolize tryptophan is unknown. In this study, the aim is to reveal the rule of tryptophan metabolism in LAB by multi-omics. The findings showed that LAB were rich in genes for tryptophan catabolism and that multiple genes were shared among LAB species. Although the number of their homologous sequences was different, they could still form the same metabolic enzyme system. The metabolomic analysis revealed that LAB were capable of producing a variety of metabolites. Strains belonging to the same species can produce the same metabolites and have similar yields. A few strains showed strain-specificity in the production of indole-3-lactic acid (ILA), indole-3-acetic acid, and 3-indolealdehyde (IAld). In the genotype-phenotype association analysis, the metabolites of LAB were found to be highly consistent with the outcomes of gene prediction, particularly ILA, indole-3-propionic acid, and indole-3-pyruvic acid. The overall prediction accuracy was more than 87% on average, which indicated the predictability of tryptophan metabolites of LAB. Additionally, genes influenced the concentration of metabolites. The levels of ILA and IAld were significantly correlated with the numbers of aromatic amino acid aminotransferase and amidase, respectively. The unique indolelactate dehydrogenase in *Ligilactobacillus salivarius* was the primary factor contributing to its large production of ILA. In summary, we demonstrated the gene distribution and production level of tryptophan metabolism in LAB and explored the correlation

between genes and phenotypes. The predictability and specificity of the tryptophan metabolites in LAB were proven. These results provide a novel genomic method for the discovery of LAB with tryptophan metabolism potential and offer experimental data for probiotics that produce specific tryptophan metabolites.

KEYWORDS

tryptophan, metabolism, lactic acid bacteria, specificity, predictability, genomics, metabolomics

1 Introduction

Tryptophan metabolites play an important role in both the development of diseases and the homeostasis of the human body's internal environment (Platten et al., 2019). The catabolism of tryptophan in mammals mainly includes the kynurenine (KYN) pathway, the serotonin pathway, and the indole derivative pathway, of which the indole derivatives can only be produced by microbes (Agus et al., 2018). The KYN pathway is the main direction of tryptophan catabolism, which consumes about 90% of tryptophan (Cervenka et al., 2017). However, previous studies found that metabolites produced along the KYN pathway can help tumors escape immune surveillance and promote their development (Offringa et al., 2022). Tryptophan can be metabolized by microorganisms into a variety of indole derivatives, which the host is unable to produce. The KYN pathway competes with this metabolic pathway. Recently, an increasing number of studies have demonstrated that indole derivatives played a significant role in preventing the onset and progression of diseases, particularly in chronic conditions like inflammatory bowel disease (Flannigan et al., 2022), atopic dermatitis (Fang et al., 2022), Alzheimer's disease (Sun et al., 2022), alcoholic liver disease (Wrzosek et al., 2021), and coronary artery disease(Li et al., 2022). Intestinal microorganisms are the key to produce indole derivatives that can alleviate chronic diseases. *In vitro* fermentation experiments showed that *Clostridium* and *Peptostreptococcus* could produce 3-indoleacrylic acid (IA) and indole-3-propionic acid (IPA) (Dodd et al., 2017; Wlodarska et al., 2017). IA was thought to ease colitis and improve intestinal epithelial barrier performance. The risk and severity of atherosclerosis were significantly negatively correlated with the serum IPA concentration in patients with coronary heart disease (Xue et al., 2022). *Bifidobacterium longum* could produce 3-indolealdehyde (IAld) to relieve symptoms of patients with atopic dermatitis by activating AhR (Fang et al., 2022). *Bifidobacterium* has also been proven to metabolize tryptophan to indole-3-lactic acid (ILA), which could help build the infant's early immune system (Laursen et al., 2021). However, ILA, IAld and indole-3-acetic acid (IAA) could be metabolized by multiple microorganisms, such as *Escherichia coli*, *Bacteroides*, *Clostridium*, and *Faecalibacterium prausnitzii* (Roager and Licht, 2018). To sum up, these intestinal microorganisms jointly regulate the complex metabolism of indole derivatives and have a profound impact on human health. Therefore, regulating the metabolism of indole derivatives by intestinal microorganisms to promote human health has become the research hotspot.

Lactic acid bacteria (LAB) are a class of bacteria that can ferment and produce lactic acid. Some LAB are considered to have prebiotic functions and have been developed as dietary supplements to be used to regulate the balance of the human intestinal environment and promote the beneficial shift of gut microbiota (De Filippis et al., 2020). Previous studies demonstrated some LAB can convert tryptophan into indole derivatives and influence human immunity (Zelante et al., 2013; Cervantes-Barragan et al., 2017). However, currently known LAB that can metabolize tryptophan are mainly *Limosilactobacillus reuteri*, and the metabolic ability of other species is unclear. Previous studies have shown that *Bifidobacterium* of the same species but from different sources have different abilities to use carbon sources (Liu et al., 2021). Therefore, it is also necessary to investigate whether the isolation source has an impact on the LAB' capacity to metabolize tryptophan. The evidence indicated that a complete metabolic enzyme system can predict any metabolite of microorganisms (Rath et al., 2017; Heinken et al., 2019). The necessity of the tryptophan metabolism gene in the transformation of indole derivatives by microorganisms has been confirmed (Hutcheson and Kosuge, 1985; Koga, 1995; Williams et al., 2014; Dodd et al., 2017; Wlodarska et al., 2017), and the bacterial tryptophan metabolism profile, jointly constructed by catabolites and enzymes, has also been improved (Roager and Licht, 2018). Therefore, it seems possible to use comparative genomic approaches to construct tryptophan metabolism enzyme spectra of different LAB and explain the tryptophan metabolism ability of LAB through association analysis with metabolomics.

In this study, we selected 148 strains from 13 LAB species. These species have been extensively studied and were considered beneficial to human wellness. Some species appeared to be involved in tryptophan metabolism. For instance, it has been demonstrated that *L. reuteri* DSM 20016 produced IAld and ILA (Zelante et al., 2013). However, in-depth research has not been reported to determine whether this metabolic capability is universal in *L. reuteri*. Therefore, in order to explore the specificity of tryptophan metabolism in these species and their prbiotic potential, we conducted the following experiments: The homology of the tryptophan metabolism genes in LAB was analyzed by using

comparative genomic approaches, and the potential metabolites were predicted. Additionally, the capacity of every strain to metabolize tryptophan *in vitro* was investigated using targeted metabolomics. Combing genomic analysis with metabolomic results according to bioinformatic methods, we demonstrated the predictability of tryptophan metabolites in LAB. We also explored the effect of the number of genes on the metabolism of tryptophan and found two kinds of indolelactate dehydrogenase that may control the high production of ILA in *Ligilactobacillus salivarius*.These results help to understand the complex metabolism of tryptophan in LAB and provide an experimental basis and new ideas for the development of probiotics.

2 Materials and methods

2.1 Selection of strains

The 148 strains used in this study were isolated from the Culture Collection of Food Microorganisms of Jiangnan University (Wuxi, China), and we sequenced the genomes of each strain individually. They are distributed in 13 species from 6 genera (Table 1). The specific information for all LAB is listed in Table S1.

2.2 DNA extraction, sequencing, and prediction of coding sequences

All LAB were cultured on MRS plates for 24 hours; single colonies were selected and cultured in MRS broth for 12–14 hours until the early stage of stabilization. The culture was removed after centrifugation at 5000 x g for 10 min, and bacterial cells were washed with 0.9% sterile saline and collected under the same centrifugation conditions. The DNA of LAB was extracted according to the operation process of the rapid bacterial genetic DNA isolation kit (Sangon Biotech Ltd., Shanghai, China).

TABLE 1 Lactic acid bacteria speices in this study.

Genus	Species	No. of Strains	Source
Lacticaseibacillus	*paracasei*	10	Human feces; Chinese pickle
Lacticaseibacillus	*rhamnosus*	9	Human feces
Lactiplantibacillus	*plantarum*	10	Human feces; Chinese pickle
Lactiplantibacillus	*pentosus*	8	Human feces; pickles
Lactobacillus	*acidophilus*	8	Human feces
Lactobacillus	*crispatus*	14	Human feces; Human vagina
Lactobacillus	*gasseri*	8	Human feces
Lactobacillus	*helveticus*	9	Fermented yak milk; Yak qula; Dairy fan
Latilactobacillus	*curvatus*	4	Human feces
Ligilactobacillus	*salivarius*	12	Human feces
Limosilactobacillus	*fermentum*	11	Human feces
Limosilactobacillus	*mucosae*	15	Human feces
Limosilactobacillus	*reuteri*	30	Fermented rice milk; Human feces

Illumina HiSeq platform (Novogene Biotech Ltd., Tianjin, China; Majorbio Biotech Ltd., Shanghai, China), which produced 2 × 150-bp pair-end read libraries, was used for genome sequencing. For each sample, the raw data were provided and then trimmed into high-quality reads with a minimum of 100 genome coverage depth (clean data). The software SOAPdenovo2 (Luo et al., 2012) was used to assemble contigs, and then we tested various Kmer values and obtained the optimal assembly result. The assembly result was then partially assembled and optimized to form scaffolds based on the relationship between paired-end reads and read overlaps.

The software Prodigal (version, 2.6.3; -p, single; -g, 11) predicted the coding sequences in all LAB genomes and translated them into protein sequences for subsequent homologous protein searching (Hyatt et al., 2010).

2.3 Identification of genes associated with tryptophan metabolism

We have identified the genes involved in microbial tryptophan metabolism and the corresponding Enzyme Commission (EC) number by consulting previous studies on microbial tryptophan metabolism (Agus et al., 2018; Roager and Licht, 2018) and the gene information displayed in the tryptophan metabolism pathway in Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp). The specific information on tryptophan metabolic enzymes involved in this study is listed in Table S2. We obtained the protein sequence corresponding to the tryptophan metabolism gene from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) RefSeq and GeneBank databases by the full name of the enzyme and EC number. The tryptophan metabolic enzymes of LAB were identified using these proteins as reference sequences.

2.4 Homology search and prediction of tryptophan metabolites

The homology analysis of LAB proteome was completed by DIAMOND (version, 2.0.14) BLASTP (identity, 30%; E value, 1e-3; subject cover, 70%; query cover, 70%; –ultra-sensitive) (Buchfink et al., 2021). The specific homology search information for all strains is listed in Table S3. Hit sequences that satisfy the aforementioned parameters are regarded as homologous sequences involved in the metabolism of tryptophan. The sequences with the highest homology were used to summarize the tryptophan metabolic enzyme results in LAB, and each strain's tryptophan metabolic products were predicted. Strains with a complete enzyme system can produce the corresponding metabolites. For instance, it is predicted that a strain will be able

to produce indole propionic acid if it possesses the homologous sequences of aromatic amino acid aminotransferase (ArAT), indolelactate dehydrogenase (*fldH*) or lactate dehydrogenase (LDH), cinnamoyl-coA: chenyllactate coA-transferase (*fldA*), phenyllactoyl-coA dehydratase alpha/beta (*fldBC*), R-phenyllactate dehydratase activator (*fldI*), and phenylacrylate reductase (*acdA*).

2.5 Extraction of tryptophan metabolites from *in vitro* fermentation

Strains from glycerol preservation tubes were transplanted to MRS plates and grew at 37°C until a single colony appeared. Single colonies were then selected and grown in MRS broth for 12 hours to the late logarithmic phase (two groups of culture media, one for further experiments and the other for determining CFU/OD). ensuring that there are 10^9 CFU of bacteria in total per milliliter. Bacterial precipitation is washed twice with physiological saline after MRS broth has been centrifuged at 4000 x g for 10 min. According to previous studies, there was a significant negative correlation between microorganisms' capacity to metabolize amino acids and their rate of growth. These catabolic reactions need to be carried out effectively under the condition of non-rapid growth of bacteria (Vieira-Silva et al., 2016). Therefore, resting cell fermentation (Dodd et al., 2017) was used to explore the tryptophan metabolism ability of LAB. Briefly, after 1 h of resuspended bacterial precipitation in potassium phosphate buffer, the ATP level reaches a constant value, indicating that the majority of the remaining substrate has been consumed (Johann Bader, 1983). The bacteria cells were separated by centrifugation and resuspended in buffer containing 1 mM tryptophan, which was similar to the concentration of tryptophan in the small intestine (Koper et al., 2022). After one hour of standing at 37°C, the supernatant needed to be immediately frozen at -80°C for future metabolomic analysis.

2.6 Metabolomics

The LAB *in vitro* fermentation supernatant samples undergone the following pretreatments: First, 100 µL of fermentation supernatant and 400 µL of pre-cooled methanol were fully mixed, and then stood at -20°C for 30 min. To remove the protein, the mixture was centrifuged at 20000 × g for 15 min. After that, 300 µL of the supernatant was vacuum-dried at 45°C. The obtained dry matter was resuspended in 100 µL of methanol dilution (water: methanol=4:1) and filtered through the 0.22 µm microporous membrane. The filtrate was tested for tryptophan metabolites using ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS).

Compounds were separated by using ACQUIRE UPLC BEH C18 column (Waters, Milford, MA, USA; 1.7 m, 2.1 100 mm) and detected by using Vanquish UHPLC Q-Exactive Plus MS (Thermo Fisher, CA, USA) with extended a dynamic range (100-300 m/z) in positive ion scanning mode. The mobile phase consisted of eluent A (acetonitrile, Supelco, Sigma-Aldrich) and eluent B (0.1% formic acid, Supelco, Sigma-Aldrich). The sample with a volume of 2 µL was injected into the mobile phase, and chromatographic separation was performed at a flow rate of 0.3 mL/min. The linear gradient of the mobile phase was as follows: 5% A and 95% B in the initial 3 min; 3–9 min, eluent A from 5% to 30%, eluent B from 95% to 70%; 9–15 min, mobile phase A rising from 30% to 100%; kept 100% mobile phase A unchanged for 15-16.5 min; in the last 3.5 min, maintained 5% mobile phase A and 95% mobile phase B for rebalancing the column. The indole derivatives metabolized by LAB were qualitatively and quantitatively analyzed using a standard curve. The metabolism of all strains has been listed in Table S4. The specific information, such as the retention time of reference materials, has been listed in Table S5.

2.7 Phylogenetic analysis

To identify the potential key players in the production of ILA, the homologous protein sequences annotated as *fldH* and without specific substrate catalytic function were used to constructed the phylogenetic tree. The phylogenetic tree of LDH was also built. MUSCLE (version, 3.8.31) was used to align all homologous sequences of *fldH* and LDH respectively (Edgar, 2004) and IQ-TREE (version 2.2.0-Linux; model, MF; bootstrap, 1000) was used to build the evolutionary tree of the sequence(Nguyen et al., 2015). iTOL is used to visualize the outcomes(Letunic and Bork, 2021). Most branches of the phylogenetic tree of *fldH* and LDH were folded to better illustrate the evolutionary distance between the unique protein sequence and other sequences, but this did not modify the topological structure of the phylogenetic tree.

2.8 Protein structure analysis

AlphaFold2 (version, 1.4; pair mode, unpaired_paired; model type, default) was used to construct the *fldH* homologous protein in *L. salivarius* FWXBH185 and the experimentally verified *fldH* (Uniprot: J7SHB8) protein structure in *C. sporogenes* ATCC 15579. We used the Pairwise Structure Alignment function (algorithm, jFATCAT-rigid) provided by PDB (https://www.rcsb.org) to establish residue-residue correspondence between *L. salivarius* FWXBH185 *fldH* and *C. sporogenes* ATCC 15579 *fldH*.

2.9 Statistical analysis and visualization

IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA) was used to calculate Spearman's rank correlation coefficient (Spearman's ρ; Benj n Hochberg false discovery rate [FDR]). The Mann-Whitney test is used to analyze whether there are metabolic differences among strains belonging to the same species but from different sources. All data sets were collated using Python 3. Graphpad Prism 9 (La Jolla, California, United States) was used for data visualization. The subsequent editing of all figures was completed with Adobe Illustrator CC2022.

3 Results

3.1 LAB are rich in genes of tryptophan catabolism

The KYN pathway, the indole pathway, and the serotonin pathway are the three main metabolic pathways for tryptophan. Only microbes are capable of producing indole derivatives. In this study, we focused on nine indole derivatives that play an important role in human health. Except for the enzyme that converts IAA into IAld, the metabolic enzyme spectrum of other indole derivatives is clear (Figure 1A).

We annotated the tryptophan metabolic genes of 148 strains (Table 1) to determine whether LAB encode these tryptophan metabolic enzymes (Table S3). As a result of LAB metabolic genome annotation, the sequence with the highest homology was displayed (Figure 1B), and all homologous sequences were in the corresponding gene count (Figure 2). ArAT, Tryptophan decarboxylase (TDC), and Tryptophan 2-monooxygenase (TMO) are three enzymes that directly use tryptophan as a substrate (Figure 1A). We found that all strains did not encode TDC, and the tryptamine (TA) metabolic pathway did not exist in LAB. The majority of strains also failed to locate the TMO homologous sequence, but all LAB contained amidase (*amiE*). Except for *Latilactobacillus curvatus*, ArAT was found in the genome of all LAB, and most (10/12 species) LAB contained multiple ArAT (Figure 2), which was reported that existed widely in *L. reuteri* previously (Ozcam et al., 2019). Tryptophan will be converted to ILA by *fldH* or LDH after being catabolized to indole-3-pyruvic acid (IPYA) by ArAT (Dodd et al., 2017; Laursen et al., 2021). The evidence of *fldH* was first found in *Clostridium*, and the previous labeling of *fldH* in *Lactobacillus* was mostly replaced by LDH (Montgomery et al., 2022), which was enriched in LAB, especially in *Limosillactobacillus* (Figure 2). LDH and *fldH* originate from the same EC number (1.1.1.-), and the protein sequence homology was ~40%. In this study, most of the homologous proteins of *fldH* in LAB were annotated as D-2-hydroxyacid dehydrogenase (HdhD) in NCBI, which were also from the same EC number as *fldH*, and the sequence homology was ~40%. This might indicate that HdhD is a neglected ILA dehydrogenase. In addition, the directly adjacent of the *fldH* and ArAT in some LAB suggested that they are functional metabolic operons (Table S3). In terms of genotype, LAB other than *L. curvatus* appeared to be able to produce ILA, but none of them created a gene bridge for the transformation of ILA into IA because they were all deficient in the *fldIBC* functional gene cluster. All LAB contained aldehyde dehydrogenase (ALD), just like ArAT. Additionally, some strains encoded indolepyruvate decarboxylase (IPD) or phenylpyruvate decarboxylase (PPD), which together contributed to the conversion of tryptophan to indoleacetic acid.

The tryptophan metabolites of LAB were then predicted using the gene annotation results (Table 2). Since *L. curvatus* contained no first-step metabolic enzyme, it was considered that it did not have any metabolic ability for indole derivatives. Other LAB maintained a high degree of similarity in the production of IPYA, TA, ILA, IA, and IPA. The predicted results of other metabolites

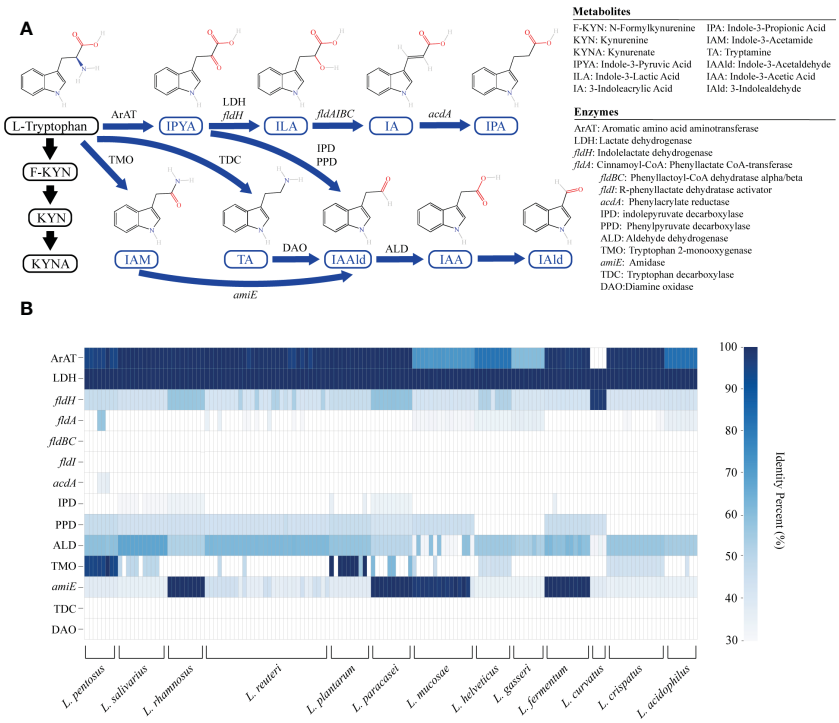

FIGURE 1
The genome of lactic acid bacteria (LAB) is rich in tryptophan metabolism genes. **(A)** The primary tryptophan metabolites and metabolic network. The host pathway is represented by black, and the microbial pathway is represented by blue. **(B)** The heatmap represents the sequence identity of the best hit of the tryptophan metabolism genes in LAB strains. Genes with more than 30% identity are considered homologous sequences.

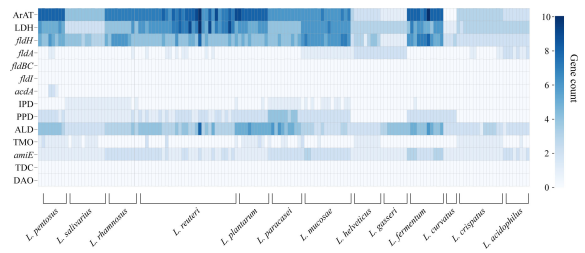

FIGURE 2
The heatmap shows the number of tryptophan metabolism genes contained in lactic acid bacteria strains, and the color depth indicates the homologous tryptophan metabolism gene count.

were species- or strain-specific. To sum up, LAB encoded a large number of tryptophan metabolic genes. LAB had genetic evidence for the metabolism of tryptophan into multiple metabolites.

3.2 The specificity of LAB in tryptophan metabolites

We performed *in vitro* experiments to confirm the outcomes of gene prediction after obtaining genomic evidence of tryptophan metabolism in LAB. The findings demonstrated that LAB could generate a wide range of tryptophan metabolites, and the majority of LAB maintained a high level of species specificity in metabolism (Figure 3A). IA (4.139-41.789 ng/mL) was found in the fermentation broth of all strains of *Limosillactobacillus mucosae* (Figure 3B), but prior genomic homology analysis had not revealed any homologous sequences with the *fldIBC* gene cluster. No strain can produce IPA, which is consistent with the result of gene prediction. However, tryptamine (20.454-58.145 ng/mL) was detected in the fermentation of all strains of *Lactobacillus helveticus* (Figure 3C), which, like IA, also lacked genetic evidence. The production of ILA demonstrated that IPYA had appeared in the fermentation process because IPYA was the only upstream product of ILA, even though we were unable to detect IPYA in the fermentation supernatant of LAB. IPYA might be rapidly converted into ILA or indole-3-acetaldehyde (IAAld), which made it difficult to enrich. As a result, we believed that LAB, except for *L. curvatus*, were capable of producing IPYA and ILA, which is consistent with the findings of gene prediction. Additionally, compared with other LAB (0-1368.567 ng/mL, mean = 102.554 ng/mL), *L. salivarius* (566.553-3864.553 ng/mL, mean = 1907.063ng/mL) had a stronger ability to metabolize ILA, especially *L. salivarius* FWXBH185 (3864.553 ng/mL) and FBJSY202 (3463.081 ng/mL) (Figure 3D). Contrary to gene prediction results, indole-3-acetamide (IAM) could not be found in all LAB fermentation broths. Its downstream metabolite, IAA, had inconsistent *in vitro* fermentation and gene prediction results, and metabolic concentration varied between species. *Lactiplantibacillus pentosus* (0-40.967 ng/mL, mean = 22.507 ng/mL) produced more IAA than other LAB (0-19.667 ng/mL, mean = 4.594 ng/mL) (Figure 3E). We also detected the concentration of IAld in the LAB fermentation broth. Except for *L. curvatus*, other LAB (2.984-332.681 ng/mL, mean = 40.266 ng/mL) could produce IAld, and the yield was relatively conservative at the species level, but some strains of *L. reuteri* had strong IAld metabolism abilities (Figure 3F), such as DYNDL2M15 (184.103 ng/mL), DYNDL8M31 (332.681 ng/mL), and FSCPS76L4 (268.246 ng/mL).

We also examined how the tryptophan metabolism of strains from various sources varied. All strains from the same species in this study had the same category of tryptophan metabolites, despite the fact that some species had multiple sources for their isolation (Table S4). The yield of ILA of *L. helveticus* DYNDL451 from dairy

TABLE 2 Predicted tryptophan metabolites of lactic acid bacteria at the species level.

Species	No. of Strains	Proportion of strains in the species that could produce the following metabolites (%)							
		IPYA	ILA	IA	IPA	IAM	TA	IAAld	IAA
L. acidophilus	8	100	100	0	0	12.5	0	0	12.5
L. crispatus	14	100	100	0	0	100	0	0	100
L. curvatus	4	0	0	0	0	0	0	0	0
L. fermentum	11	100	100	0	0	0	0	100	100
L. gasseri	8	100	100	0	0	0	0	0	0
L. helveticus	9	100	100	0	0	88.9	0	0	88.9
L. mucosae	15	100	100	0	0	13.3	0	100	100
L. paracasei	10	100	100	0	0	40	0	100	100
L. plantarum	10	100	100	0	0	80	0	100	100
L. reuteri	30	100	100	0	0	6.7	0	100	100
L. rhamnosus	9	100	100	0	0	0	0	100	100
L. salivarius	12	100	100	0	0	66.7	0	100	100
L. pentosus	8	100	100	0	0	100	0	100	100

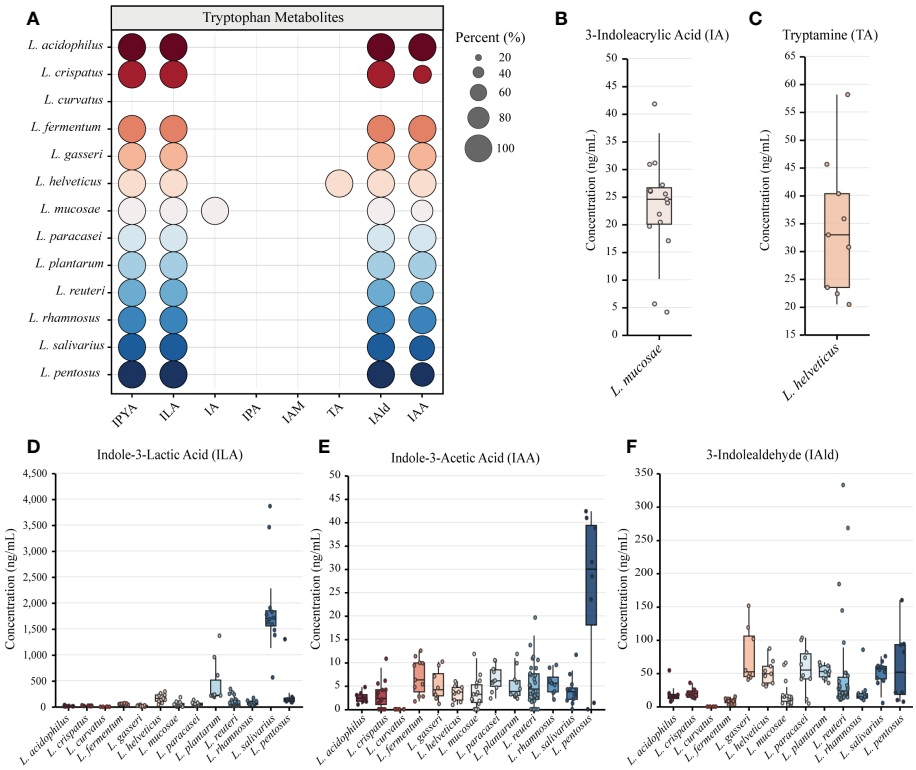

FIGURE 3
Metabolomics revealed the tryptophan metabolism ability of lactic acid bacteria (LAB). **(A)** LAB species metabolize a variety of indole derivatives. The proportion of strains within a species that produce metabolites is indicated by the size of the circle. Different colors represent different species. **(B–F)** The concentrations of all tryptophan metabolites detected in the fermentation broth of different strains are shown by box plots. All data were collected by repeating three experiments and taking the average of the results. Tukey's honestly significant difference test was used to exclude discrete points.

fan was lower when compared to the strains isolated from fenmented yak milk and yak qula. The results of Mann-Whitney test showed that the IAld metabolic level of *L. reuteri* from fermented rice milk was significantly higher than that of the strain isolated from human feces. There was no significant difference in the tryptophan metabolism level of strains from different sources in other species (Figure S1).

3.3 Accuracy of prediction by genomic-metabolomic association analysis

To confirm the gene prediction, we used the metabolism results of LAB. In LAB, only *L. helveticus*, whose TA metabolic gene and true metabolic level could not correspond (Figure 4A). Additionally, *L. mucase* produced IA, although no homologous complete *fldAIBC* gene cluster was found in all (Figure 4B). IAM and IAA's actual metabolisms and gene predictions both revealed species-specificity, and the two metabolites' gene prediction results were less than 80% accurate. The gene prediction results and metabolism of the three metabolites IPYA, ILA, and IPA in all LAB strains are completely consistent, and the accuracy of gene prediction is 100% (Figure 4C). The average accuracy of gene prediction is more than 88%.

3.4 Effect of gene diversity on the concentration of tryptophan metabolites in LAB

3.4.1 Gene count

We then investigated the correlation between the quantity of genes and the concentration of metabolites using Spearman's ρ (Figure 5A). The significance would be shown only when the correlation is greater than 0.3. The number of homologous sequences of ArAT was significantly positively correlated with the ILA production of LAB, but Spearman's ρ was only 0.31, which was a relatively weak correlation. The concentration of IAld was significantly negatively correlated with the number of *amiE*. In conclusion, the homologous genes of tryptophan metabolism found in LAB could be used to predict the ability of LAB to metabolize tryptophan. Additionally, the number of genes may control how metabolites are produced. These findings suggest that genotype and phenotype are strongly correlated.

3.4.2 The IPYA to ILA conversion is primarily controlled by *fldH*

ILA is currently the most researched indole derivative. It can alleviate the occurrence and development of various diseases and regulate immune homeostasis (Zelante et al., 2013; Cervantes-

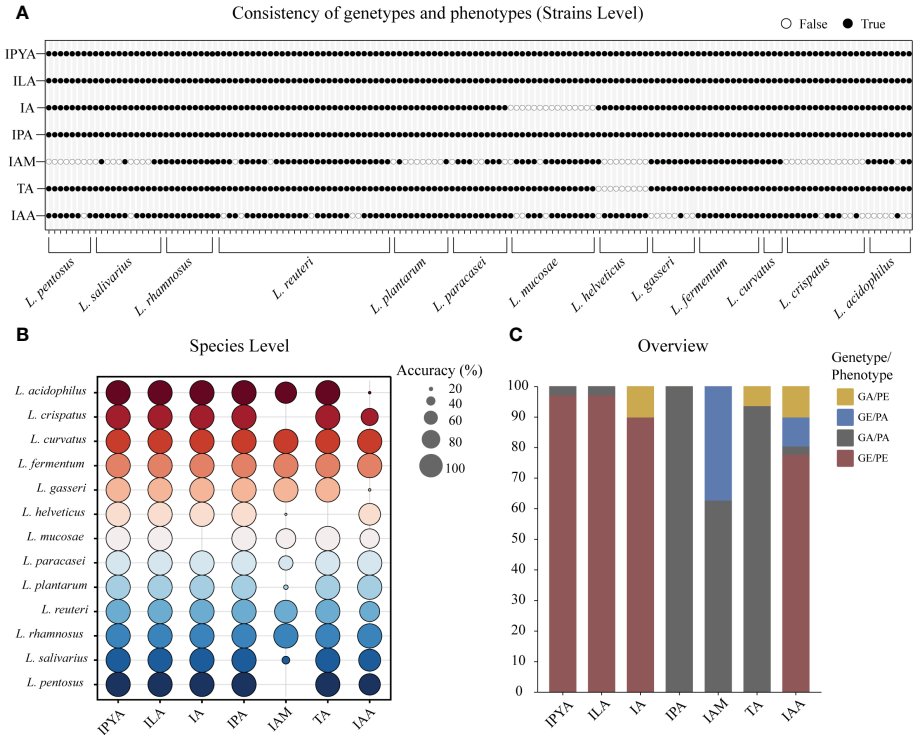

FIGURE 4
The metabolites of lactic acid bacteria (LAB) predicted by homologous genes had high consistency with actual production. **(A)** The binary diagram illustrates whether the predicted products and metabolites of each strain are consistent. White filling denotes the opposite of the two results, black filling indicates the predicted results are consistent to the metabolic results. **(B)** The consistency of genotype and phenotype is reflected at the species level. The accuracy of the metabolite prediction within a species increases with the size of the prediction circle. Different colors represent different species. **(C)** The stacked column chart provides an overview of the consistency between the metabolomics-based actual metabolites and the genomics-based predicted metabolites in LAB. Phenotype absence (PA); Phenotype presence (PE); Gene presence (GE); Gene absence (GA).

Barragan et al., 2017; Laursen et al., 2021). In this study, *L. salivarius* demonstrated exceptionally high ILA metabolism capacity (Figure 3D). Although the above result showed that the content of ILA was positively correlated with the number of ArAT, the correlation was weak, and the number of ArAT in *L. salivarius* was not the highest among LAB, being far less than that in *Limitsilactobacillus* species. Previous research had shown that a specific LDH controlled ILA production in *Bifidobacterium* (Laursen et al., 2021), whereas *fldH* controlled the metabolic processes of *C. spologenes* ATCC 15579, which could produce a significant amount of ILA at resting cells (Dodd et al., 2017). We, hypothesized that LDH or *fldH* might be the enzyme in charge of regulating ILA production in LAB. The phylogenetic analysis of all *fldH* in LAB revealed that the *fldH* encoded by the *L. salivarius* independently clustered (here called type 1, type 2, type 3, and type 4 *fldH*). Type 4 *fldH* had close genetic distance and high affinity with other LAB. The other three types of *fldH* were far from the fldH of other LAB, suggesting that they may have the unique potential to efficiently convert IPYA into ILA. However, type 1 and type 2 *fldH* could be found in all strains of *L. salivarius*, but type 3 *fldH* only existed in the genomes of 7 strains of *L. salivarius* (Figure 5B). The phylogenetic analysis of LDH did not reveal a comparable phenomenon. There are no independent gene clusters, and the genetic distances between LDH of *L. salivarius* and LDH of other LAB were very close (Figure S2). Then, we compared the sequence homology, constructed the protein structure and compared the protein structure similarity of the *fldH* (Uniprot: J7SHB8) of *C. spologenes* ATCC 15579 (here called reference *fldH*) and type 1-4 *fldH* of *L. salivarius* FWXBH185, which was the largest production of ILA in all LAB. Type 1, type 2, type 3, and type 4 *fldH* shared 43.6%, 40.6%, 38.5%, and 35.3% homology with reference *fldH*, respectively (Table 3). In protein structure alignment, the type 1-3 *fldH* models could well overlap the reference *fldH* protein model (Figure 6), which had low root mean square deviation (RMSD) and high template modeling score (TM-score).They might possess the same substrate catalytic center. However, type 4 *fldH* showed poor structural consistency with reference *fldH*, with RMSD ~2.26 and TM-score ~0.85 (Table 3).

4 Discussion

The tryptophan metabolism of microorganisms is a complex metabolic network. Numerous enzymes have an impact on the metabolites in this pathway (Table S2). To produce the corresponding metabolites, a complete metabolic enzyme system is required (Dodd et al., 2017). Previous studies have found a small amount of gene evidence for tryptophan metabolism-related enzymes encoded in LAB, but only ArAT has been experimentally confirmed at present(Zelante et al., 2013; Cervantes-Barragan et al., 2017; Montgomery et al., 2022). It is widely present in *L. reuteri* and has numerous gene copies. The likelihood that LAB also codes for other

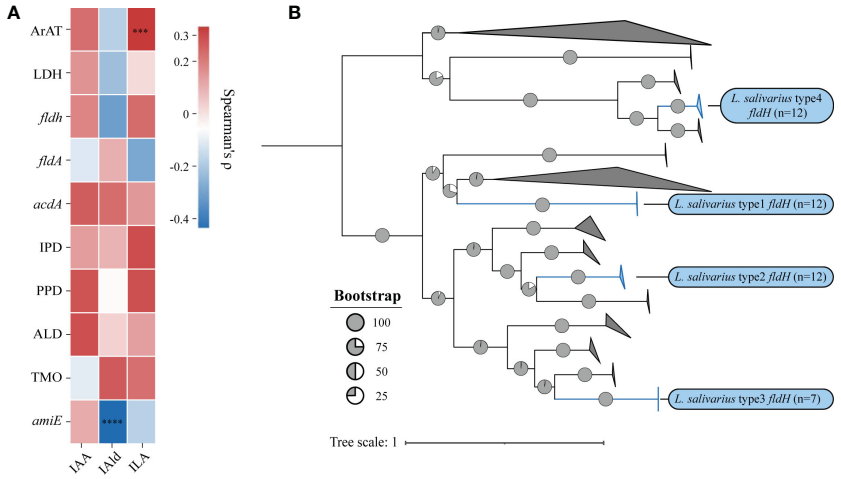

FIGURE 5
(A) The heatmap represents the correlation between the number of genes and the concentration of metabolites, and the statistical significance will be marked only when Spearman's ρ (Benjamin Hochberg false discovery rate [FDR]) is greater than 0.3.***p = 0.0003, ****p < 0.0001. **(B)** Phylogenetic trees were built using the indolelactate dehydrogenase (*fldH*) homologous sequences of all lactic acid bacteria. Without modifying the topological structure of phylogenetic trees, the folded branches were used to facilitate the display of two different types of *fldH* sequences of *L. salivarius*. The folded cluster of *fldH* from *L. salivarius* was marked blue.

tryptophan-metabolizing enzymes, in addition to ArAT, depends on the homology evidence of the corresponding enzymes from other bacteria. In this section, we widened the range of reference sequences among species and genera and carried out a homologous search for sequences in the LAB genome. LAB contained a large number of tryptophan metabolism genes according to the results of gene counting. According to the gene annotation results of the sequence with the highest homology, whether most tryptophan metabolizing enzymes can be detected from the genome is species-specific. All LAB had the metabolic enzymes *amiE* and ALD, but not all LAB had their upstream tryptophan metabolism genes encoded. ALD and *amiE*, however, are not indole derivative-specific catalytic proteins (Racker, 1949; Bray et al., 1950), they have additional substrates in other metabolic pathways, indicating that they have additional roles in LAB.

In metabolomics, although we did not detect IPYA in the fermentation broth, as was also the case in earlier studies, it is the only starting point of the ILA metabolic pathway, and the existence of ILA could prove that IPYA appears briefly *in vitro* fermentation. The metabolic level of ILA remained highly species-specific. Although the production of ILA in different *L. salavarius* strains fluctuated greatly, their levels were still significantly higher than those of other LAB strains. Especially FWXBH185 and FBJSY202, which could produce large amounts of ILA, may have great potential to maintain human immune balance (Laursen et al., 2021). IAA and IAM metabolism in LAB deviated from gene predictions, and there was strain specificity. Although we predicted some strains would produce IAM, we were unable to locate any pertinent evidence during the *in vitro* fermentation. IAA also has annotation genes but no phenotype. These results might indicate that IAM and IAA were used as intermediate metabolites in the fermentation process, especially IAM, which was the first step metabolite. As the precursor of IAld (De Mello et al., 1980), IAA might also be difficult to enrich during fermentation. The metabolism of TA and IA in LAB was species-specific, and there was no homologous gene in the LAB genome, but some species produced these metabolites during fermentation. This demonstrates that the availability of some tryptophan metabolic genes in existing open databases is still limited and that to increase the hit rate of the query sequence, the reference sequence range needs to be expanded (Rath et al., 2018). However, although there were deviations between the predicted results of some genes and the phenotype, the average accuracy rate was more than 88%, which indicated that genes could predict the metabolites of LAB to a certain extent. In addition, we tracked the metabolism of IAld in LAB. It can activate the AhR receptor and regulate the level of IL-22 in cells to alleviate the symptoms of host colitis (Renga et al., 2022), which plays an important role in the human immune system. *L. reuteri*

TABLE 3 The *fldH* structure alignment between *Ligilactobacillus salivarius* FWXBH185 and *Clostridium sporogenes* ATCC 15579.

Type ID	Loci	RMSD	TM-score	Sequence Identity (%)	Reference Coverage (%)	Target Coverage (%)
Type1 *fldH*	Scaffold7_93500 92511	1.71	0.93	43.6	99	99
Type2 *fldH*	Scaffold3_94834 95829	1.82	0.93	40.6	99	99
Type3 *fldH*	Scaffold17_13829 14821	1.85	0.93	38.5	99	99
Type4 *fldH*	Scaffold1_494215 495171	2.26	0.85	35.3	93	97

RSMD, root mean square deviation; TM-score, template modeling score.

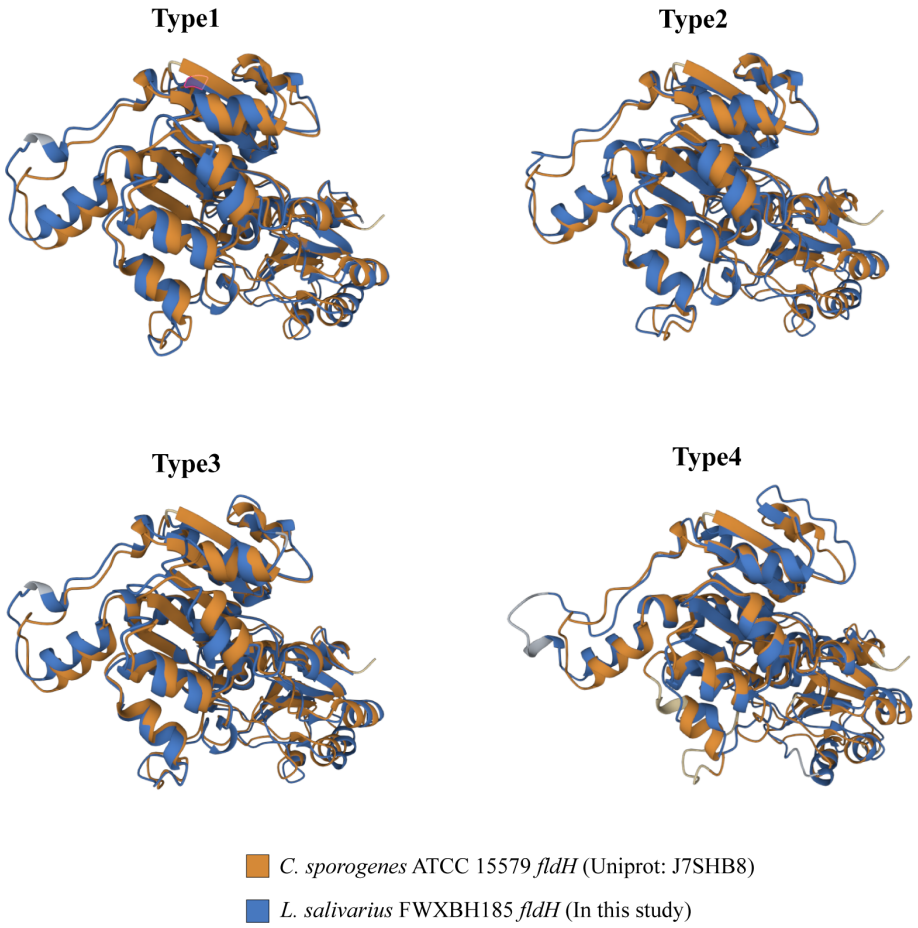

FIGURE 6
All *fldH* of *L. salivarius* FWXBH185 was compared with the reference *fldH* (Uniprot: J7SHB8) of *C. sporogenes* ATCC 15579 *via* protein structure alignment. The proteins were overlapped to show the similarity of the spatial structure of the two *fldH* proteins. The structure of *C. sporogenes* ATCC 15579 *fldH* was marked with yellow. The *fldH* of *L. salivarius* FWXBH185 was marked with blue, and the type of fldH was marked on the top of the structure.

DYNDL8M31, FSCPS76L4 and DYNDL2M15 metabolized a large amount of IAld, and these strains might become the focus of probiotic development in the future. The results of metabolomics also revealed the regulation of tryptophan metabolism in LAB of the same species but from different sources. Different sources of separation did not appear to alter the types of tryptophan metabolites that LAB produce, but they may have an impact on some LAB' metabolic ability. It is important to note that despite the fact that the Mann-Whitney test revealed a significant difference between the production of IAld by *L. reuteri* from fermented rice milk and other strains, due to the small number of samples, this statistical analysis may be influenced by the results of DYNDL8M31, which could produce a high yield of IAld. In general, the types of indole derivatives produced by LAB are not affected by the isolation source, but the relationship between the isolation source and the production of tryptophan metabolites by LAB needs to be explained through more experimental data. Additionally, the growth environment of bacteria has a direct impact on their metabolism of tryptophan. According to earlier research, *Bifidobacterium* could only convert tryptophan to ILA in MRS (Laursen et al., 2021). However, in M9 medium, which has much fewer nutrients than MRS, *Bifidobacterium* can produce a variety of indole derivatives (Fang et al., 2022). This indicates that the various nutrients that are available to bacteria in the intricate environment of the human intestine will also influence their catabolism. There are many nutrients in the proximal colon. All microorganisms are under pressure to grow quickly in order to occupy a more favorable niche. In the proximal colon, microorganisms' primary mode of catabolism is glycolysis (Roager et al., 2016). In the distal colon, carbohydrate is gradually consumed completely, and protein becomes the main substrate of bacterial catabolism. The previous study demonstrated the distal colon had more than four times the concentration of phenolic compounds produced by the breakdown of aromatic amino acids than the proximal colon (Smith and Macfarlane, 1996). These findings suggest that the resting cell culture approach employed in this study might be more effective than the nutrient-rich fermentation system for producing these tryptophan metabolites. However, bacterial tryptophan metabolism does not only occur in the colon; for example, *Lactobacillus johnsonii* could also decompose tryptophan in the stomach of mice (Zelante et al., 2013).Therefore, the rule of microbial tryptophan metabolism in the human digestive tract needs to be further explored by more realistic simulated intestinal experiments. We attempted to investigate how genes affect the generation of LAB tryptophan metabolites. In the correlation analysis between the number of genes and the yield, we discovered that IAld

was significantly negatively correlated with the upstream enzyme *amiE*, which was responsible for catalyzing the production of IAA. IAld production was significantly positively correlated with ILA production in the intra-group correlation analysis (Figure S3). This suggests that there might be multiple metabolic pathways for IAld. There is still no experimental proof explaining the precise chemical changes and corresponding catalytic enzyme system in the process of IAA conversion to IAld, even though many studies believe that IAA is the precursor to IAld (Montgomery et al., 2022). The upstream products of IAld and its corresponding metabolic pathway still need experimental proof. The number of ILA and ArAT in LAB was positively correlated, but its Spearman's ρ was only 0.31, which was a relatively weak correlation. Compared with *Limosillactobacillus* species, *L. salivarius* does not have a large amount of ArAT enrichment. However, its ILA metabolic yield was the highest among all LAB species. This suggested that while ArAT might control ILA production, it was not the crucial factor. Previous studies have proven that both LDH and *fldH* control the production of ILA (Dodd et al., 2017; Laursen et al., 2021). We therefore thought that the homologous protein of LDH and *fldH* may be the enzyme that regulates the high production of ILA in *L. salivarius*. In the phylogenetic analysis of *fldH* homologous proteins, we discovered that three types of *fldH* sequences (type 1-3 *fldH*) of *L. salivarius* were clustered separately and maintained a relatively large genetic distance from the *fldH* of other LAB. Phylogenetic analysis of LDH did not present a similar phenomenon. The metabolic enzymes in the same cluster are thought to have similar roles and catalytic capabilities in phylogenetic analysis, according to earlier research (Laursen et al., 2021). Then, we built protein structures of four types *fldH* of *L. salivarius* FWXBH185, which were used for protein sequence and structural alignment. We discovered that the reference *fldH* and type 1-3 *fldH* protein models can overlap well and both have high TM-scores and low RMSD. This indicates that they might have similar catalytic centers and perform similar functions as reference *fldH*, so that IPYA can be efficiently converted into ILA. However, although type 3 *fldH* had a relatively long genetic distance from all other *fldH* of LAB and had a similar structure with reference *fldH*, it could only be found in 7 *L. salivarius* strains. Since all *L. salivarius* can produce a large number of ILA, this may indicate that type 3 *fldH* is not the enzyme that mainly regulates the level of ILA metabolism in *L. salivarius*. Therefore, We speculate that type 1 and type 2 *fldH* may lead to the ability of *L.salivarius* to metabolize ILA at a high level. The precise function of these two types of proteins in the ILA pathway needs to be demonstrated through experiments in the future.

In conclusion, we emphasize the necessity of using multi-omics to sort out the complex tryptophan metabolism. We discovered through metabolomics that, in addition to IAA, the other tryptophan metabolites of LAB have high species specificity, the metabolic concentration of most strains within a species remains constant and only a small number of strains have strain specificity. Combined with genomic analysis, it was revealed that the tryptophan metabolites of LAB were predictable, and the number of genes and special metabolic genes could regulate the tryptophan metabolism of LAB. These findings offer novel perspectives and experimental evidence that will help the development of probiotics that produce particular tryptophan metabolites.

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.

Author contributions

Specific author contributions: TP: investigation, data analysis, writing-original draft, visualization, and methodology; ZP: methodology; ZF: investigation; HW: validation and visualization; JZhu: data analysis; ZH: validation, resources; JZha: funding acquisition and resources; WC: resources, funding acquisition, validation, and data curation; WL: supervision, writing–review and editing, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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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/fcimb.2023.1154346/full#supplementary-material

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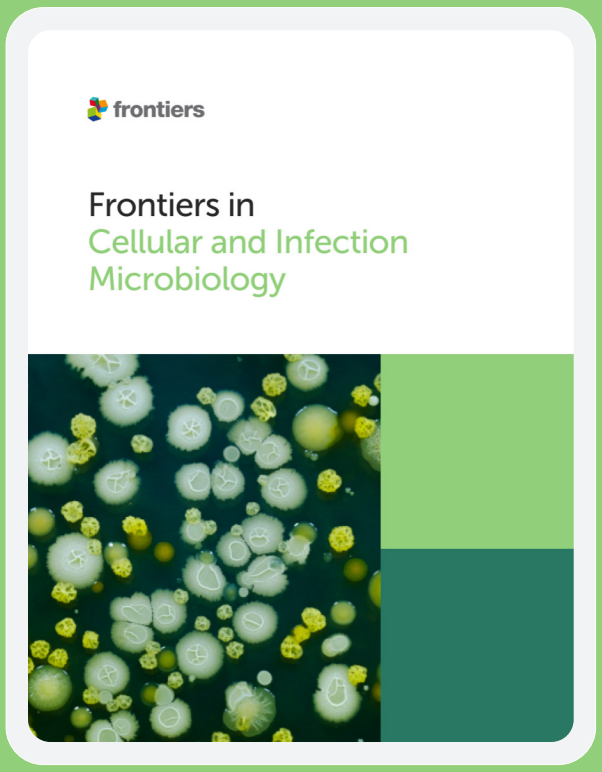

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