THE ROLE OF DIETARY INTERVENTIONS IN THE REGULATION OF HOST-MICROBE INTERACTIONS: VOLUME II,
2nd Edition

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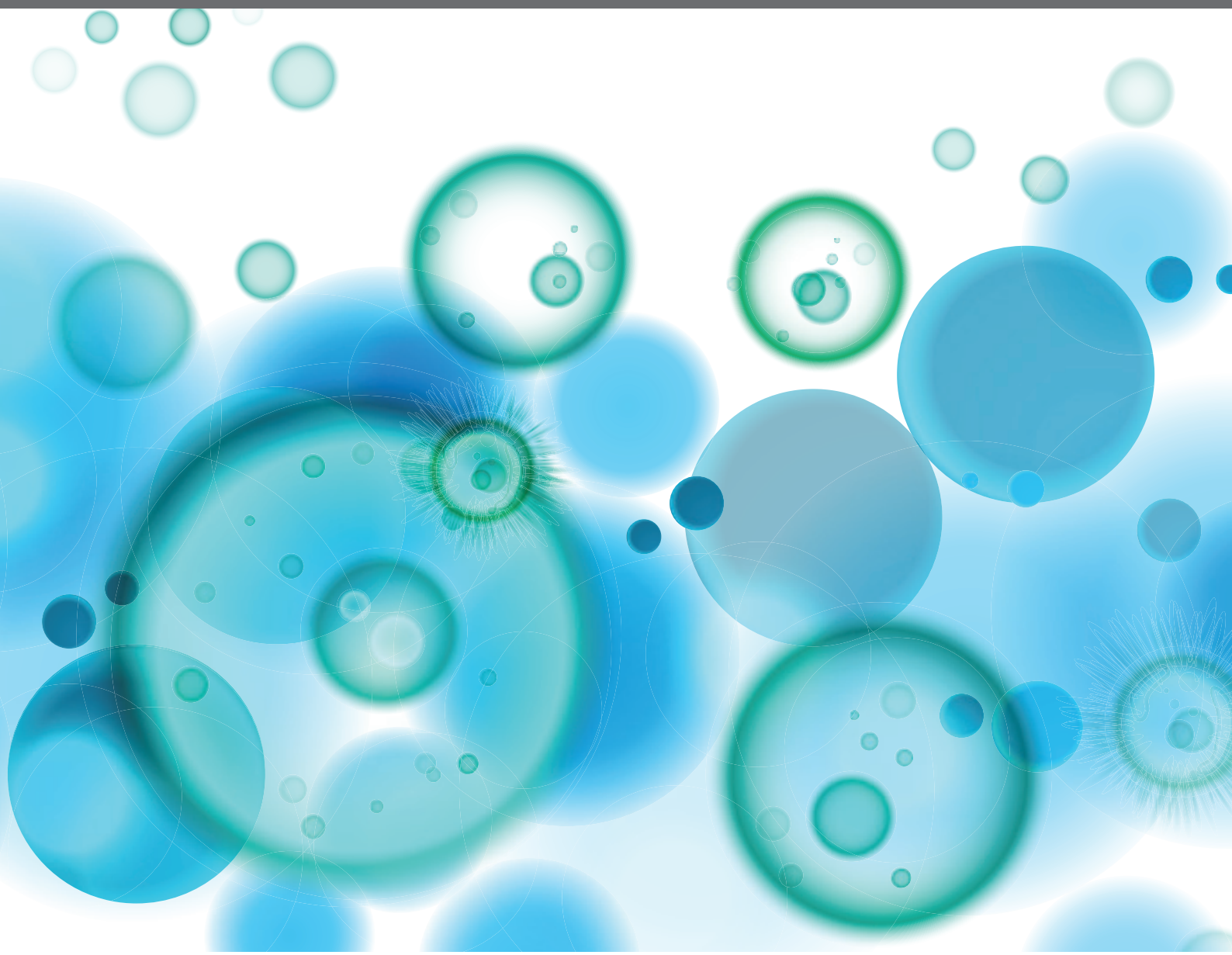

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THE ROLE OF DIETARY INTERVENTIONS IN THE REGULATION OF HOST-MICROBE INTERACTIONS: VOLUME II, 2nd Edition

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Editorial: The role of dietary interventions in the regulation of host-microbe interactions: Volume II

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Editorial on the Research Topic
The role of dietary interventions in the regulation of host-microbe interactions: Volume II

Intestinal microbiota, a complex ecosystem of microbes that inhabits and critically maintains homeostasis of the gastrointestinal tract, exerts a major impact on host physiological, nutritional and immunological processes. Among the multiple factors related with intestinal microbiota, diet is regarded as the most important determinants. Dietary intervention mainly regulates the composition of intestinal microbiota by ingesting probiotics/prebiotics, dietary fiber, etc., and then improves host immunity, metabolic processes and nutrients bioavailability, inhibits oxidative stress and inflammatory pathways to achieve the purpose of improving host health. Therefore, in the present Research Topic, we have collected eleven articles related to the regulation of intestinal microbiota and host health through dietary interventions, including nine original research articles, one prospective clinical study and one review in the fields of human systemic diseases (allergy, hyperuricemia, hyperlipidemic), gastrointestinal diseases (colorectal polyps), neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease (PD)), and animal husbandry (calves, broiler feed, weaned piglets, chickens).

Systemic diseases are diseases that affect all organs and systems of the body, which are often chronic diseases such as allergy and diabetes. Intestinal microbiota plays an important role in the development of systemic diseases. Based on β-lactoglobulin-induced allergy mice model, Tian et al. explored the mechanism of *Bifidobacterium animalis* KV9 (KV9) and *Lactobacillus vaginalis* FN3 (FN3) on alleviating allergic

reactions and regulating immune cell function. KV9 and FN3 intervention activated the toll-like receptor 4-NF-kB signaling pathway in intestinal dendritic cells, resulting in an increase in interleukin-12 secretion and a decrease in interleukin-4 secretion, which have the potential to promote T-cell differentiation into T helper type 1 cells.

Hyperuricemia is a systemic disease in which excess uric acid (UA) is present in the blood, increasing risk of chronic kidney disease and gout. Using a hyperuricemia mouse model, Cao et al. examined the impact of probiotics - *L. paracasei* X11 on UA metabolism. By correcting the proportion of *Bacteroidetes* to *Firmicutes* to promote the intestinal microbiota homeostasis, *L. paracasei* inhibited the renal pro-inflammatory cytokine IL-1, restored normal levels of hepatic metabolic enzymes (adenosine deaminase, xanthine oxidase), transporter protein expression (GLUT9, NPT1, and URAT1), and lowered serum UA by 52.45%. In addition, prebiotics and probiotics can reduce metabolic syndrome by regulating intestinal microbiota. Verified by *in vitro* screening, Pi et al. found that oligosaccharide as a prebiotic improved the growth of live combined *Enterococcus faecium* and *Bacillus subtilis* (LCBE). Based on a hyperlipidemic mouse model, LCBE combined with oligosaccharide diet significantly reduced plasma cholesterol levels, lowered the *Firmicutes/Bacteroidetes* ratio and increased the relative abundance of *Akkermansia* and *Bifidobacteria*, which was proven to help avoid functional gastrointestinal disorders.

Gastrointestinal disorders are diseases of the human digestive system, closely related to the disruption of intestinal microbiota homeostasis, and therefore are the main targets of dietary interventions. Pan et al. characterized the distribution and diversity of mucin-degrading bacteria in the human gut. Mucin-degrading bacteria were widely distributed in human intestinal, mainly *Bacteroides spp*, which reduced the inflammatory response brought on by *E. coli* by inhibiting the NF-κB pathway and enhanced the epithelial tight junction. In another prospective clinical study led by Liu et al., patients after intestinal polypectomy received oral *B. animalis* MH-2 to assess its effect on postoperative symptoms (pain, bloating, difficult defecation). The results showed that MH-2 helped restore intestinal microbiota diversity by increasing the relative abundance of *Bifidobacterium*, while decreasing the relative abundance of *Clostridium* spp., thereby alleviated difficult defecation and shortened recovery time.

Neurodegenerative diseases, like AD, are debilitating, progressive, neurodegenerative conditions, which directly associated with the dysbiosis of intestinal microbiota. Using an amyloid-β-induced AD mouse model, environmental enrichment (EE) training together with the *B. breve* CCFM1025 intervention were examined by Zhu et al. for their ability to reduce neuroinflammation and cognitive impairment. Uptake of EE+*B. breve* CCFM1025 dramatically raised *B. longum*'s relative abundance, decreased *B. pseudocatenulatum*'s relative abundance, and reduced 5-hydroxyindole acetic acid levels, which can mimic the composition of healthy brain and improve cognitive performance.

Beyond the application in human health, dietary interventions also play an important role in animal husbandry. As a common disease in livestock, diarrhoea caused by pathogenic *Escherichia coli* can disrupt the intestinal barrier in newborn calves. He et al. established calf diarrhea model by taking pathogenic *E. coli* O1 orally to inhibit the proliferation of probiotic bacteria, such as *Butyricoccus* and *Lactobacillus*, which significantly increased serum IL-6 level, indicating impaired intestinal barrier function and immune function. In addition, Cao et al. proposed an effective solution-dietary addition of potassium magnesium sulfateon (PMS). PMS significantly increased the abundance of intestinal microbiota, especially *Ruminococcaceae* and *Peptostreptococcaceae*, and inhibited interleukin-1β level and promoted IgM level, which enhanced the antioxidant capacity and modify intestinal immunity of weaned piglets.

To reduce the impact of dietary sources of pathogenic bacteria and toxins, Wang et al. added activated charcoal-herb extractum complex (CHC) to broiler feed. CHC intake increased the abundance of probiotic bacteria (*Romboutsia* and *Lactobacillus*) and reduced the abundance of the pathogenic bacteria (*Alistipes*), which in turn reduced serum levels of interleukin-1β and interferon-γ, exhibiting beneficial effects on immune status and intestinal microbiota composition. In addition, Amevor et al. proposed that the addition of quercetin and vitamin E to chicken feed could promote egg production and immunity. Quercetin partially abrogated the disruption of the intestinal microbiota by increasing *Lactobacillus* abundance and decreasing *Ruminicoccaceae* abundance. Vitamin E supplementation increased *Rikenellaceae*, which promoted fermentation of glucose, mannose and lactose to form intestinal-beneficial organic acids.

PD is a progressive, degenerative disorder that affects 10 million people worldwide. Zhu et al. reviewed recent studies related to the role of intestinal microbiota on the development of PD. Dysbiotic intestinal microbiota can increase intestinal permeability, worsened neuroinflammation, abnormal aggregation of α-synuclein fibrils, oxidative stress, and reduced neurotransmitter production. Dietary intervention based on probiotics/prebiotics can alter the composition of the intestinal microbiota and modulate the microbiota-gut-brain axis for the PD treatment.

In conclusion, this Research Topic provides readers with an overview of the impact of dietary intervention on the regulation of intestinal microbiota and host health, further elucidating the interaction between diet and intestinal microbiota. However, there are still many difficulties in achieving precision medical therapy through dietary interventions until the physiological and molecular mechanisms behind can be elucidated in depth. In order to address these issues, multi-omics technologies such as microbiomics, metabolomics and proteomics should be jointly utilized to study the detailed relationship between intestinal microbiota and diet and related diseases.

Author contributions

QS, YG, and HT wrote this article, TC and HT revised this article. 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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BRIEF RESEARCH REPORT
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Effects of Live Combined *Bacillus subtilis* and *Enterococcus faecium* on Gut Microbiota Composition in C57BL/6 Mice and in Humans

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Probiotics, prebiotics, and synbiotics can alleviate metabolic syndrome by altering the composition of the gut microbiota. Live combined *Enterococcus faecium* and *Bacillus subtilis* has been indicated to promote growth and reduce inflammation in animal models. However, the modulatory effects of live combined *B. subtilis* R-179 and *E. faecium* R-026 (LCBE) on human microbiota remain unclear. The current study examined the growth of these two strains in the presence of various oligosaccharides and assessed the effects of this probiotic mixture on human and murine gut microbiota *in vitro* and *in vivo*. Oligosaccharides improved the growth of *E. faecium* R-026 and *B. subtilis* R-179 as well as increased their production of short-chain fatty acids. *E. faecium* R-026 or *B. subtilis* R-179 co-incubated with *Bifidobacterium* and *Clostridium* significantly increased the number of the anaerobic bacteria *Bifidobacterium longum* and *Clostridium butyricum* by *in vitro* fermentation. Moreover, LCBE significantly reduced plasma cholesterol levels in mouse models of hyperlipidemia. LCBE combined with galacto-oligosaccharides led to a significant decrease in the Firmicutes/Bacteroidetes ratio and a significant increase in the relative abundance of *Akkermansia* and *Bifidobacteria* after treating mice with LCBE (0.23 g/day) for eight weeks. Furthermore, *in vitro* fermentation also showed that both the single strains and the two-strain mixture modulated human gut microbiota, resulting in increased *Lactobacillus* and *Bifidobacteria*, and decreased *Escherichia-Shigella*. Overall, these results suggest that LCBE can improve host health by reducing the level of cholesterol in mouse models by modifying the composition of the gut microbiota.

Keywords: probiotics, prebiotics, short-chain fatty acids, gut, microbiota

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INTRODUCTION

Probiotics are live microorganisms that may confer potential health benefits to hosts when administered orally (Cunningham et al., 2021) and are thought to promote host health through the production of beneficial enzymes, organic acids, vitamins, and nontoxic antibacterial substances. Many studies have reported that probiotics improve host health by modulating the gut microbiota. Some probiotics have been used to treat gastrointestinal diseases including constipation, diarrhea,

infection, necrotizing enterocolitis, inflammatory bowel disease, and colon cancer (Liang et al., 2021).

Probiotics can consist of a single bacterial strain or a mixture of strains and can also be combined with prebiotics. Based on a limited number of studies, multi-strain probiotics appear to show greater efficacy than those with single strains. For example, a combination of *Bacillus subtilis* and *Saccharomyces boulardii* was shown to improve digestion, growth, gastrointestinal health, and the overall health status of weaned piglets (Goktas et al., 2021). Similarly, administration of *Bifidobacterium animalis* subsp. *lactis* BB12 with *Enterococcus faecium* L3 significantly ameliorated signs and symptoms of allergic arthritis (Anania et al., 2021).

Several studies have shown that *B. subtilis*, an aerobic bacterium, can utilize a substantial amount of free oxygen in the gut for its proliferation. Dietary supplementation with *B. subtilis* KN-42 has been shown to limit the proliferation of aerobic pathogens and enhance the growth of beneficial anaerobic bacteria such as *Bifidobacterium* and *Lactobacillus*, thus improving the growth performance and digestive tract health of animals (Hu et al., 2014). *Enterococcus faecium* is widely used as a feed probiotic supplement and may have clinical benefits such as suppressing diarrhea as well as improving growth performance and composition of the gut microbiota (Satokari, 2019).

Previous research has shown the beneficial effects of a combined live *B. subtilis* R-179 and *E. faecium* R-026 on host health (Guo et al., 2018; Wang et al., 2021). Live combined *B. subtilis* and *E. faecium* (LCBE) strains are approved for use as animal feed additives (Jensen and Bjørnvad, 2019). LCBE has been reported to ameliorate colitis in a murine model (Hu et al., 2014), and has shown a protective effect in polymicrobial sepsis through the activation and transformation of macrophages and mast cells (Shi et al., 2017b; Guo et al., 2018). LCBE has also shown to regulate the performance, immune status, and gut microbiota of lactating sows (Huang et al., 2021). Furthermore, administration of LCBE capsules with lactulose for the treatment of functional constipation is being explored in clinical studies (Li et al., 2012). However, the underlying mechanisms of these beneficial effects of LCBE on host health are not well characterized. Moreover, little is known regarding the effects of live combined strains on gut microbiota.

This study aimed to explore the relationship between LCBE and other anaerobic probiotics such as *Bifidobacterium* and *Clostridium*. The potential benefits of LCBE were explored and the ability of the probiotics to utilize prebiotics and their compatibility with other probiotics were assessed.

MATERIALS AND METHODS

Probiotics Strains and Growth Conditions

B. subtilis R179 and *E. faecium* R-026 strains were obtained from a commercial probiotic product made by Medilac-S (Beijing Hanmi Pharmaceutical Co. Ltd., China). LCBE contained *E. faecium* (1.35×10^8 CFU/g) and *B. subitlis* (1.5×10^7 CFU/g). *B. subtilis* R-179 strains were cultured in Luria-Bertani broth (LB) medium while *E. faecium* R-026 strains were cultured overnight at 37°C in brain heart infusion medium. *Bifidobacterium longum* CECT 7894 and *Clostridium butyricum* MS1 were grown in reinforced clostridial medium (RCM) under anaerobic conditions. Overnight cultures of probiotics strains were used to inoculate the coculture fermentation and batch culture fermentation. RCM medium was used as the coculture fermentation medium of probiotics strains while basic growth medium VI supplemented with 0.8% (*w*/*v*) soluble starch was prepared and used for batch fermentation. Each oligosaccharide as the sole carbon source was dissolved into basic growth medium and autoclaved. The oligosaccharide prebiotics used were: galacto-oligosaccharides (GOS), mannose-oligosaccharides (MOS), isomalto-oligosaccharide (IMO; 95%), fructo-oligosaccharides (FOS; 95%) and resistant dextrin (RDX) purchased from Baolingbao Biological Co. (Shandong, China). Additionally, inulin powder (INU; 95%) was purchased from Fengning Pingan High-tech Industrial Co. Ltd. (China); stachyose powder (STE; 90%) was purchased from Tianmei Biotechnology Co. Ltd. (Xi'an, China); lactulose (LAU) was acquired from Beijing Hanmi Pharmaceutical Co. Ltd. (Beijing, China); raffinose (RAF) was acquired from Aladdin (Shanghai, China); and polyglucan type I (PG1), mannitol (MAI), xylitol (XYI), and sorbitol (SBI) were purchased from Sigma-Aldrich.

In Vitro Coculture and Viable Counts of Probiotic Bacteria

To assess the interaction among four probiotics (*B. fidobacterium, C. butyrium, B. subtilis* R179 and *E. faecium* R-026), four batch experiments were carried on two different strain combinations. The stage and level of bacterial growth were assessed by measuring the optical density of samples using a MicroScreen 16-HT machine (Gering, Tianjin, China). Viable colony counts of anaerobic probiotics were determined using RCM agars in an ElectroTek AW 400TG Anaerobic Workstation (ElectroTek, West Yorkshire, United Kingdom). Four bacterial cultures were grown overnight in RCM medium until they reached the exponential phase. Then, *B. subtilis* R-179 and *E. faecium* R-026 were cocultured with *B. fidobacterium* and *C. butyricum* in a 1:1 ratio (*v*/*v*). Individual bacteria (500 μL) were used as a negative control. The individual bacteria (500 μL) and 1 mL of the two-strain mixture (500 μL of each bacterial strain) were transferred to a 5 mL anaerobic bottle with RCM liquid medium. After 24 h, 1 mL of the cocultures and individual bacteria were removed and homogenized in a 1:10 dilution with phosphate-buffered saline (PBS). Values of colony forming units (CFU)/mL were determined by serial dilution and plating on RCM agar medium. *B. subtilis* and *E. faecium* were streaked on LB medium plates and incubated overnight at 37°C under aerobic conditions. *B. longum* and *C. butyric* were streaked on RCM medium plates and incubated overnight at 37°C under anaerobic conditions. All experiments were independently performed in duplicate.

Preparation of Probiotics and Experimental Design

Probiotics strains were cultured for 6–8 h in the appropriate media to reach the optimum cell division stage (log phase). The culture was then washed twice using PBS to remove all culture media. Equal counts (1×10^8 CFU) of newly prepared bacterial cells were pooled in four groups; (1) Control group: human feces slurry only; (2) *B. subtilis* R-179 group: *B. subtilis* R-179 strain with human feces slurry; (3) Enterococci: *E. faecium* R-026 strain with human feces slurry; and (4) Probiotic complex: *B. subtilis* R-179 and *E. faecium* R-026 complex with human feces slurry.

Measurement of Short-Chain Fatty Acids

Six short-chain fatty acids (SCFA; propionate, acetate, valerate, isobutyrate, isovalerate, and butyrate) were quantified using the gas chromatography technique (Shimadzu, GC-2010 Plus, Japan). Specifically, a DB-FFAP chromatographic column (Agilent, USA) with a hydrogen flame ionization detector was used. Crotonic acid (trans-2-butenoic acid) was used as the internal standard.

Redox Potential Measurements

After filtration of the fermentation cultures, the redox state of the fermentation supernatant was measured by a redox electrode with an Ag/AgCl reference electrode (Unisense, Aarhus Denmark). The electrode was calibrated with saturated quinhydrone buffer solutions of pH 4 and pH 7 at 30°C. The value of the redox potential was defined relative to the standard hydrogen electrode. Redox data were collected thrice after the detection of baseline stability.

Animals and Experimental Design

Forty male C57BL/6 mice (eight-week-old) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were housed in an specific pathogen free (SPF) animal room under a controlled environment (temperature: 25 ± 2°C; relative humidity: 50 ± 5%; 12/12 h light/dark cycle). After acclimatization for one week, mice were randomly divided into five groups (n = 8 per group). Except for the control group (N) that received standard chow, the mice were fed a high-fat diet for eight weeks to model hyperlipidemia, which included the model group (M) and three treatment groups (MG, MP, and MPG groups). Mice in the MG group were administered GOS (0.23 g/day) by gavage, mice in the MP group were administered LCBE (0.23 g/day) by gavage, and mice in the MPG group were administered a mixture of equivalent doses of LCBE and GOS dissolved in physiological saline. All chow was purchased from Jiangsu Xietong Biology Co. Ltd. (Nanjing, China). The N and M groups were administered saline (0.1 mL/10 g/day) by gavage. Mice were administered with LCBE and/or GOS and fed the high-fat diet for eight weeks after reaching the hyperlipidemia standard. The average body weight of the model group was 20% higher than the normal group, and the total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were significantly increased compared with the control group. At the end of week 8, all mice were fasted overnight and anesthetized with isoflurane. Blood samples were collected from their orbital vein into tubules containing heparin sodium. Fresh feces from each mouse were collected in sterile tubules for gut microbiota analyses. All animal research was approved by the Committee for Animal Ethics of Zhejiang Academy of Agricultural Sciences.

Analysis of Body Weight Gain and Serum Lipid Levels

Serum levels of TC, TG, HDL-C, and LDL-C in mice were assayed using an automatic biochemical analyzer *Chemray 800* (Rayto Life and Analytical Sciences Co. Ltd.) according to the manufacturer's instructions.

Human Gut Microbiota Culture and Fermentation

Ten healthy participants that self-reported no use of antibiotics in at least the three-month period immediately preceding the study were enrolled after providing written informed consent. The study was approved by the Ethics Committee of the Hangzhou Center for Disease Control and Prevention (No. 202047). Gut microbiota was cultured from fresh fecal samples using an automatic fecal system as described previously (Liu et al., 2020). Every sample was diluted with 10% PBS, and a feces slurry was injected into the fermentation chamber. The experiment was divided into the following four groups: control (without probiotics), EF group (supplemented with *E. faecium*), BS group (supplemented with *B. subtilis*), and complex group (supplemented with *E. faecium* and *B. subtilis*). The diluted fecal suspension was made into 10% (*w*/*v*) slurries. Batch fermentation was conducted at 37°C for 24 h using 10% fecal slurry in anaerobic penicillin vials. The cultures were centrifuged and immediately persevered at -80°C for further analysis of microbial community composition.

DNA Extraction and 16s rRNA Sequencing of Fermentation Samples

Microbial DNA was extracted from 250 mg fecal samples using the Qiagen DNA extraction kit (Qiagen, Germany) following the manufacturer's protocol. Polymerase chain reaction (PCR) was used to amplify the V3-V4 region of the 16S ribosomal RNA gene, and the primer pair 343F: 5'- TACGGRAGGCAGCAG -3' and 798R: 5'- AGGGTATCTAATCCT-3' was used for amplification. The PCR conditions were as follows: initial denaturation for 2 min at 95°C, 20 cycles of denaturation (94°C for 30 sec), annealing (48°C for 30 sec), and extension (72°C for 2 min). The DNA sequencing was conducted on an Illumina MiSeq PE300 system platform operated by Shanghai OE Biotech Technology Co. Ltd. (Shanghai, China).

Sequence Processing and Bioinformatics Analysis

Sequences were processed by bar-codes using QIIME (version 1.8). Vsearch software with a 97% similarity cutoff was used to remove primer sequences from clean reads as well as to perform clustering to generate operational taxonomic units (OTUs)

(Edgar, 2010). The QIIME package was used to select the representative reads of each OTU. All representative reads were annotated, then blasted on the Silva database (version 123; Greengenes) for 16s rDNA using the RDP classifier with a confidence threshold set at 70% (Wang et al., 2007). Mothur was used to calculate alpha diversities, Shannon and Simpson indices, and richness (observed number of OTUs). The Vegan package was used to calculate phylogenetic measures of beta diversity based on genus level abundance profiles. Alpha diversity was calculated by indices of Shannon, Chao1, PD whole tree, Simpson, and observed species. A principal coordinate analysis (PCoA) plot was plotted using the ggplot2 package based on unweighted UniFrac distances. All consensus sequence data of humans and mice were submitted to the National Center for Biotechnology Information Short Read Archive under accession no. SRP233155 and PRJNA753235, respectively.

Statistical Analysis

Data were presented as mean ± standard deviation. Statistical analyses were performed using SPSS software. Intergroup differences were assessed using one-way analysis of variance, followed by Turkey's *post hoc* test. P values < 0.05 were considered as statistical significance. Linear discriminant analysis (LDA) of effect size (LEfSe) was used to determine the most discriminant taxa and predicted functions (Kyoto Encyclopedia of Genes and Genomes pathways) between the two groups. Wilcoxon rank sum test and LDA analysis were used to measure the LEfSe of the abundant taxon. Two filters, i.e., $P < 0.05$ and LDA score > 2 were used.

RESULTS

Coculture of *B. subtilis* R-179 and *E. faecium* With Probiotics and Bacteria Commonly Found in the Gut

The growth of *E. faecium* R-026 and *B. subtilis* R-179 was examined in the presence of prebiotics commonly found in the gut (**Figure 1**). *E. faecium* R-026 grew rapidly to the exponential phase and reached a stationary phase after 4 h, whereas *B. subtilis* R-179 exhibited a slow increase in growth rate for 24 h. *E. faecium* R-026 showed the most rapid growth in the presence of GOS, followed by MOS, INU, and FOS (**Figure 1A**) compared with the control group. Similarly, *B. subtilis* R-179 (**Figure 1B**) showed the fastest growth in the presence of GOS, FOS, INU, and MOS.

Next, the growth of *E. faecium* R-026 and *B. subtilis* R-179 was examined when cocultured with anaerobic probiotic strains commonly found in gut, i.e., *Bifidobacterium longum* and *Clostridium butyrium*. Both these bacterial strains are under investigation for clinical use. The viability of *E. faecium* R-026 or *B. subtilis* R-179 was not affected by coculture with either *B. longum* or *C. butyricum* (**Table 1**). Additionally, the viability *B. longum* increased from 10.5 ± 0.2 log CFU/mL to 10.7 ± 0.9 log CFU/mL in the presence of *B. subtilis* R-179 and from 10.2 ± 0.2 log CFU/mL to 10.3 ± 0.1 log CFU/mL in the presence of *E. faecium* R-026. Similarly, the viability of *C. butyricum* increased from 2.6 ± 0.2 log CFU/mL to 7.1 ± 0.2 log CFU/mL when cocultured with *B. subtilis* R-179 and from 6.0 ± 1.0 log CFU/mL to 10.4 ± 0.9 log CFU/mL when cocultured with *E. faecium* R-026.

Production of SCFAs

SCFAs produced by gut bacteria are generally thought to be beneficial to the host. Therefore, the production of SCFAs by *E. faecium* R-026 and *B. subtilis* R-179 were examined in the presence of various oligosaccharides (**Figure 2**). After 24 h of culture, both strains were found to have produced a substantial amount of acetate in the presence of RAF, LAU, MAI, and SBI. Similarly, both strains produced a substantial amount of propionate in the presence of GOS, FOS, and MOS. However, butyrate production by both strains was most prominent in the presence of INU, FOS, and RAF. Coculture of *E. faecium* R-026 and *B. subtilis* R-179 produced the highest amount of acetate in the presence of SBI, RAF, and INU, the highest amount of propionate in the presence of MOS, GOS, and IMO, and the highest amount of butyrate in the presence of GOS, PG1, and RDX.

Additionally, SCFA production by *B. subtilis* R-179 or *E. faecium* R-026 was detected after coculture with anaerobic probiotic strains, i.e., *B. longum* or *C. butyricum*. An increase

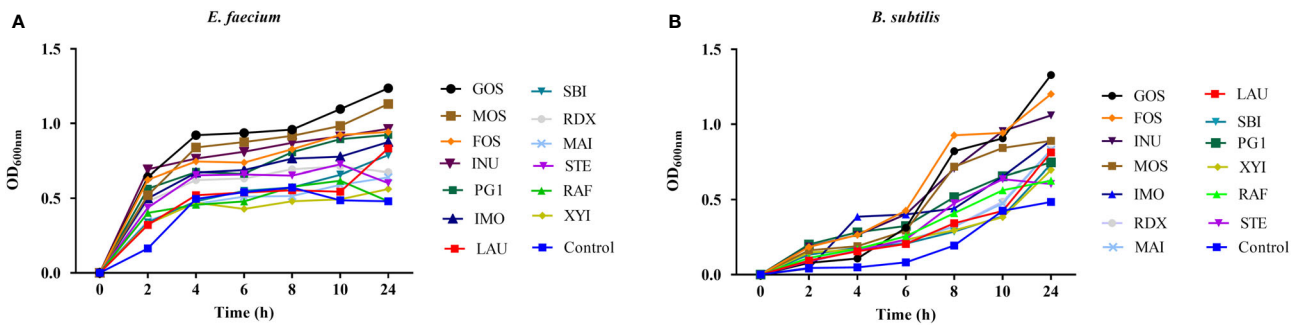

FIGURE 1 | Growth kinetics of *Enterococcus faecium* **(A)** and *Bacillus subtilis* **(B)** when cultured with different oligosaccharides, as measured by optical density (OD) values during 24 h of an incubation period in batch fermentation. basal medium (YCFA), fruto-oligosaccharides (FOS), lactulose (LAU), raffinose (RAF), stachyose (STE), galacto-oligosaccharides (GOS), mannose-oligosaccharides (MOS), isomalto-oligosaccharide (IMO), inulin (INU), polyglucan type 1 (PG1), xylitol (XYI), sorbitol (SBI), mannitol (MAI), resistance dextrin (RDX).

TABLE 1 | Viability bacterial counting for individual and mixed cultures of *Enterococcus faecium* or *Bacillus subtilis* cocultured with *Bifidobacterium longum* or *Clostridium butyricum*.

Group	Strains	Viable count (log CFU/mL)	
1		*B. subtilis*	*B. longum*
	B. subtilis	9.6 ± 0.1	0
	B. longum	0	10.5 ± 0.9
	Mixed culture (*B. subtilis* + *B. longum*)	10.2 ± 0.1	10.7 ± 0.9
2		*B. subtilis*	*C. butyrium*
	B. subtilis	10.4 ± 0.9	0
	C. butyrium	0	7.1 ± 0.2
	Mixed culture (*B. subtilis* + *C. butyrium*)	10.8 ± 0.3	11.4 ± 0.2*
3		*E. faecium*	*B. longum*
	E. faecium	10.8 ± 0.2	0
	B. longum	0	10.2 ± 0.2
	Mixed culture (*E. faecium* + *B. longum*)	10.2 ± 0.2	10.3 ± 0.1
4		*E. faecium*	*C. butyrium*
	E. faecium	10.1 ± 0.2	0
	C. butyrium	0	7.1 ± 0.2
	Mixed culture (*E. faecium* + *C. butyrium*)	10.3 ± 0.1	10.4 ± 0.9*

*Indicates significant difference between the mean values within a column ($P < 0.05$). CFU, colony forming units.

in acetate was more pronounced in the coculture with *B. longum*, while an increase in butyrate was more pronounced in the coculture with *C. butyricum* (**Figure S1**).

Redox Potential Changes During Probiotic Fermentation

To further understand the effects of *B. subtilis* R-179, *E. faecium* R-026, and their live combination on the chemical environment in the gut, the redox potential was then examined 24 h after culturing the strains anaerobically in a separate or combined system. Lower redox potentials were observed in all cultures; of note, the lowest redox potential was observed in the live combination culture vs cultures of individual strains (**Table 2**).

Effects of LCBE on Body Weight and Plasma Lipids

Next, the potential effects of LCBE were examined using a murine model of obesity. Groups of mice were fed a high-fat diet and given GOS, LCBE, or GOS with LCBE. Mice in all groups fed with the high-fat diet showed a significant increase in body weight compared with mice fed with a standard diet. However, TC levels in mice fed with high-fat diet that received

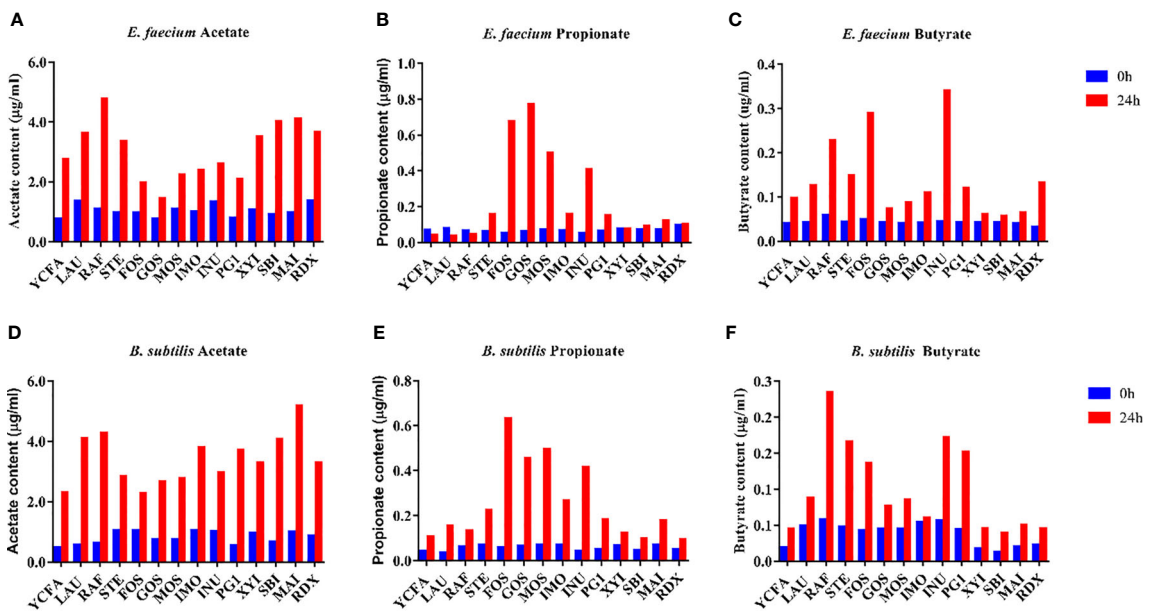

FIGURE 2 | Short-chain fatty acid (SCFA) content after culture of *Enterococcus faecium* **(A–C)** and *Bacillus subtilis* **(D–F)** with different oligosaccharides after 24 h fermentation. basal medium (YCFA), fruto-oligosaccharides (FOS), lactulose (LAU), raffinose (RAF), stachyose (STE), galacto-oligosaccharides (GOS), mannose-oligosaccharides (MOS), isomalto-oligosaccharide (IMO), inulin (INU), polyglucan type 1 (PG1), xylitol (XYI), sorbitol (SBI), mannitol (MAI), resistance dextrin (RDX).

TABLE 2 | Change of redox potential after culture of *Bacillus subtilis* and *Enterococcus faecium* alone and in combination.

Groups	Redox [redox (mv)]
Control group (blank medium)	550.5 ± 4.42[a]
B. subtilis group	439.7 ± 7.78[b]
E. faecium group	430.7 ± 2.78[b]
Mixed group of *B. subtilis* and *E. faecium*	353.5 ± 1.03[c]

Same letters between the groups indicate no significant difference ($P > 0.05$), and different letters indicate statistically significant differences ($P < 0.05$).

the combination of GOS and LCBE were significantly lower compared with mice fed the high-fat diet alone. There was no significant difference in TC levels between mice on a high-fat diet that received GOS or LCBE alone compared with mice on a high-fat diet alone. No significant differences in TG, HDL-C, or LDL-C levels were noted among the mice on a high-fat diet that received GOS, LCBE, or a combination of GOS and LCBE, and mice on a high-fat diet alone (**Figure 3**).

Effects of Different Dietary Interventions on Murine Gut Microbiota

The relative abundance of bacterial taxa at the phylum and genus levels were then examined in all five groups of mice, i.e., mice on standard diet, mice on high-fat diet alone, and mice on high-fat diet receiving GOS, LCBE, or a combination of GOS and LCBE. At the phylum level, Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria, and Proteobacteria were the major phyla identified (bacterial proportion >1.0%) among the five groups.

There were significant differences in the ratio of Firmicutes/Bacteriodetes (F/B) between these groups. Compared with mice given standard show, mice fed with the high-fat diet alone showed an increased F/B ratio (0.4 and 1.8, respectively). Treatment with GOS (F/B value= 1.0) or LCBE (F/B value = 1.4) reduced the F/B ratio. Of note, the lowest F/B ratio was noted in mice on a high-fat diet that received the combination of GOS and LCBE (F/B value = 0.3).

At the genus level, *Akkermansia* and *Lachnospiraceae_NK4A136* were the major genera (bacterial proportion >1%) in the gut microbiota of mice. A high-fat diet significantly increased the relative abundance of *Lactobacillus*, *Alloprevotella*, *Faecallibaculum*, and *Enterococcus* compared with mice on a standard diet, and the increased abundance of these genera was attenuated in mice receiving GOS, LCBE, or a combination of GOS and LCBE (**Figure 4B**). Moreover, a significant increase in the abundance of *Akkermansia* and *Bifidobacteria* as well as a decrease in the abundance of *Lachnospiraceae* NK4A136 was noted in mice receiving GOS, LCBE, or a combination of GOS and LCBE compared with mice on a standard diet (**Figure 4A**).

Effects of *B. subtilis* R-179 and *E. faecium* R-026 on Human Fecal Microbiota

The effects of the probiotic strains on human fecal bacterial communities were compared using a batch fermentation system inoculated with ten human fecal samples. Using high-throughput sequencing, a total of 1,002,166,296 clean reads were obtained from 36 samples after filtering. The value of good coverage in each sample was higher than 0.99. This implies that the 16S rRNA gene from every library represented the largest bacteria proportion. Further analyses found that only *B. subtilis* R-179 treatment improved the diversity of gut microbiota, although no significant differences in alpha-diversity were noted between the control group, the probiotic interventions, and the two-strain combined treatment (**Figure S2**). Moreover, PCoA plots of unweighted UniFrac indicated no clear separation between the control and any of the probiotic treatment groups.

The bacterial compositions after probiotic treatment in the *in vitro* fermentation systems were analyzed by high-throughput sequencing. The overall microbiota structure at the top 30 phylum level is shown in **Figure 5**. The results revealed that the Proteobacteria phylum was the dominant phylum with the control medium in the absence of probiotics strains. Treatment with *E. faecium* R-026, *B. subtilis* R-179, or LCBE significantly improved the abundance of Actinobacteria and Firmicutes. The

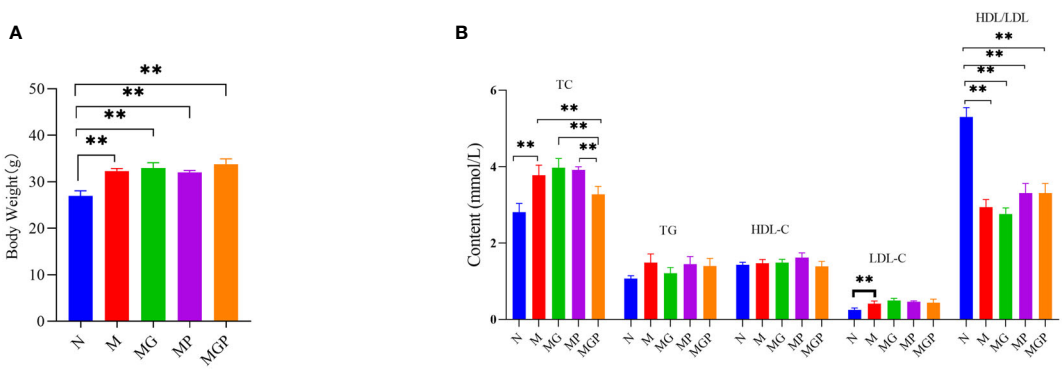

FIGURE 3 | Effects of live culture of *Enterococcus faecium* and *Bacillus subtilis* (LCBE) and galacto-oligosaccharides (GOS) on body weight **(A)** and plasma metabolites **(B)** in mice. N represents the group of mice fed with a standard diet; M represents the group of mice fed with the high-fat diet; MG represents the group of mice fed with the high-fat diet and GOS; MP represents the group of mice fed with the high-fat diet and probiotic mixture; MPG represents the group of mice fed with the high-fat diet, GOS and probiotic mixture. **Indicates significant difference compared with M group ($P < 0.01$).

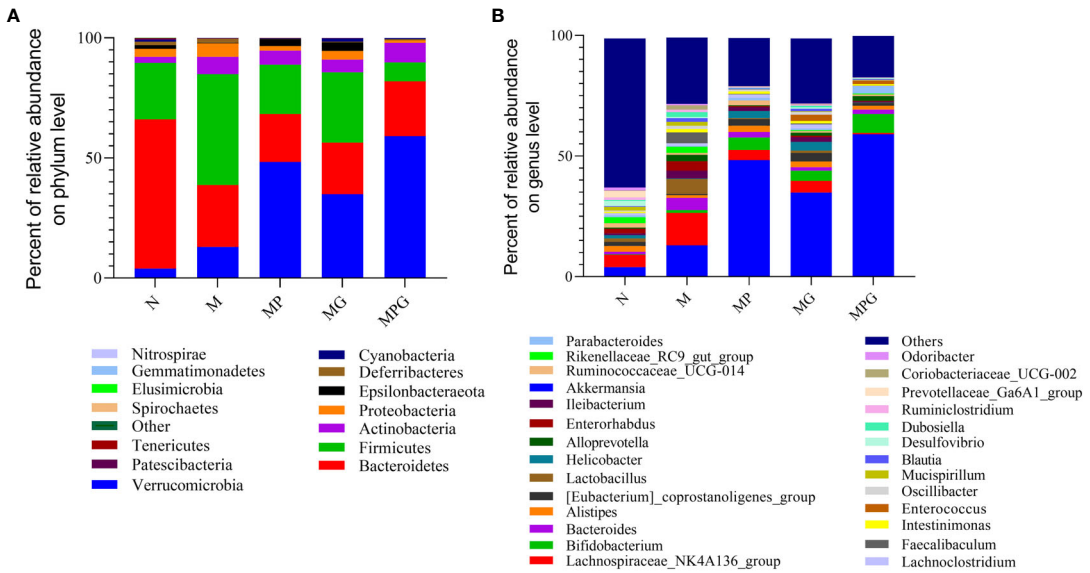

FIGURE 4 | The effect of live culture of *Enterococcus faecium* and *Bacillus subtilis* (LCBE) and galacto-oligosaccharides (GOS) on murine microbial composition. **(A)** Relative abundance of bacteria at phylum level; **(B)** Relative abundance of bacteria at genus level under different treatments. The ordinate represents the species name, the color gradient indicates the proportion of the species.

population of the Firmicutes phylum increased from 25.94% to 51.40% when cocultured with *B. subtilis* R-179, to 44.25% when cocultured with *E. faecium* R-026, and to 51.57% when cocultured with LCBE. In contrast, a reduction in the abundance of Proteobacter and Fusobacteria phyla was noted.

At the genus level, the abundance of *Bacteroidetes*, *Escherichia-Shigella*, *Bacteroids*, and *Parabacteroides* decreased when cocultured with probiotics (*E. faecium* R-026 alone, *B. subtilis* R-179 alone and LCBE) compared to the control group. Moreover, the abundance of–*Lactobacillus*, *Prevotella_9*, *Bifdobacterium*, *Enterococcus*, *Lactococcus* and *Faecalibacterium* at the genus level were higher when cocultured with probiotics compared to the control group. Abundance of *Bacillus* genus members was also higher when cocultured with *B. subtilis* R-179 or LCBE, although this effect was attributed to *B. subtilis* R-179 with the combined treatment. LEfSe analysis showed that coculture with *B. subtilis* R-179 alone increased *Lactobacillates*, *Lactobacillaceae*, *Lactobacillus*, *Enterococcus*, and *Pseudomonadles*, while coculture with *E. faecium* R-026 alone increased the abundance of *Bacilli*, Firmicutes, *Bacillales*, *Bacillaceae*, *Bacillusensia*, *Eubacterium coprostanoligenes*, and *Bacteroidales* S24-7 (**Figure S3**). In contrast, coculture with LCBE significantly increased *Bacillus*, Firmicutes and *Lactobacillates*, but decreased Proteobacteria, *Enterobacteraceae*, *Gammaprotecobacteria* and *Escherichia-Shigella* populations (**Figure 5**).

SCFAs analyses revealed that coculture with *E. faecium* R-026 alone or LCBE significantly increased the levels of isobutyrate. However, there were no significant alternations in the production of acetate, propionate, butyrate, isovalerate, or valerate in any of the treatment groups (**Table 3**). This indicated that microbiome regulation may increase the production of SCFAs in the gut.

DISCUSSION

A healthy gut microbiota is essential for the well-being of the host. For example, *Bacillus* has been found to promote the production of diverse digestive enzymes and over 45 kinds of antibacterial compounds that suppress the growth of pathogenic bacteria (Peng et al., 2021). *Enterococcus* has been used as a dietary supplement to suppress harmful microorganisms (Chen et al., 2014). *B. subtilis* R-179 and *E. faecium* R-026 have been considered probiotics for a long time (Ben Braïek and Smaoui, 2019; Sun et al., 2019). This study aimed to assess the probiotic effects of *E. faecium* R-026, *B. subtilis* R-179, and LCBE, the live combination of the two strains. The growth pattern of the two strains was first examined and the growth of both strains was found to be accelerated in the presence of oligosaccharides commonly found in the gut. Additionally, the growth pattern of the two strains was not affected by anaerobic strains of bacteria in the gut. Conversely, the growth of anaerobic strains, e.g., *Bifidobacterium* and *Clostridium*, was not affected by the presence of either *E. faecium* R-026 or *B. subtilis* R-179. It was noted that when each strain or LCBE was cultured in the presence of GOS or FOS, the production of butyrate and propionate was significantly increased. These results indicated it is highly likely that *E. faecium* R-026, *B. subtilis* R-179, or the live combination of the two strains may adapt well to the gut microenvironment, and that the addition of these strains to the microbiome may increase the production of SCFAs that are beneficial to the host as well as facilitate the growth of other strains of bacteria that are common in the gut. Of note, it was also found that human fecal bacterial composition was altered after coculture with either strain or LCBE, indicating that the LCBE may modify the micro-environment of the gut *via* modification of the microbiome.

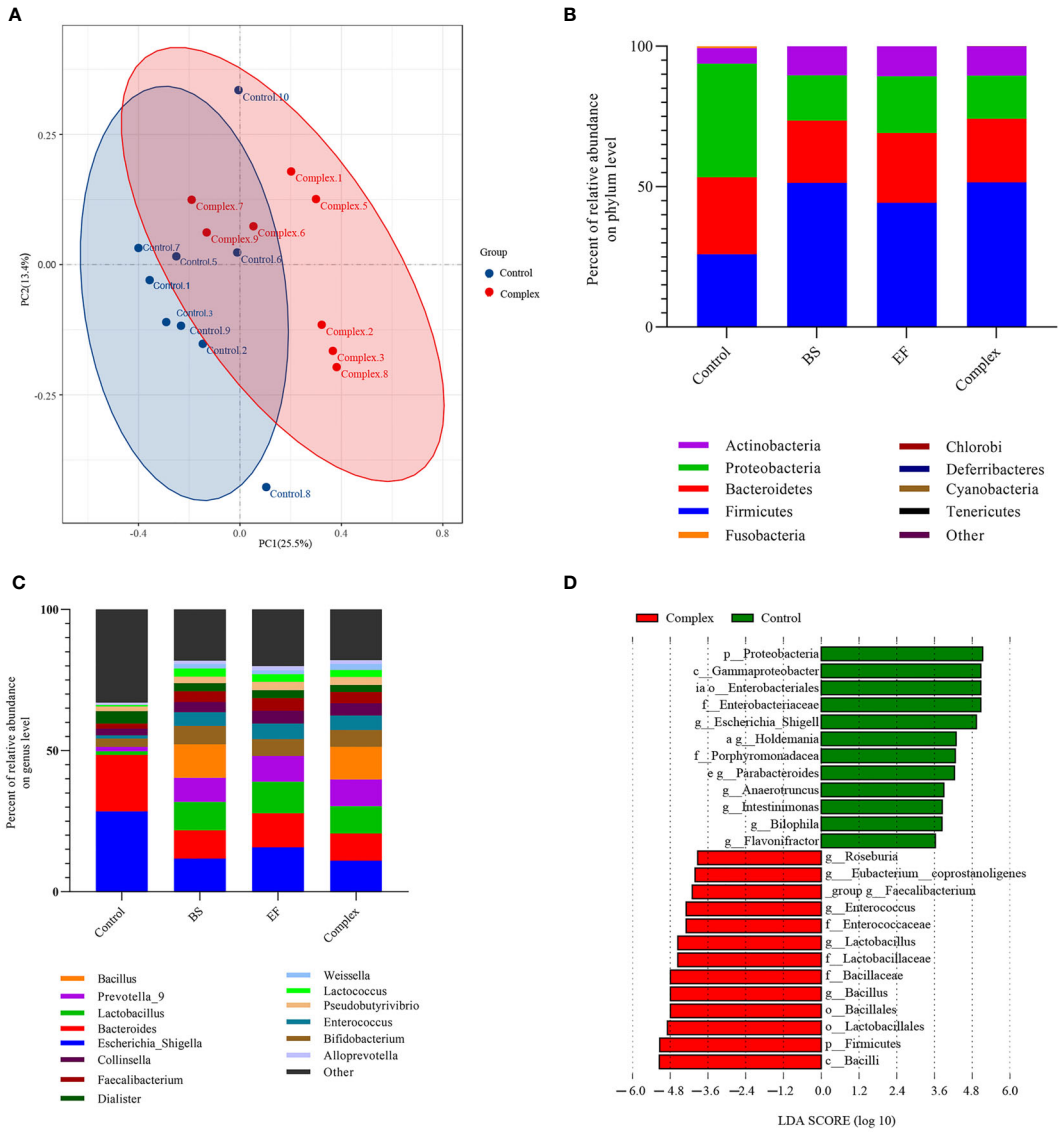

FIGURE 5 | Probiotic treatment differentially changes the human fecal microbiota *in vitro* fermentation after 24 h of anaerobic incubation. **(A)** Principal coordinate analysis (PCoA) of unweighted-UniFrac distances of all human fecal microbiome samples; **(B, C)** Major phyla and genus changes upon *Bacillus subtilis*, *Enterococcus faecium* and probiotic complex treatment over time up to 24 h; **(D)** Microbial cladogram indicating microbial clustering of human fecal microbiota in probiotic complex compared to control treatment. BS represents the *Bacillus subtilis* group, EF represents the *Enterococcus faecium* group.

The gut microbiota contributes to the regulation of the chemical environment in the gut. Ben Braïek and Smaoui discovered that oxygen consumption in the colon *via* the process of oxidation is a major cause of antibiotic-triggered dysbiosis of the microbiota (Ben Braïek and Smaoui, 2019). Increased redox potential in the gut may indicate an intestinal inflammatory state or malnutrition. Additionally, antibiotic-induced gut microbiota alterations can disturb the redox dynamics in the gut and result in the overgrowth of facultative anaerobes such as Enterobacteriaceae (Shi et al., 2017a). It was observed in the current study that the redox potential was decreased during the coculture with *E. faecium* R-026 and *B. subtilis* R-179 under anaerobic conditions (**Table 2**), and the results support the hypothesis that redox dynamics can be altered by a specific bacterial taxon present within the intestinal microbiota.

In addition, LCBE combined with GOS was shown to attenuate an increase in TC levels in the plasma; however, no significant differences in TG, HDL-C, or LDL-C levels were observed in mice fed with a high-fat diet. In the current study, an increase in the F/B ratio was noted in mice on a high-fat diet due to an increased abundance of Firmicutes and decreased abundance of Bacteroidetes. This phenomenon has been reported to contribute to the pathogenesis of colonic inflammation in patients with inflammatory bowel disease and

TABLE 3 | Short-chain fatty acid (SCFA) concentration of different probiotic treatments after 24 h fermentation.

Probiotics treatment	SCFA (mM)					
	Acetate	Propoinic acid	Butyrate	Isovalentic	Isobutyrate	Valentic
Control group	13.65 ± 7.21	4.01 ± 3.50	0.29 ± 0.19	0.05 ± 0.02	0.04 ± 0.03	0.03 ± 0.04
Bacillus group	14.74 ± 6.62	4.91 ± 4.35	0.42 ± 0.22	0.07 ± 0.03	0.06 ± 0.04	0.04 ± 0.05
Enterococcus group	11.27 ± 5.88	4.26 ± 4.08	0.08 ± 0.07	0.04 ± 0.04	0.31 ± 0.17*	0.08 ± 0.02
Complex group	12.12 ± 5.83	4.61 ± 5.04	0.26 ± 0.21	0.08 ± 0.03	0.21 ± 0.30	0.07 ± 0.07

*Indicates significant difference between mean values within a column ($P < 0.05$).

other functional gastrointestinal disorders (Walker et al., 2011). Administration of LCBE with GOS in mice on a high-fat diet attenuated this increase, and it is highly likely that the combination of LCBE and GOS promoted the growth of *Bifidobacterium*. This observation is consistent with previous reports that demonstrated the important role of the *Bifidobacterium* population in preventing the development of obesity and insulin resistance (Seganfredo et al., 2017). Similarly, at the genus level, mice administered with both LCBE and GOS showed an increased abundance of the genera *Lachnospiraceae* NK4A136, a strain that has been reported to improve host health (Stadlbauer et al., 2020). Moreover, the reduced abundance of the genus *Dialister* was observed, a strain associated with IBS and spondyloarthritis (Tito et al., 2017; Lopetuso et al., 2018). Therefore, LCBE may promote the growth of beneficial bacteria and suppress the growth of harmful bacteria in the gut.

The use of probiotic bacterial strains has been explored in clinical studies for the treatment or prevention of diarrhea and hepatopathy. Among these, *B. subtilis* R-179 has shown some potential as a treatment for oral candidiasis due to its ability to inhibit the growth and proliferation of *Candida* spp. (Zhao et al., 2016). Moreover, a live combination of *B. subtilis* R-179 and *E. faecium* R-026 has been used as a probiotic supplementation to inhibit the growth of *Heliobacter pylori* (Stein, 2005). Additionally, *B. subtilis* R-179 and *E. faecium* R-026 can also restore the entire fecal microbiota after the use of antibiotics and thus play a role in ameliorating secondary infection in clinical patients (Lu et al., 2014). The current study indicated that LCBE may adapt well to the gut microenvironment and promote SCFA production and the growth of other bacterial strains that are beneficial to the host. Furthermore, LCBE conferred some benefits to mice on a high-fat diet and may modify the composition of the gut microbiota, and thereby provide further health benefits to the host. Further studies are required to confirm the potential benefits of LCBE on host health.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The research was approved by the Committee for Animal Ethics of Zhejiang Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

WL, GZ, and XP designed the study, performed data analysis, and wrote the manuscript. WL and XP conducted the experiments. DF and WT collected all fecal samples. All authors read and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.821662/full#supplementary-material

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Effect of Early Pathogenic *Escherichia coli* Infection on the Intestinal Barrier and Immune Function in Newborn Calves

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We studied the effect of early pathogenic *Escherichia coli* infection on newborn calves' intestinal barrier and immune function. A total of 64 newborn Holstein male calves (40–43 kg) were divided into two groups: normal (NG) and test (TG), each with 32 heads. At the beginning of the experiment, the TG calves were orally administered pathogenic *E. coli* O1 (2.5×10^{11} CFU/mL, 100 mL) to establish a calf diarrhea model. In contrast, the NG calves were given the same amount of normal saline. During the 30 d trial period, the feeding and management of the two groups remained constant. Enzyme-linked immunosorbent assay, quantification PCR, and high-throughput 16S rRNA sequencing technology were used to detect indicators related to the intestinal barrier and immune function in the calf serum and tissues. Pathogenic *E. coli* O1 had a significant effect on calf diarrhea in the TG; it increased the bovine diamine oxidase ($P < 0.05$) and endotoxin levels in the serum and decreased ($P < 0.05$) the intestinal trefoil factor ($P < 0.05$), *Occludin, Claudin-1*, and *Zonula Occludens 1 (ZO-1)* levels in the colon tissue, as well as downregulated the mRNA expression of *Occludin, Claudin-1*,and *ZO-1* in the colon mucosa, leading to increased intestinal permeability and impaired intestinal barrier function. Additionally, pathogenic *E. coli* had a significant impact on the diversity of colonic microbial flora, increasing the relative abundance of Proteobacteria at the phylum level and decreasing the levels of Firmicutes and Bacteroides. At the genus level, the relative abundance of *Escherichia* and *Shigella* in the TG increased significantly ($P < 0.05$), whereas that of Bacteroides, *Butyricicoccus*, Rikenellaceae_RC9_gut_group, *Blautia*, and *Lactobacillus* was significantly decreased ($P < 0.05$). In addition, the level of IL-6 in the serum of the TG calves was significantly increased ($P < 0.05$), whereas the IL-4 and IL-10 levels were significantly decreased ($P < 0.05$), compared to those in the NG calves. Thus, pathogenic *E. coli* induced diarrhea early in life disrupts intestinal barrier and impairs immune function in calves.

Keywords: pathogenic *Escherichia coli* O1, calf, colonic microflora, intestinal barrier, immune function

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1 INTRODUCTION

With the continuous development of the cattle industry, calf diarrhea has brought substantial economic losses. Bacterial diarrhea accounts for 30% of calf diarrhea. Pathogenic *Escherichia coli* is the most common causative agent of bacterial diarrhea (Alomari et al., 2021). The balance of intestinal flora is the essential for animal health, and imbalance will result in various problems such as diarrhea, reduced digestibility, and nutrient digestion and absorption (Kim et al., 2021). Pathogenic *E. coli* O1 destroys the intestinal barrier and increases inflammatory factor levels and intestinal permeability, resulting in the outflow of macromolecular substances and diarrhea (Jia et al., 2018). The intestinal tract of newborn calves lacks a stable structure, and the intestinal flora is unstable, making it more vulnerable to pathogenic microorganisms and causing intestinal diseases. Many microorganisms colonize the gastrointestinal tract of newborn calves from the external environment. Once the intestinal microbial barrier is compromised, many pathogenic bacteria can colonize the intestinal tract and cause inflammation (Qin et al., 2010).

The animal intestine is not only an important site for digestion and absorption but also the largest immune organ of the body (Pabst et al., 2008). As an important site for the body to interact with the external environment, it can protect the body from foreign pathogenic microorganisms and improve its immunity (Hiippala et al., 2018). Changes in the intestinal barrier affect nutrient absorption and allow for the invasion of harmful substances (Jacobi and Jack, 2012). Intestinal barrier dysfunction can result in various intestinal diseases, such as diarrhea, inflammatory bowel disease, ischemic disease, Crohn's disease, and food allergies (Kucharzik et al., 2001). The intestinal barrier is a complex defense system that serves as the first line of defense against pathogen invasion. It comprises a mechanical barrier, chemical barrier, microbial barrier, and immune barrier (Wells et al., 2017). The intestinal mechanical barrier is composed of intestinal epithelial cells (Poritz et al., 2004). The chemical barrier consists of mucus secreted by intestinal epithelial cells, digestive juices, and antibacterial substances in the intestinal cavity. The normal microbial flora and the intestinal mucosa work together to prevent the colonization of foreign pathogenic bacteria while maintaining the intestinal micro-ecological balance, forming a microbial barrier. The immune barrier consists of antibodies secreted by intestinal epithelial cells and gut-associated lymphoid tissue (Anderson et al., 2012). The intestinal barrier is crucial for the intestinal function of an organism. Damage to any aspect can lead to intestinal dysfunction.

TJ proteins are essential components of the intestinal mechanical barrier that form the foundation of its structure and can strengthen its function. The blocking proteins occludin, claudin, Zonula Occludens (ZO)ZO-1, and ZO-2 are all important members of TJs (Nusrat et al., 2000). Occludin is the first complete membrane protein located in tightly connected fibrils. It plays an important role in the regulation of cell permeability. A change or decrease in occludin levels increases the permeability of intestinal epithelial cells and allows for the entry of macromolecular substances. Thus, occludin can be used as the main detection index of intestinal tract damage (Mazzon et al., 2002). In addition, occludin is combined with a zipper through the outer ring to produce a tight paracellular closure and participate in the signaling pathway formed by TJs (Le et al., 2018; Sharma et al., 2018). When pathogens invade TJs, toll-like receptors (TLRs) are activated in the intestinal mucosa and bind to multiple ligands, thereby functioning as pro-inflammatory mediators to stimulate epithelial cells and send alarm signals. The activation of pro-inflammatory mediators allows innate immune cells and adhesion molecules to regulate the entry of inflammatory cells and even transport them through the epithelium into the intestinal lumen (Medzhitov and Janeway, 2002). Intestinal trefoil factor (ITF) is a protease resistance factor secreted by goblet cells into the lumen of the small and large intestines. When intestinal damage increases significantly, ITF can prevent intestinal epithelial damage and promote repair (Wu et al., 2021). The intestinal mucosa is a highly complex structure that plays an essential role in the relationship between the host and the environment, regulating the interaction between bacteria and host cells, and affecting the absorption of nutrients.

Therefore, this study developed a diarrhea model to study the effects of early pathogenic *E.coli* infection-induced diarrhea on the intestinal barrier and immune function of calves and provided a theoretical foundation for the healthy breeding of calves and the prevention of pathogenic *E. coli*-induced diarrhea.

2 MATERIALS AND METHODS

2.1 Preparation of Pathogenic *E. coli* O1

Pathogenic *E. coli* O1 was inoculated from laboratory stocks onto nutrient agar and incubated at 37°C for 24 h. Thereafter, a single colony from the agar culture was inoculated in nutrient broth, followed by incubation at 37°C for 24 h on a shaker. The optical density (OD) at 600 nm of the bacterial culture in the logarithmic growth phase was measured using a microplate reader to determine the required concentration. The culture was stored in a suspension at 4°C in a refrigerator, and eosin-methylene blue medium was used for strain detection.

2.2 Grouping of Experimental Animals

Sixty-four newborn Holstein male calves (40–43 kg) were randomly divided into a normal group (NG) and a test group (TG), each comprising 32 cows. At the beginning of the experiment, the calves in the TG were orally administered pathogenic *E. coli* O1 (2.5×10^{11} CFU/mL, 100 mL) to establish a calf diarrhea model, and the NG calves were orally administered the same volume of normal saline. The feeding and management of both groups remained constant, and the trial period lasted 30 d.

2.3 Ethics Statement

The calf test was evaluated and approved by the Animal Care and Use Committee of the Inner Mongolia Agricultural University (Hohhot, China). The experimental program was conducted in

strict accordance with the National Standard Guidelines for the Ethical Review of Animal Welfare (GB/T 35892-2018).

2.4 Observation of Calves' Clinical Symptoms and Histopathological Analysis

The calf feces were scored during the test period using Teixeira's fecal scoring standard (**Table S1**) (Teixeira et al., 2015). The scoring results were combined with the observations of appetite, body condition, and coat color to evaluate the mental state.

The calves were slaughtered according to welfare standards, and their colons were immediately collected and fixed in 4% paraformaldehyde for histopathological examination. The tissue was trimmed, dehydrated, transparent, paraffin-infiltrated and embedded to make paraffin sections. Finally, the tissue morphology was observed by hematoxylin-eosin (HE) routine staining.

2.5 Determination of Intestinal Permeability

Blood samples (10 mL) were collected before morning feeding aged 12 h, 24 h, 36 h, 48 h, 72 h, 5 d, 10 d and 30 d. The blood sample was left standing for 30 min, and centrifuged at 3,000 r/min for 15 min to obtain serum, which was stored at -20°C. After blood collection, four calves from each group were slaughtered. During the slaughter process, a section of the colon from each calf was aseptically collected in a 2-mL cryotube and stored at -80°C. Then, 0.1 g sample of the stored tissue was placed in an autoclave-sterilized mortar, quickly frozen with a small volume of liquid nitrogen, and ground into powder. Thereafter, 0.9 mL of sterile physiological saline was added and mixed well to prepare a tissue homogenate (10%). After treatment with an ultrasonic cell pulverizer, the homogenate was centrifuged at 3,000 r/min for 15 min, and the supernatant was collected. Enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Enzyme Immunology Industry Co., Ltd., Jiangsu, China) were used to measure the levels of diamine oxidase (DAO) and endotoxin (ET) in the serum as well as the contents of occludin, claudin-1, ZO-1, and ITF in the supernatant to assess intestinal permeability.

2.6 Measurement of mRNA Expression of TJ Proteins in the Colon Mucosa

The sampling times for measuring mRNA expression of TJ proteins in colon mucosa were the same as those mentioned in Section 2.5. During the slaughter process, clean and sterile glass slides were used to scrape the colonic mucosa and store it in a 2 mL cryotube at -80°C. The mRNA expression levels of the colonic TJ proteins *Claudin-1*, *Occludin*, and *ZO-1* were measured using quantitative fluorescence PCR.

First, 50–100 mg of colon mucosa was weighed, cryopreserved at -80°C, and lysed with TRIzol reagent to extract total RNA. The quality of the total RNA was determined based on the OD260/OD280 ratio (R-value); a value ranging from 1.8 to 2.0 indicated RNA purity, lower than 1.8 indicated protein contamination, and higher than 2.0 indicated RNA degradation. Thereafter, the PrimeScriptTM RT Master Mix kit (Takara Bio, Japan) was used to reverse-transcribe the extracted total RNA into cDNA. Next, the primers for the TJ-related proteins *Claudin-1*, *Occludin*, *ZO-1*, and the internal reference gene ACTB were used, according to the manufacturer's instructions. The sequences of each primer are listed in **Table 1**. Primers were synthesized by Skyray Biotech (Hohhot, China). The primers were diluted according to the manufacturer's instructions, packed separately, and stored at -20°C for later use. The three genes claudin-1, occludin, and *ZO-1* were subjected to qPCR with ACTB as the internal reference gene. The qPCR components are listed in **Table S2**, and the reaction conditions are listed in **Table S3**. Each sample was analyzed in triplicate. The average Ct value and ΔCt value of the internal reference gene β-actin and TJ proteins were calculated as follows:

$$\Delta\text{Ct} = \text{target gene Ct value} - \text{internal reference gene Ct value}$$

$$\Delta\Delta\text{Ct} = (\text{TG target gene Ct value} - \text{TG internal reference gene Ct value}) - (\text{NG target gene Ct value-NG internal reference gene Ct value})$$

Furthermore, the 2-ΔΔCt method was used to calculate the relative expression of the target gene.

2.7 DNA Extraction and High-Throughput 16S rDNA Gene Sequencing

Genomic DNA from the microbial cells was extracted using the E.Z.N.A.® soil DNA kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's protocol. The quality and integrity of the extracted DNA were assessed using agarose gel electrophoresis (1% gel), and the DNA concentration and purity were determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA) (Wu et al., 2021).

TABLE 1 | Sequences of primers used for assessing gene expression of *Claudin-1, Occludin* and *ZO-1*.

Gene name	Gene accession number	Primer sequence	Primer size(bp)
ACTB	NM_173979.3	F: CATCGTCCACCGCAAAT R: GCCATGCCAATCTCATCTC	103
CLAUDIN-1	NM_001001854.2	F: CCCGTGCCTTGATGGTGATTGG R: CATCTTCTGTGCCTCGTCGTCTTC	110
OCCLUDIN	NM_001082433.2	F: CCCGTGCCTTGATGGTGATTGG R: CCATAGCCATAACCGTAGCCATAGC	143
ZO-1	XM_024982009.1	F: GCATGATGATCGTCTGTCCTACCTG R: CCGCCTTCTGTGTCTGTGTCTTC	108

The primer sequences of the hypervariable V3–V4 region of bacterial 16S rDNA gene are 5'-ACTCCTACGGGAGG CAGCAG-3' (forward) and 5'-GGACTACHVGGGTW TCTAAT-3' (reverse) (Wu et al., 2021). The PCR conditions for amplifying the 16S rDNA gene are as follows: initial denaturation at 95°C for 1 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were detected using agarose gel electrophoresis (2% gel), purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using the Quantus™ Fluorometer (Promega, USA) (Chen et al., 2021). The purified amplicons were sequenced using the Illumina MiSeq PE300/NovaSeq PE250 platform (Illumina, San Diego, CA, USA), according to the standard protocol of Meggie Biological Co., Ltd. (Shanghai, China) (Chen et al., 2021).

Sequences obtained from the MiSeq platform were subjected to quality control and filtering. After samples were distinguished, operational classification unit (OTU) cluster analysis and species taxonomy analysis were performed. Sequences with similarity (≥ 97%) were classified into the same OTU. Based on the OTU cluster analysis results, the diversity index of OTU, namely Chao1 and ACE (community richness), Shannon and Simpson indices (diversity), and the Good's coverage (sequencing depth) can be assessed (Aricha et al., 2021). Based on the taxonomic information, statistical analysis of the community structure (presented as heat maps and bar graphs) and linear discriminant analysis effect (LEfSe) can be carried out at various classification levels (Segata et al., 2011). Based on the above analysis, in-depth statistical and visual analyses of the community composition and phylogenetic information of multiple samples, such as multivariate analysis and significant difference test, were performed. Sequencing steps, database construction, and statistical analyses were conducted at the Shanghai Meggie Biomedical Technology Co., Ltd.

2.8 Determination of Immune Indices of Calf Serum

The method of serum preparation for determining immune indices of calf serum was the same as that described in section 2.5. Commercial ELISA kits (Jiangsu Enzyme Immunological Industry Co., Ltd., Jiangsu, China) were used to measure IL-4, IL-6, and IL-10 levels in the serum and detect the body's immune function.

2.9 Statistical Analysis

Excel 2016 software was used to perform preliminary processing of the experimental data, SPSS 20.0 software was used for one-way analysis of variance. The measurement results are expressed as mean ± standard deviation; $P<0.05$ indicates a significant difference, $P<0.01$ indicates a highly significant difference, and $P>0.05$ indicates that the difference is not significant.

3 RESULTS

3.1 Effects of Pathogenic *E. coli* O1 on the Clinical Symptoms and Histomorphology of Newborn Calves

The scoring results are shown in **Figure S1**. Diarrhea symptoms appeared in the calves of the TG 12 h after an oral administration of a suspension of pathogenic *E. coli* O1. The calves' feces were watery, gradually changing from light yellow to gray-white, with a foul odor. The calves also exhibited, eyeball depression, and loss of appetite. The calves in the NG did not exhibit diarrhea symptoms.

The histopathological results are shown in **Figure 1**. The intestinal villi of TG calves were broken and shed, indicating that the structure of intestinal villi was damaged by inflammation. The intestinal villi of NG calves are arranged neatly and tightly, and the intestinal mucosa is intact. It showed that the intestinal model of calves infected with pathogenic *E.coli* O1 was successfully established.

3.2 Effect of Pathogenic *E. coli* O1 on the Intestinal Permeability of Newborn Calves

As shown in **Table 2**, the level of DAO in the colons of the TG calves was significantly higher ($P < 0.05$) than in the NG calves. The level of ET in the colon of the TG calves increased significantly ($P < 0.05$) 12h compared to the NG calves.

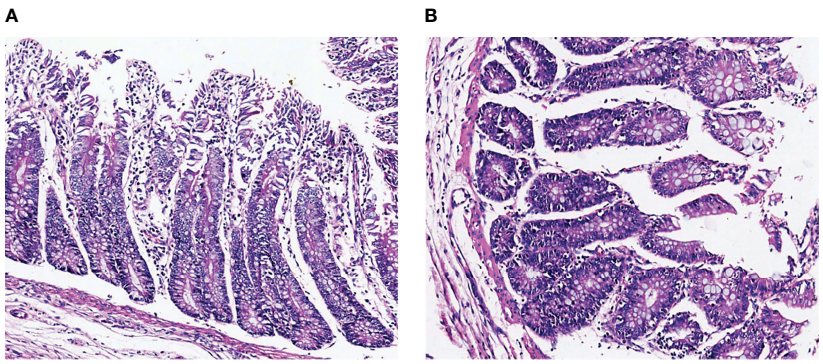

FIGURE 1 | Representative H&E staining microscopic images of colon tissues in different groups. **(A)** NG, normal group, **(B)** TG, test group.

TABLE 2 | Effect of pathogenic *E. coli* O1 on diamine oxidase (DAO) and endotoxin (ET) in the serum of calves.

Time	Group	DAO (pg/mL)	ET (EU/mL)
12 h	NG	12.85 ± 0.57^{b}	9.02 ± 0.47^{a}
	TG	15.35 ± 0.69^{a}	9.08 ± 0.86^{a}
24 h	NG	13.06 ± 0.48^{b}	10.94 ± 0.66^{b}
	TG	15.60 ± 0.67^{a}	12.03 ± 0.33^{a}
36 h	NG	14.30 ± 0.34^{b}	10.60 ± 0.53^{b}
	TG	15.85 ± 0.57^{a}	12.49 ± 0.57^{a}
48 h	NG	14.70 ± 0.46^{b}	10.08 ± 0.57^{b}
	TG	15.57 ± 0.39^{a}	12.08 ± 0.44^{a}
72 h	NG	15.01 ± 0.56^{b}	10.78 ± 0.52^{b}
	TG	16.00 ± 0.25^{a}	13.59 ± 0.73^{a}
5 d	NG	14.08 ± 0.52^{b}	10.65 ± 0.38^{b}
	TG	15.88 ± 0.47^{a}	13.33 ± 0.38^{a}
10 d	NG	14.42 ± 0.63^{b}	13.17 ± 0.30^{b}
	TG	15.96 ± 0.14^{a}	14.42 ± 0.36^{a}
30 d	NG	15.16 ± 1.05^{b}	14.90 ± 0.21^{b}
	TG	16.87 ± 0.58^{a}	15.44 ± 0.24^{a}

Different lowercase letters indicate a significant difference (P<0.05), while the same letter indicates no significant difference (P>;0.05), compared with the normal group(NG). TG, test group.

Furthermore, the DAO and ET levels showed an upward trend with increasing age in TG and NG.

Table 3 shows that the levels of claudin-1, occludin, and ZO-1 in the colons of the calves in the TG were significantly reduced ($P < 0.05$) at each sampling time point during the entire study period when compared to those in the NG.

In addition, at each sampling time point, the ITF levels in the colons of the TG calves were significantly lower than those in the NG ($P < 0.05$) (**Table 4**). The ITF concentration in both the TG and NG gradually decreased over time.

3.3 Effect of Pathogenic *E. coli* O1 on mRNA Expression of Colonic TJ Proteins in Newborn Calves

Table 5 and **Figure 2** show the effect of pathogenic *E. coli* O1 on the mRNA expression levels of colonic TJ proteins in newborn calves. The mRNA expression levels of *Claudin-1, Occludin* and *ZO-1* in the TG calves colons were significantly lower than those of the NG calves at 24 h, 36 h, 48 h, 72 h, and 10 d ($P < 0.05$).

3.4 Effect of Pathogenic *E. coli* O1 on Colonic Microflora of Newborn Calves

Based on the results of paired-end sequencing of the V3–V4 regions of 16S rRNA, 64 samples were analyzed. From the NG and TG, we obtained 2,935,442 effective sequences, and each sample had 30,490 effective sequences. The dilution curve shown in **Figure 2** indicates that the sequencing data were sufficient for subsequent detection. The data were clustered by OTUs based on 97% similarity, yielding 1,638 OTUs. The smooth decline of the curve in **Figure 3** indicates that the sample's species diversity was high. In the NG, the numbers of unique OTUs at 12 h, 24 h, 36 h, 48 h, 72 h, 5 d, 10 d, and 30 d of the experiment were 75, 24, 26, 82, 162, 20, 44, and 71, respectively, whereas the corresponding values in the TG were 5, 4, 39, 4, 14, 19, 21, and 61, respectively (**Figure 3**).

Figure 4 depicts the effects of pathogenic *E. coli* O1 on the α-diversity of calf colon microbial flora, and the sequencing coverage rates of the NG and TG were 99.72%–99.92%. The Sobs, Ace, and Chao indices of the TG were lower than those of the NG, and the difference was significant at 12 h ($P < 0.05$). Because the Ace and Chao indices are directly proportional to microbial diversity, our findings indicate that the diversity of intestinal flora of newborn calves increased with age. However, when pathogenic *E. coli* O1 was present, the microbial flora diversity of the calf colon decreased. Shannon and Simpson are microbial diversity indices, increasing the Shannon index increases community diversity while decreasing the Simpson index. In this study, the Shannon index of the TG was lower than that of the NG, and the difference was significant ($P < 0.05$), particularly at 12 h, 24 h, 36 h, 10 d, and 30 d. The Shannon index increased with calf age, whereas the Simpson index decreased, with significant differences at 12 h, 24 h, 36 h, and 5 d ($P < 0.05$). The results showed that the calves' microbial

TABLE 3 | Effect of pathogenic *E.coli* O1 on the contents of *Claudin-1, Occludin* and *ZO-1* in the colon of calves.

Time	Group	*Claudin-1* (pg/mL)	*Occludin* (ng/mL)	*ZO-1* (ng/mL)
12 h	NG	55.32 ± 2.66^{a}	626.72 ± 48.30^{a}	498.20 ± 32.91^{a}
	TG	46.61 ± 2.04^{b}	534.68 ± 29.24^{b}	461.45 ± 20.20^{a}
24 h	NG	54.97 ± 2.83^{a}	734.26 ± 100.63^{a}	539.23 ± 30.96^{a}
	TG	48.79 ± 2.28^{b}	509.49 ± 24.17^{b}	417.86 ± 35.03^{b}
36 h	NG	55.49 ± 1.94^{a}	670.32 ± 86.73^{a}	527.26 ± 42.29^{a}
	TG	49.40 ± 2.83^{b}	539.53 ± 37.10^{b}	462.30 ± 14.90^{b}
48 h	NG	57.49 ± 2.21^{a}	520.15 ± 32.65^{a}	420.42 ± 22.74^{a}
	TG	46.09 ± 0.77^{b}	453.30 ± 10.67^{b}	364.01 ± 19.62^{b}
72 h	NG	57.75 ± 1.51^{a}	646.10 ± 120.69^{a}	528.97 ± 39.58^{a}
	TG	49.92 ± 4.75^{b}	488.18 ± 23.97^{b}	429.82 ± 16.98^{b}
5 d	NG	60.28 ± 2.50^{a}	650.94 ± 92.05^{a}	475.98 ± 25.05^{a}
	TG	50.53 ± 2.02^{b}	530.81 ± 38.41^{a}	404.18 ± 22.42^{b}
10 d	NG	61.06 ± 2.32^{a}	546.31 ± 13.19^{a}	445.21 ± 17.77^{a}
	TG	53.05 ± 2.11^{b}	428.11 ± 62.20^{b}	381.10 ± 14.06^{b}
30 d	NG	62.63 ± 2.56^{a}	636.41 ± 102.65^{a}	525.55 ± 36.29^{a}
	TG	53.14 ± 2.48^{b}	457.18 ± 71.55^{b}	428.97 ± 30.63^{b}

Different lowercase letters indicate a significant difference (P<0.05), while the same letter indicates no significant difference (P>;0.05), compared with the normal group(NG). TG, test group.

TABLE 4 | Effect of pathogenic *E.coli* O1 on Intestinal trefoil factor (ITF) content in calf colon tissue (ng/mL).

Time	NG	TG
12 h	16.58 ± 1.37^{a}	13.59 ± 0.78^{b}
24 h	16.94 ± 0.93^{a}	13.95 ± 1.18^{b}
36 h	16.28 ± 1.55^{a}	13.84 ± 0.86^{b}
48 h	15.82 ± 1.93^{a}	12.17 ± 0.91^{b}
72 h	15.97 ± 2.38^{a}	12.58 ± 0.82^{b}
5 d	15.82 ± 1.73^{a}	12.58 ± 1.87^{b}
10 d	14.96 ± 1.18^{a}	12.78 ± 0.67^{b}
30 d	14.45 ± 0.89^{a}	12.37 ± 0.77^{b}

Different lowercase letters indicate a significant difference (P<0.05), while the same letter indicates no significant difference (P>;0.05), compared with the normal group(NG). TG, test group.

diversity increased after birth; however, pathogenic *E. coli* O1 infection decreased calf colon microbial flora richness and diversity.

The effect of pathogenic *E. coli* O1 on microflora level in the calf colon is shown in **Figures 5** and **6**. Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, and Epsilonbacteraeota were the dominant taxa in both the NG and TG with the remaining taxa accounting for less than 1%. Pathogenic *E. coli* O1 altered the microbial structure of the calf colon, decreasing the relative abundance of Firmicutes, Bacteroidetes, Actinobacteria, and Epsilonbacteraeota while increasing the relative abundance of Proteobacteria. The relative abundance of Proteobacteria in the TG increased significantly ($P < 0.05$) while Firmicutes and Bacteroidetes decreased significantly ($P < 0.05$) compared to the NG.

To investigate the differences between pathogenic *E. coli* O1 and the calf colon's dominant microbial flora, we selected the top 10 species of calf colon microorganisms in each group for analysis; the relative abundance of different species is shown in **Figure 6**. As shown in **Figure 7**, calf diarrhea induced by pathogenic *E. coli* O1 resulted in a change in microbial abundance in the colon. The TG had a significantly higher ($P < 0.05$) relative abundance of *Escherichia* and *Shigella* and significantly lower ($P < 0.05$) relative abundance of *Bacteroides*, *Butyricicoccus*, *Rikenellaceae_RC9_gut_group*, *Blautia*, and *Lactobacillus* than the NG.

The species with significant differences in abundance (i.e., biomarkers) can be identified using a comparative analysis between and within groups in the LDA effect size (LefSe) analysis; the larger the LDA score, the greater its influence. As shown in **Figure 8**, based on the classification level from phylum to genus, 18 different types of microorganisms were present in the colons in different groups, and the LDA scores were all greater than four. The taxa in the TG with significant differences in abundance compared to the NG were from the phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales, family Enterobacteriaceae, and genera *Escherichia Shigella*. The taxa in the NG with significant differences in abundance compared to the TG were from the phyla Firmicutes and Bacteroidetes, classes Clostridiales, Clostridia, and Bacteroidia, orders Bacteroidales and Pseudomonadales, families *Bacteroidaceae*, *Lachnospiraceae*, and *Pseudomonadaceae*, and genera Bacteroides, Butyricicoccus, and *Pseudomonas*.

3.5 Effect of Pathogenic *E. coli* O1 on Immune Function of Newborn Calves

As shown in **Table 6**, the concentration of IL-4 in the TG increased gradually; however, IL-4 concentration in the TG was significantly lower than that in the NG at the same time point ($P < 0.05$). Compared to the NG, the IL-6 level in the TG increased significantly ($P < 0.05$) at each sampling time point. At all-time points except for the 36th hour, the IL-10 level in the TG was significantly lower than that in the NG ($P < 0.05$). Although the difference between the two groups was not significant at the 36th hour, the IL-10 level in the TG was still lower than that in the NG. Furthermore, the IL-10 concentration in the TG gradually increased.

TABLE 5 | Effect of pathogenic *E.coli* O1 on the expression of *Claudin-1, Occludin and ZO-1 gene* mRNA in the colon of newborn calves.

Time	Group	*Claudin-1*	*Occludin*	*ZO-1*
12 h	NG	1.00 ± 0.04^{a}	1.00 ± 0.10^{a}	1.19 ± 0.17^{a}
	TG	0.75 ± 0.00^{b}	0.77 ± 0.07^{b}	1.00 ± 0.10^{a}
24 h	NG	1.04 ± 0.05^{a}	1.00 ± 0.01^{a}	1.21 ± 0.01^{a}
	TG	0.47 ± 0.01^{b}	0.80 ± 0.03^{b}	0.94 ± 0.01^{b}
36 h	NG	1.58 ± 0.07^{a}	1.08 ± 0.01^{a}	1.06 ± 0.03^{a}
	TG	0.97 ± 0.06^{b}	0.10 ± 0.01^{b}	0.97 ± 0.02^{b}
48 h	NG	1.51 ± 0.16^{a}	1.00 ± 0.10^{a}	1.00 ± 0.04^{a}
	TG	1.00 ± 0.03^{b}	0.10 ± 0.01^{b}	0.38 ± 0.01^{b}
72 h	NG	1.18 ± 0.03^{a}	1.26 ± 0.01^{a}	1.00 ± 0.01^{a}
	TG	0.94 ± 0.05^{b}	1.01 ± 0.01^{b}	0.90 ± 0.03^{b}
5 d	NG	1.14 ± 0.05^{a}	2.16 ± 0.23^{a}	1.00 ± 0.04^{a}
	TG	1.00 ± 0.11^{a}	1.01 ± 0.17^{b}	0.92 ± 0.04^{a}
10 d	NG	1.01 ± 0.16^{a}	1.00 ± 0.09^{a}	1.00 ± 0.01^{a}
	TG	0.46 ± 0.02^{b}	0.01 ± 0.00^{b}	0.10 ± 0.01^{b}
30 d	NG	1.00 ± 0.08^{a}	1.02 ± 0.23^{a}	1.01 ± 0.14^{a}
	TG	0.34 ± 0.01^{b}	0.93 ± 0.12^{a}	0.93 ± 0.06^{a}

Different lowercase letters indicate a significant difference (P<0.05), while the same letter indicates no significant difference (P>;0.05), compared with the normal group(NG). TG, test group.

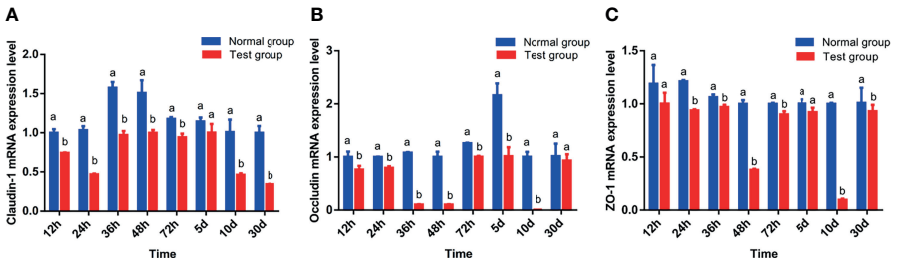

FIGURE 2 | Effect of pathogenic *Escherichia coli* O1 on tight junction protein mRNA expression in the colon of newborn calves. **(A–C)** show the expression levels of *Claudin-1*, *Occludin*, and *ZO-1*, respectively. Compared with the normal group (NG), different letters show significant differences ($P < 0.05$). The same letter indicates no significant difference ($P > 0.05$).

4 DISCUSSION

Calf diarrhea has long been considered as one of the most serious diseases in the breeding industry, necessitating early prevention. Calf diarrhea is caused by various factors, the most common of which are bacteria. Bacterial diarrhea in calves is primarily caused by pathogenic *E. coli* The composition of ruminant gastrointestinal microorganisms influences the animals' health and production performance (Uyeno et al., 2015; Celi et al., 2017). Maternal and environmental microorganisms rapidly colonize the gastrointestinal tract of calves before and after birth (Collado et al., 2012; Malmuthuge et al., 2015). Intestinal flora plays a regulatory role in host digestion, metabolism, immunity, and other physiological functions and is considered a new functional organ (Ringel-Kulka, 2012; Wei et al., 2021). The changes in intestinal flora have been linked to inflammatory bowel disease, obesity, colorectal cancer, type 2 diabetes, and intestinal dysfunction (Sabatino et al., 2017; Chen et al., 2020). The changes in the composition of calves' intestinal flora destroy the immune barrier function mediated by intestinal microbes, increasing the susceptibility of the intestinal tract to pathogenic bacteria and endangering the animals' health. Similarly, a pathogenic bacterial infection also affects the composition of the intestinal flora. For example, enteritis caused by *Salmonella enterica serovar Enteritidis* infection alters the structure of poultry intestinal flora and increases the relative abundance of lactic acid bacteria in the chicken caecum (Videnska et al., 2013). Therefore, in the present study, we used pathogenic *E. coli* O1 to induce diarrhea in calves and revealed its mechanism of action on the colonic mucosal barrier, intestinal flora, and immune function. The colonization of pathogenic bacteria in the calf intestine causes inflammation, damages the intestinal barrier, alters the structure of the intestinal flora, and reduces immune function. ELISA, qPCR, and high-throughput 16S rRNA sequencing technology were used to study the intestinal barrier, microflora structure, and immune function of calves with pathogenic *E. coli* O1-induced diarrhea.

Intestinal permeability is commonly used as an indicator to evaluate intestinal barrier function. In inflammatory bowel disease, intestinal permeability is a key factor for judging the

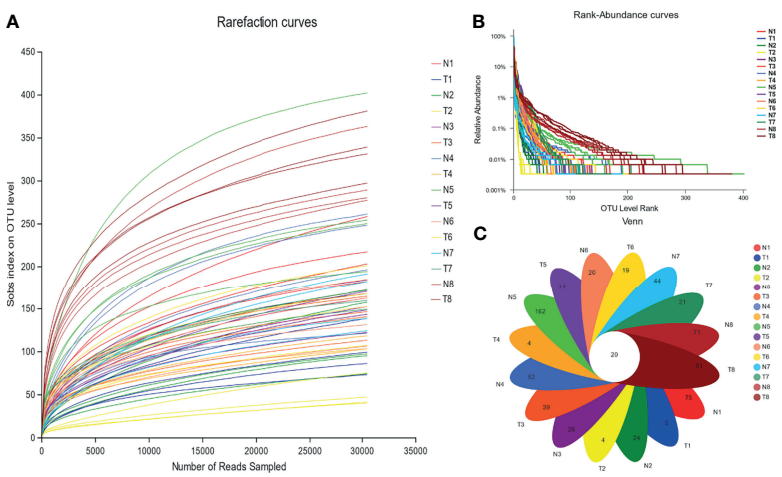

FIGURE 3 | Abundance of colonic mucosal flora based on OTU level. **(A)** Rarefaction curve, **(B)** rank-abundance curves, and **(C)** Venn diagram. 1 = 12 h; 2 = 24 h; 3 = 36 h; 4 = 48 h; 5 = 72 h; 6 = 5 d; 7 = 10 d; 8 = 30 d; N= normal group (NG); T= test group (TG).

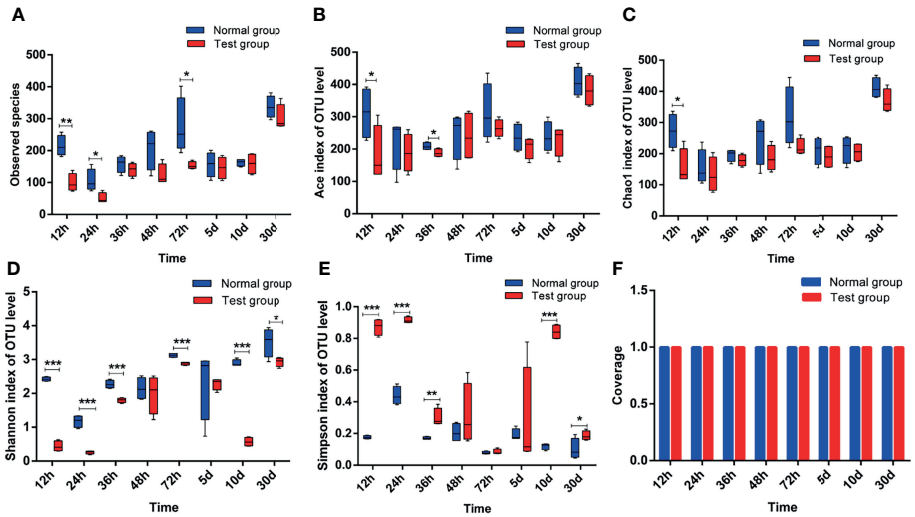

FIGURE 4 | Analysis of alpha-diversity index of colon contents in calves. **(A)** Sobs index, **(B)** Ace index, **(C)** Chao1 index, **(D)** Shannon index, **(E)** Simpson index, and **(F)** Coverage index. *0.01<*P*<=0.05, **0.001<*P*<=0.01, ***P<=0.001.

normal or pathological state of the gastrointestinal tract (Usuda et al., 2021). DAO is the main index used to assess the integrity of the intestinal mechanical barrier and the degree of mucosal villus injury (Fukudome et al., 2014). When the intestinal mucosal barrier is damaged, intestinal permeability increases, and DAO is released into the blood. DAO levels in the peripheral blood can reflect the degree of intestinal mucosal damage, and an elevated its increased level indicates that the intestinal epithelium is damaged (Crasper et al., 2016). In this study, the DAO of the TG was significantly higher than that of the NG, indicating that the TG calf intestinal mucosa was damaged. The concentration of ET concentration can indirectly reflect the state of intestinal health (Wang et al., 2021). ET enters the liver *via* the circulatory system and stimulates the production of inflammatory factors. Increased concentrations of ET cause intestinal capillary shrinkage and damage to the intestinal barrier function. In the present study, the ET concentration in the TG calves was significantly increased, indicating that their intestinal tract had suffered some damage.

Occludin and claudin, two important intestinal epithelial TJ proteins, coexist in large quantities on the animal intestinal cell membrane, forming a selective barrier to increase the permeability, adhesion, and migration between cells (Berkes et al., 2003). The concentrations of *occludin and claudin-1* in the TG were significantly lower than those in the NG. *ZO-1* not only participates in cell material transport but also interacts directly with actin *via* its C-terminal domain. The N-terminal PDZ domain directly interacts with other ZO proteins and claudins to promote the molecular link between the cytoskeleton and TJ complex (Muza et al., 2001). Calves in the

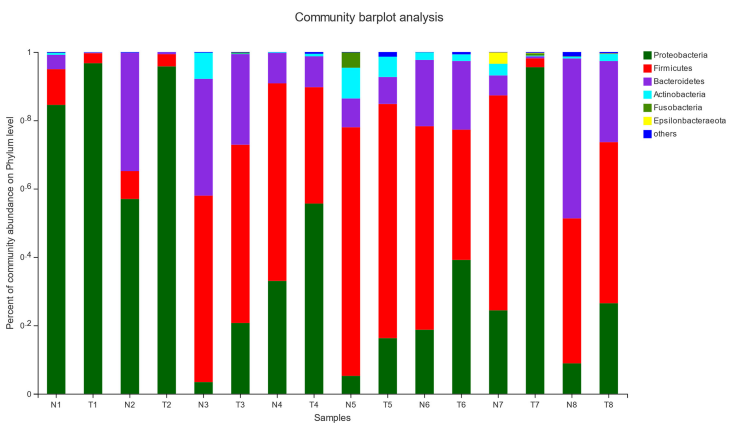

FIGURE 5 | Abundance of colonic mucosa flora at phylum level (%). 1 = 12 h; 2 = 24 h; 3 = 36 h; 4 = 48 h; 5 = 72 h; 6 = 5 d; 7 = 10 d; 8 = 30 d; N = normal group (NG); T = test group (TG).

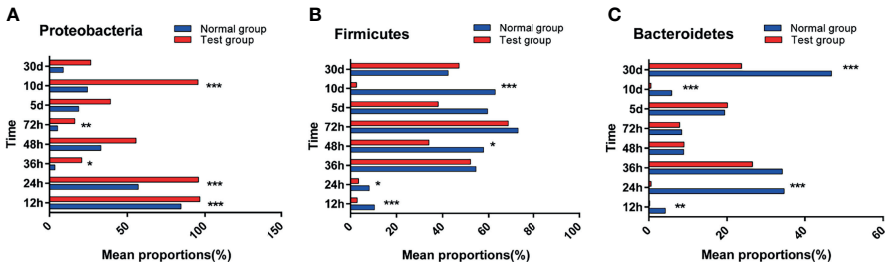

FIGURE 6 | Abundance significance of dominant flora in supraportal colonic mucosa at the phylum level (%). **(A)** Proteobacteria, **(B)** Firmicutes, and **(C)** Bacteroidetes abundance. *0.01< *P* <=0.05, **0.001 < *P* <=0.01, ***P* <=0.001.

TG had significantly lower *ZO-1* levels than those in the NG. Pathogenic *E. coli* can destroy the intestinal TJs of calves by destroying the stability of epithelial TJ proteins and decreasing claudin-1 levels, affecting intestinal permeability (Awad et al., 2017). In this study, it was found that pathogenic *E. coli* O1 significantly reduced the mRNA and protein expression levels of *Claudin-1, Occludin*, and *ZO-1* in the calf colon increased the concentration of DAO and ET, increased the permeability of calf intestinal mucosa, and destroyed the intestinal barrier function of the calf intestine.

ITF is found in various mammalian tissues, primarily in the mucosal cells of animals' small and large intestines, where it is synthesized and secreted by intestinal mucosal goblet cells. ITF is an intestinal mucosal protective factor that can promote cell proliferation and rebuild regional epithelial cells; additionally, it has a protective effect on cells (Taupin et al., 2000). ITF can enhance superficial cell resistance, decrease epithelial cell permeability, strengthen the cell-to-cell connection in intestinal mucosal injury, promote epithelial cell repair and growth by promoting cell migration, inhibit apoptosis, and form a mucin layer with mucin, thereby enhancing the function of the intestinal mucosa (Vocka et al., 2015). Furthermore, some studies have revealed that ITF inhibits the secretion of intestinal proinflammatory factors in the intestinal tract, thereby alleviating the inflammatory response (Zhang et al., 2003). In this study, the ITF concentration in the TG was found to be significantly lower than that in the NG. This result indicates that pathogenic *E. coli* O1 can decrease ITF levels in intestinal tissue and, thus, the ITF-mediated repairing effect on cells and the intestinal mucosal barrier and ITF binding with mucin weaken ITF's repair function when intestinal inflammation occurs.

Intestinal microorganisms are inseparable from the intestinal mucosal barrier, and as a part of it, they play an important role in the defense and immune function against bacteria. This experiment used high-throughput sequencing technology to perform paired-end sequencing on the V3–V4 regions of 16S rRNA to study the effect of pathogenic *E. coli* O1 on calf colon

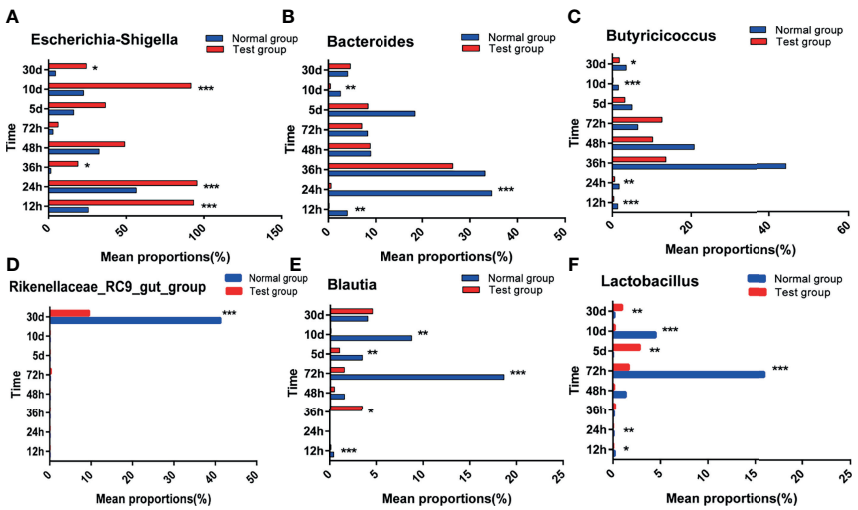

FIGURE 7 | Abundance significance of dominant flora in supraportal colonic mucosa at the genus level(%). **(A)** *Escherichia-Shigella*, **(B)** Bacteroides, **(C)** Butyricicoccus, **(D)** Rikenellaceae_RC9_gut_group, **(E)** Blautia, and **(F)** *Lactobacillus*.*0.01< *P* <=0.05, **0.001< *P* <=0.01, ***P* <=0.001.

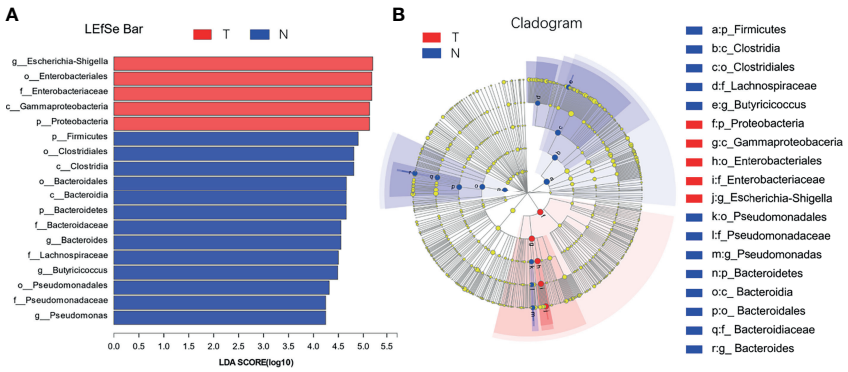

FIGURE 8 | LDA discrimination in calf colon. **(A)** Length of the histogram represents the influence of significantly different species, and different colors represent different samples, **(B)** Circle from inside to outside indicates the level of phylogeny from genus to phylum.

microbes and further understand the mechanism of the intestinal flora. The results showed that pathogenic *E. coli* O1 could reduce the richness and diversity of calf colon flora. Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria dominated at the phylum level, is consistent with previous research (Ley et al., 2008). Studies have revealed that the abundance of the bacteria phyla level in mammals fluctuated and was influenced by many factors including animal species, diet, and the mother; however, the phyla Firmicutes and Bacteroides were dominant, followed by Fusobacterium, Proteobacterium, and Actinomycetes (Ley et al., 2008). Related studies have revealed that Proteobacteria is the main phylum in the intestinal tract and is used as a gold indicator to measure the balance of intestinal flora because of its high species richness. In addition, Proteobacteria is closely related to enteritis, immune imbalance, and flora imbalance (Chinen and Rudensky, 2012; Shin et al., 2015). Our results show that with an increase in calf age, the intestinal flora gradually matured and the abundance of Proteobacteria decreased substantially. The abundance of Proteobacteria in the TG was significantly higher than that in the NG. In contrast, the abundance of Firmicutes and Bacteroides was significantly decreased in the TG compared to that in the NG. Significant differences in the microflora composition were observed at the genus level between the two groups, including differences in the levels of *E. coli Shigella, Bacteroides, Butyricoccus, Rikenellaceae _ RC9 _ gut_ group, Blautia*, and *Lactobacillus*. *Enterobacteriaceae* includes facultative anaerobic bacteria that can affect the intestinal microflora of the host. Under normal conditions, the abundance of, Enterobacteriaceae in the intestinal tract is low; when it increases, it aggravates inflammation; therefore, it is typically used as a signal indicator of disease-causing microorganisms (Lupp et al., 2007). In colitis patients, the abundance of *Butyricicoccus* spp., which produces butyric acid and improves intestinal permeability, decreases in patients with colitis (Devriese et al., 2017). The abundance of *E. coli Shigella* in the TG was significantly higher than that in the NG, whereas the abundance of *Bacteroides, Butyricoccus, Rikenellaceae _ RC9 _ gut _ group, Blautia*, and *Lactobacillus*

TABLE 6 | Effect of pathogenic *E. coli* O1 on IL-4, IL-6, and IL-10 in calf serum (pg/mL).

Time	Group	IL-4	IL-6	IL-10
12 h	NG	33.93 ± 0.97^{a}	386.75 ± 15.28^{b}	90.84 ± 3.27^{a}
	TG	32.02 ± 0.86^{b}	416.79 ± 13.07^{a}	83.62 ± 2.22^{b}
24 h	NG	37.92 ± 2.05^{a}	430.32 ± 17.72^{b}	94.41 ± 3.64^{a}
	TG	32.90 ± 2.73^{b}	451.89 ± 21.01^{a}	86.97 ± 2.22^{b}
36 h	NG	36.19 ± 0.98^{a}	469.23 ± 8.11^{b}	82.78 ± 4.83^{a}
	TG	31.42 ± 1.87^{b}	378.72 ± 15.81^{a}	75.97 ± 3.77^{a}
48 h	NG	31.35 ± 1.24^{a}	356.72 ± 12.88^{b}	92.42 ± 5.13^{a}
	TG	28.04 ± 1.16^{b}	434.55 ± 20.90^{a}	82.57 ± 5.13^{b}
72 h	NG	35.23 ± 1.07^{a}	456.97 ± 27.22^{b}	89.38 ± 3.53^{a}
	TG	31.92 ± 0.81^{b}	485.73 ± 22.08^{a}	80.05 ± 3.11^{b}
5 d	NG	40.31 ± 1.63^{a}	401.14 ± 21.73^{b}	76.18 ± 4.18^{a}
	TG	32.59 ± 2.95^{b}	487.00 ± 22.66^{a}	68.11 ± 4.19^{b}
10 d	NG	36.57 ± 1.63^{a}	412.13 ± 30.59^{b}	83.20 ± 5.76^{a}
	TG	33.43 ± 1.71^{b}	520.41 ± 30.47^{a}	73.45 ± 2.97^{b}
30 d	NG	38.52 ± 1.53^{a}	451.89 ± 14.45^{b}	95.24 ± 3.18^{a}
	TG	35.55 ± 1.23^{b}	515.34 ± 16.13^{a}	79.53 ± 6.22^{b}

Different lowercase letters indicate a significant difference (P<0.05), while the same letter indicates no significant difference (P>;0.05), compared with the normal group(NG). TG, test group.

in the TG were significantly lower than in the NG, which was consistent with previous research findings. In this study, pathogenic *E. coli* O1 induced an inflammatory response in the calf colon, resulting in changes in the colon microbial composition: the abundance of commensal bacteria decreased, while the abundance of harmful bacteria increased.

Cytokines have various functions, and their overall function can either promote or inhibit inflammation (Lai et al., 2017). For example, IL-6 promotes inflammation, whereas IL-10 inhibits inflammation (Jin et al., 2015). Moreover, IL-10 can transmit negative feedback signals, inhibit the activated immune system after inflammation, inactivate macrophages, and reduce cytokines produced by T cells (Youngah et al., 2013). High IL-6 levels promote inflammatory responses, whereas low IL-10 levels weaken the protective effects of inflammation (Youngah et al., 2013; Lai et al., 2017). After the inflammatory cytokine diffuses into the tissue, it activates local macrophages, fibroblasts, and endothelial cells, which are successively activated to produce mediators, promoting the overall immune response (Tejada-Simon and Pestka, 1999).

IL-4 is a pleiotropic growth factor produced by $CD4^+$ T-cell subsets known as TH2 cells and basophils, which can activate T cells, B cells, and thymocytes (Van Kampen et al., 2005). Studies have revealed that IL-4 can inhibit monocytes' production of IL1-, TNF-α, and IL-6 and their production of IL-2.It plays a critical role in regulating the intestinal immune response and inhibiting intestinal inflammation (Mittal et al., 2005). IL-6, which is secreted by T and B cells, is a multifunctional pleiotropic cytokine with an immune response (Kishimoto, 2010) and a key component of the inflammatory mediator network. It is the first cytokine produced after a bacterial infection and stimulates the anti-apoptosis gene cascade in T cells after binding with soluble receptors, which leads to resulting in a continuous accumulation of T cells in the intestinal mucosa and a persistent inflammatory response. TGF-β and IL-6 can stimulate the production of IL-10 by TH-17 cells. IL-10 is a cytokine that inhibits inflammation, transmits negative feedback signals, activates the immune system after inflammation, inhibits the stimulation of TH1 cells by macrophages, and the production of pro-inflammatory immune factors such as IL-6 and IL-1 (Fiorentino et al., 1991). IL-10 is secreted by TH2 cells and plays a key immunomodulatory role in controlling the intestinal antigen stimulation response; it can terminate the inflammatory response and restore the tolerance of T cells to intestinal bacteria (Lammers et al., 2003). Studies have revealed that IL-10 production is induced in TH1 cells under strong inflammatory stimulation (McGeachy et al., 2007). In the present study, the concentration of the pro-inflammatory cytokine IL-6 in the serum and colon tissue of the TG was significantly higher than that of the NG, which was consistent with the experimental results obtained by Sheldon et al. (2014). It can be inferred that the introduction of pathogenic *E. coli* O1 into calves increased the concentration of the pro-inflammatory cytokine IL-6, thereby leading to diarrhea that triggered the inflammatory response. At the same time, the concentrations of IL-10 and IL-4 in the TG were significantly lower than those in the NG; this result indicates that pathogenic *E. coli* O1 disrupted the balance between TH1 and TH2 cells and inhibited TH2 cells from secreting anti-inflammatory factors, thereby reducing the humoral immunity against foreign pathogens.

5 CONCLUSIONS

The following conclusions were drawn: colonization of pathogenic *E. coli* O1 can cause inflammation, significantly reducing the mRNA expression and protein levels of *claudin-1, occludin*, and *ZO-1*, while increasing the concentration of DAO and ET in the calf colon, thereby increasing the intestinal permeability of the calf colon and compromising the intestinal barrier function. Furthermore, after pathogenic E. coli O1 infection, the microflora composition in the calf colon changes, with an increase in the abundance of harmful bacteria and a decrease in the abundance of commensal bacteria. Pathogenic *E. coli* O1 infection increases the pro-inflammatory cytokine IL-6. It decreases the levels of the anti-inflammatory cytokines IL-10 and IL-4 in the calf serum, thereby disrupting the balance between TH1 and TH2 cells, inhibiting TH2 cells from secreting anti-inflammatory factors, and lowering the body's immune function. The present study provides insights into the effects of *E. coli*-induced diarrhea on calf immune function and intestinal flora. In the future, we will investigate the mechanism of intestinal mucosal barrier damage caused by pathogenic *E.coli* infection using the TLR4/NF-κB signaling pathway. We believe that the study will help to improve the economic benefits associated with calf breeding.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available in NCBI under accession number PRJNA785755.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the Inner Mongolia Agricultural University (Hohhot, China). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: CA. Methodology: LH, HS, HA, JZ, BL, CZ, and YC. Software: LH. Validation: CA. Formal analysis: LH. Investigation: LH. Resources: CA and CW. Data curation: LH. Writing—original draft preparation: LH. Writing—review and editing: CA and HS. Visualization: LH. Supervision: CA. Project administration: BL. Funding acquisition: CA. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.818276/full#supplementary-material

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Beneficial Alteration in Growth Performance, Immune Status, and Intestinal Microbiota by Supplementation of Activated Charcoal-Herb Extractum Complex in Broilers

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This study aimed to examine the effects of activated charcoal-herb extractum complex (CHC) on the growth performance of broilers, inflammatory status, microbiota, and their relationships. A total of 864 1-day-old Arbor Acres male broilers (41.83 ± 0.64 g) were distributed to eight dietary treatments with six replicates (18 birds per replicate), which were a corn-soybean meal-based diet (NCON); basal diets supplemented with 250, 500, 750, or 1,000 mg/kg CHC, and three positive controls; basal diets supplemented with 200 mg/kg antibacterial peptide (AMP), 200 mg/kg calsporin (Probio) or 500 mg/kg montmorillonite. The study period was 42 days including the starter (day 0–21) and grower (day 22–42) phases. Compared with the NCON group, CHC supplementation (optimal dose of 500 mg/kg) increased ($p < 0.05$) growth performance and tended to increase feed conversion rate in broilers. CHC (optimal dose of 500 mg/kg) decreased the level of the interleukin-1β (IL-1β) and interferon-γ (IFN-γ) in serum and improved the levels of immunoglobulins A (IgA) and immunoglobulins A (IgM) in serum, and secretory immunoglobulin A (SIgA) in the mucosa of duodenum and jejunum ($p < 0.05$). In the ileum, CHC supplementation decreased community abundance represented by lower Sobs, Chao 1, Ace, and Shannon compared with NCON ($p < 0.05$). At the phylum level, CHC supplementation increased the abundance of Firmicutes, while decreasing the abundance of Bacteroidetes in ileum and cecum ($p < 0.05$). At the genus level, compared with the NCON group, CHC markedly reduced ($p < 0.05$) the abundances of pathogenic bacteria *Alistipes* in the ileum, which were negatively associated with the levels of SIgA and IL-1β in ileum mucosa. In conclusion, CHC had beneficial effects on growth performance, immune status, and intestinal microbiota composition. CHC had dual functions of absorption like clays and antibacterial like antibacterial peptides.

Keywords: activated charcoal-herb extractum complex, broiler, immune, growth performance, microbiota activated charcoal-herb extractum complex, microbiota

INTRODUCTION

Various factors related to diet, infectious disease pathogens, and environment have a negative impact on the delicate balance among components of the chicken gut and subsequently impair growth rate and feed conversion efficiency (Hughes, 2005). In addition, toxins cause disease when fed to broilers in contaminated feedstuffs (Ochieng et al., 2021). Feedstuffs and feed can be contaminated with toxins in farms, post-harvesting, or during storage (Patriarca and Fernández Pinto, 2017). Over the past eight decades, antibiotics have been used in poultry production to increase productivity and efficiency (Mehdi et al., 2018). However, scientific evidence suggests that the continuous use of antibiotics in feed and/or water has led to the development of antibiotic resistance in pathogenic bacteria (Forgetta et al., 2012), and the presence of antibiotic residues in animal products and the environment (Gonzalez Ronquillo and Angeles Hernandez, 2017). These outcomes of continuous antibiotic use can compromise human and animal health (Diarra et al., 2010). Therefore, there is an urgent need to find effective additives that are both benefit growth performance and weaken gut harmful bacteria.

Activated charcoal-herb extractum complex (CHC) is produced by active charcoal's sorption of extractum from Chinese herbs. Consequently, CHC possesses dual functions of absorption and antibacterial properties (Wang et al., 2019). Activated charcoal has been proven to absorb mycotoxins in feed (Jindal et al., 1994; Burchacka et al., 2019; Santos and van Eerden, 2021) and improve the growth performance of broilers (Oso et al., 2014). Activated charcoal could clear the immune suppression induced by mycotoxins (Khatoon et al., 2018; Bhatti et al., 2021). Chinese herbs (such as *Pulsatilla chinensis*, *Portulaca oleracea L.*, *Artemisia argyi Folium*, and *Pteris multifida Poir*) have been used widely in China clinically for the treatment of digestive infections including enteritis, bacillary dysentery, and intestinal amoebiasis owing to its anti-bacterial, anti-inflammatory, and immunomodulatory activities (Wang and Wang, 2010; Liu et al., 2011; Wang and Lv, 2016; Iranshahy et al., 2017; Lan et al., 2020). CHC can reduce diarrhea in weaned pigs (Wang et al., 2019), but the effects of CHC on growth performance, immune response, and intestinal microbial composition in broilers have not been investigated.

Charcoal-herb extractum complex has two primary properties of interest. First, CHC contains activated charcoal that metabolizes or absorbs toxins in the gastrointestinal tract and prevents absorption and entrance of toxins to the liver (Zellner et al., 2019). Second, Chinese medicines are effective treatments for various infectious diseases (Wang and Yang, 2011). However, during the production of CHC, cross-reactions among active radicals may negatively affect the functionality of the activated charcoal and Chinese medicines. Thus, we selected three antibiotic alternatives with clear functional mechanisms as positive controls to compare to CHC activities. First, montmorillonite (MMT) is widely used as a feed additive due to its capacity to bind mycotoxins in animal feed and the gastrointestinal tract (Liu et al., 2021). Second, an antibacterial peptide (AMP) is a prebiotic product that contains secondary metabolites of *Bacillus licheniformis* and has strong inhibitory effects on gram-positive bacteria (Chen et al., 2021). Finally, *B. subtilis* strain (Probio) can favor the growth of beneficial microbiota and lactic acid-producing bacteria (Alakomi et al., 2000; Hoa et al., 2000).

In this study, we examined the effects of CHC on the growth performance of broilers and the potential effects of CHC on the absorption of toxins and antibacterial effects in the gastrointestinal tract, and the related immune status of broilers.

MATERIALS AND METHODS

This experiment was carried out at the National Feed Engineering Technology Research Center at the Ministry of Agriculture Feed Industry Center Animal Testing Base (Hebei, China). All procedures here were conducted in accordance with the Chinese Guidelines for Animal Welfare and approved by the Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee of China Agricultural University (AW503800710-2-2).

Preparation of Charcoal-Herb Extractum Complex

An activated charcoal-herb extractum complex was obtained from the Fujian Baicaoshaung Biotechnology Co., Ltd. (Nanping, China). The manufacture of CHC was reported in a previous paper (Wang et al., 2019). In brief, CHC is formed by active charcoal sorption of Chinese herbs extractum. Analysis of elemental composition demonstrated that approximately 90% of CHC was carbon. Activated carbon was prepared by crushing and screening cedarwood and pine wood, followed by carbonization and activation at high temperatures (120–130°C). After that, the activated carbon was screened and only the part with the size range of 0.18 to 0.25 mm was retained for the next step. The mixture of traditional Chinese herbs included a raw plant of *Pulsatilla chinensis* (dried rhizome), *Portulaca oleracea L.* (dried whole plant), *Artemisia argyi Folium* (dried whole plant), and *Pteris multifida Poir* (dried whole plant) in the ratio of 7:8:10:6. The plant mixture was washed and soaked in the five-folds volume of water for 12 h. Subsequently, the plant mixture was decocted twice at 100°C under normal pressure, each for 1 h, then the decoction was filtered and collected, followed by concentration under a low pressure to a small volume (∼0.2-fold volume of initial plant volume) of Chinese herbs extractum. Finally, Chinese herb extractum were mixed with activated carbon in a proportion of 1:9 for 8 h and then dried to make CHC product. AMP was purchased from Qilu Pharmaceutical Co., Ltd. (Shandong, China). Probio (*B. subtilis* strain C-3102, Calsporin) was purchased from Shanghai Muguan Enterprise Development Co., Ltd. (Shanghai, China). MMT was purchased from Guangzhou Jingmu Feed Co., Ltd. (Guangzhou, China).

Experiment Animals, Design, and Diets

Arbor Acres male broilers (n = 864; one day of age; initial BW = 41.83 ± 0.64 g) were allocated randomly to eight treatment groups, with six replicate pens per treatment and

18 broiler chickens per pen. Dietary treatments were: (1) negative control: corn-soybean meal-based diet (NCON), (2) NCON with 200 mg/kg Antibacterial peptide (AMP), (3) NCON with 200 mg/kg calsporin (Probio, equivalent to adding 2.0×10^9 CFU/g of viable *B. subtilis*), (4) NCON with 500 mg/kg montmorillonite (MMT), and (5) NCON with 250, 500, 750, or 1,000 mg/kg CHC. The optimal supplemental level was selected according to the growth performance in CHC treatments compared to NCON. CHC treatment with optimal dose was used as representative CHC group to analyze intestinal microbial composition with the controls.

The 42-day experimental period included a starter phase (day 0–21) and a grower phase (day 22–42). The basal diet was formulated to meet or exceed estimates of the nutrient requirements of broilers (**Table 1**) suggested by the (National Research Council [NRC], 1994). Prior to the mixing process, the feed ingredients have been weighed and ground. The ingredients, composed less than 1% (w/w) of the diet (vitamins, minerals, and CHC), were mixed well with soybean meal in advance. Finally, all ingredients were all put into a feed blender and mixed well. The broiler shed contained equipment for ventilation, heating, and lighting. Every wire-mesh cage (90 cm × 90 cm × 75 cm) housed six chicks, each equipped with two automatic teat waterers and a round feeder (diameter: 37 cm). A lighting program with 24-h continuous light was used throughout the trial. All broiler chickens had *ad libitum* access to feed and water. Environmental temperature for 1-day-old broilers was controlled to about 33°C and then gradually decreased to 24°C at week 3 (the temperature was lowered to 3°C every week), which was maintained at this temperature thereafter. Broilers were vaccinated with Newcastle disease vaccine on day 7 and vaccinated with an inactivated infectious bursal disease vaccine on day 14.

TABLE 1 | Composition and nutrient concentration of basal diets (%, as-fed basis).

Item	Starter (0–21 day)	Grower (22–42 day)
Ingredients,%		
Corn	59.85	60.80
Soybean meal	30.13	28.63
Fish meal	4.00	2.77
Soybean oil	2.75	4.54
Dicalcium phosphate	0.88	1.00
Limestone	1.45	1.38
DL-Methionine, 98%	0.14	0.08
Salt	0.30	0.30
Vitamin-mineral premix[a]	0.50	0.50
Total	100.00	100.00
Nutrient concentration[b]		
Digestible energy, kcal/kg	3,050	3,150
Crude protein	21.46	20.00
Calcium	1.00	0.95
Total phosphorus	0.68	0.67
Methionine	0.50	0.41
Lysine	1.13	1.04
Mycotoxin level, measured[c]		
DON, μg/kg	1128.17	1082.96
ZEA, μg/kg	255.81	240.34
AFB1, μg/kg	3.72	4.29

[a]Premix provided the following per kg of feed: vitamin A, 10,000 IU; vitamin D3, 3,000 IU; vitamin E, 24 mg; vitamin K_3, 2.1 mg; vitamin B_{12}, 2 mg; riboflavin, 5.0 mg; pantothenic acid, 15 mg; niacin, 40 mg; choline chloride, 500 mg; folic acid, 0.9 mg; vitamin B_6, 3.0 mg; biotin, 0.05 mg; Mn (from $MnSO_4 \cdot H_2O$), 70 mg; Fe (from $FeSO_4 \cdot H_2O$), 80 mg; Zn (from $ZnSO_4 \cdot H_2O$), 100 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 18.8 mg; I (from KI), 0.35 mg; Se (from Na_2SeO_3), 0.30 mg. [b]Values were calculated according to NRC (1994). [c]Values were measured by UPLC-MS/MS analysis. AFB1, aflatoxin B1; DON, deoxynivalenol; ZEA, zearalenone.

Sample Collection and Processing

The body weight of birds was recorded on days 1, 21, and 42 of the experiment after 12 h withdrawing of feed but not water. Body weight gain (BWG) was calculated for 1–21, 21–42, and 1–42 days periods. Feed intake (FI) was recorded for the same periods and feed conversion ratio (FCR) was calculated after correcting for mortalities. Two phases of feed samples were collected, and stored at −20°C prior to mycotoxin analysis.

On days 21 and 42, one broiler closest to the average BW of each pen was selected for blood sampling. Blood samples were collected from the jugular vein. Serum was collected after centrifugation at 1,300 × *g* for 10 min at 4°C and stored (−20°C) for analysis of immune and inflammatory indices. On day 42, after blood collection, birds from each treatment (n = 6) were weighed and then killed by cervical decapitation.

On day 42, the small intestine was divided into three parts: the duodenum (from the pylorus to the ligament of Treitz), the jejunum (from the distal portion of the duodenal loop to Meckel's diverticulum), and the ileum (anterior portion of the ileocecal junction). The intestine was sliced open lengthwise and gently rinsed in ice-cold PBS. Mucosa from the mid-duodenum, mid-jejunum, and mid-ileum of birds from each treatment (n = 6) was scraped with a glass slide. The glass slide with scrapings was rapidly placed in liquid nitrogen for measurement of intestinal immune and inflammatory indices. Gut contents from the ileum, cecum, and colon of birds from each treatment (n = 5) were collected into sterile plastic bags and immediately chilled on dry ice for analysis of intestinal microbiota.

Mycotoxin Content

Mycotoxin content was measured using ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. Briefly, the feed sample was extracted according to the QuEChERS method with slightly modified (Oplatowska-Stachowiak et al., 2015). The analysis was performed using the Acquity UPLC I-class system (Waters, Milford, MA, United States) and mass spectrometer (Xevo TQ-S, Waters) according to Kolawole et al. (2020).

Inflammatory and Immune Status

Serum immunoglobulins A, M, and G (IgA, IgM, and IgG) were measured with an automatic biochemical analyzer (Hitachi 7600, Hitachi High-Technologies Corporation, Tokyo, Japan), following the instructions that accompanied commercially available kits for each immunoglobulin (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Concentrations of interleukin-1β (IL-1β) and interferon-γ (IFN-γ) in serum and

mucosa of duodenum, jejunum, and ileum were determined by enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Intestinal secretory immunoglobulin A (SIgA) concentrations were assayed using an Sn-69513-type immune counter (Shanghai Nucleus Annular Photoelectric Instrument Co., Ltd., Shanghai, China).

Microbiota Analysis

Microbial DNA was extracted from ileal, cecal, and colonic digesta using the E.Z.N.A.® stool DNA kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions. Markers and adaptor-linked universal primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) targeting the V3-V4 region were used to amplify microbial 16S rRNA. The PCR amplification of the 16S rRNA gene was performed as follows: 95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min. The 2% agarose gel was used for the detection of purity of PCR products, and the products were purified with the AxyPrep DNA Gel Extraction kit (Axygen Biosciences, Union City, CA, United States). Purified amplicons were pooled in equimolar proportions and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, United States). The processing of sequencing data was conducted as previously described (Wang et al., 2021). Sequenced raw reads were deposited into the NCBI sequence read archive database (Accession Number: PRJNA794039).

After demultiplexing, the resulting sequences were merged with FLASH (v 1.2.11) (Magoč and Salzberg, 2011) and quality filtered with fastp (v 0.19.6) (Chen et al., 2018). Then the high-quality sequences were de-noised using DADA2 (Callahan et al., 2016) plugin in the QIIME 2 (version 2020.2) pipeline with recommended parameters, which obtains single nucleotide resolution based on error profiles within samples (Bolyen et al., 2019). DADA2 denoised sequences are usually called amplicon sequence variants (ASVs). ASVs were aligned with MAFFT and used to construct a phylogeny with FastTree2. Taxonomic assignment of ASVs was performed using the Naive bayes consensus taxonomy classifier implemented in QIIME 2 and the SILVA 16S rRNA database (v138). Analyses of the 16S rRNA microbiome sequencing data was performed using the free online platform of Majorbio Cloud Platform (cloud.majorbio.com).

Statistical Measurements

Growth Performance and Immunological Parameters Analysis

The GLM procedure of SAS (SAS Institute Inc., Cary, NC, United States) was used for data analysis. Each pen served as an experimental unit for broiler growth performance. Individual broiler was considered the experimental unit for inflammation and immune status data. Duncan's multiple comparisons were used for analyzing differences between groups. Coefficients for unequally spaced contrasts were generated by the interactive matrix algebra procedure (IML) of SAS. Then, orthogonal polynomial contrast was applied to assess the linear and quadratic responses of CHC. Differences were regarded statistically significant at $p < 0.05$, and $0.05 \leq p < 0.10$ was indicative of a differential trend.

Microbiota Analysis

The α-diversity indices, including the Shannon diversity index (Shannon), ACE estimator (Ace), Chao 1 estimator (Chao 1), and observed richness (Sobs) were expressed as mean and compared among NCON, CHC, AMP, Probio, and MMT. For α-diversity, the Kruskal–Wallis test was used for comparing multiple groups, and Mann–Whitney *U* test was used for two groups. Differences between β-diversity indices were determined by the ANOSIM test. Principal coordinates analysis (PCoA) plots were generated using the "ggplot2" packages of the R software (version 3.3.1). Also, we performed Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) using 999 permutations based on Bray–Curtis distances using the Adonis function in the package "vegan" in R software (version 3.3.1). The significant differential bacteria at phylum and genus level were determined by using analysis of the composition of microbiomes (ANCOM). $p < 0.05$ was considered statistically significant for the overall effect, and false discovery rate (FDR) < 0.10 was significant for individual contrasts in order to incorporate a stringent false discovery rate (Feye et al., 2019). Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was conducted to find differences in the relative abundance of bacteria among treatments. LEfSe scores were used to measure the consistency of differences in relative abundance between the taxa analyzed in the groups (NCON vs. CHC vs. AMP vs. Probio vs. MMT). The higher score, the higher consistency. The significant taxa were set at LDA score > 3.5 and $p < 0.05$. Spearman correlation coefficient among bacterial taxa and the concentrations of immune indices and inflammatory cytokines were calculated using the "Hmisc" packages of the R software (version 3.3.1), and the visualization work was done using "pheatmap" packages of R software. Functions of microbial community from ileal, cecal, and colonic digesta were predicted by Reconstruction of Unobserved States (PICRUSt2) analysis. Significant differences in microbiological communities among groups were evaluated by ANOSIM with the R package, "vegan."

RESULTS

Growth Performance

During the starter phase, compared with the NCON group, supplementation of 500 mg/kg CHC in diets increased BWG ($p < 0.01$, **Table 2**). As the level of CHC in the diet increased, BWG tended to increase linearly ($p = 0.058$) and responded quadratically ($p = 0.030$). Compared with the AMP and Probio groups, dietary supplementation of 500 mg/kg CHC increased BWG ($p < 0.01$), and tended to improve FCR ($p = 0.062$). There was no difference in growth performance among broilers fed positive controls and those fed 0, 250, 750, or 1,000 mg/kg CHC ($p > 0.05$).

During the grower phase, growth performance was not affected by CHC treatment. Compared with AMP, Probio, and

TABLE 2 | Effects of CHC on the growth performance of broilers (n = 6)[1].

Item	AMP	Probio	MMT	CHC, mg/kg					SEM[2]	*p* value		
				0	250	500	750	1,000		ANOVA[3]	Linear[4]	Quadratic[5]
1–21 day												
BWG, g	307.6^{b}	325.3^{b}	329.7^{ab}	323.5^{b}	330.3^{ab}	370.1^{a}	342.9^{ab}	345.0^{ab}	9.80	<0.01	0.058	0.03
FI, g	492.1	563.2	578.1	499.8	496.6	552.3	539.3	520.7	27.41	0.219	0.322	0.323
FCR	1.6	1.74	1.76	1.55	1.49	1.49	1.57	1.50	0.073	0.062	0.929	0.801
22–42 day												
BWG, g	1106.7	1063.6	1088.4	995.5	1108.2	1204.3	1048.3	1029.3	43.75	0.067	0.952	<0.01
FI, g	1992.1	1999.5	1959.1	1927.2	1896.7	1963.8	1863.7	1838.0	48.95	0.206	0.037	0.18
FCR	1.81	1.90	1.80	1.98	1.72	1.64	1.78	1.80	0.074	0.089	0.196	<0.01
1–42 day												
BWG, g	1414.3^{ab}	1388.8^{ab}	1418.1^{ab}	1319.1^{b}	1438.4^{ab}	1574.4^{a}	1391.2^{ab}	1374.3^{ab}	45.36	0.024	0.62	<0.01
FI, g	2463.5	2492.7	2490.9	2457.1	2423.6	2549. 9	2435.7	2386.0	52.42	0.510	0.312	0.079
FCR	1.75	1.81	1.76	1.88	1.69	1.62	1.75	1.74	0.054	0.081	0.196	<0.01

[1]Experimental diets were a corn-soybean meal basal diets (NCON) and basal diets supplemented with 250, 500, 750, or 1,000 mg/kg activated charcoal-herb extractum complex (CHC). Three additional diets containing 200 mg/kg antibacterial peptide (AMP), 200 mg/kg calsporin (Probio) or basal diets with 500 mg/kg montmorillonite (MMT) were set as the positive controls. [2]Values listed for means and pooled SEM of all data are actual data. [3]Statistical significance was determined using one-way ANOVA among all groups (including the three positive controls). [4,5]Linear and quadratic effects of added CHC levels, except for the positive controls (AMP, Probio, MMT). [a,b]Means within rows with different letter superscripts differ ($p < 0.05$). BWG average daily gain, FI average daily feed intake, FCR feed to gain ratio.

MMT groups, feeding 500 mg/kg CHC tended to improve BWG ($p = 0.067$) and FCR ($p = 0.089$) of broilers.

Over the whole experimental period, BWG and FCR quadratically responded with an increasing level of CHC ($p < 0.01$). Compared with the NCON group, dietary CHC supplementation at a dosage of 500 mg/kg increased BWG ($p = 0.024$), while other doses showed no effect ($p > 0.05$). In addition, supplementation of 500 mg/kg CHC tended to improve FCR ($p = 0.081$), compared with three positive controls.

Serum Immunological Parameters

Concentrations of serum IgA ($p < 0.01$) on day 21 when feeding 500 mg/kg and 750 mg/kg CHC were higher than that in NCON (**Table 3**). Concentrations of serum IgA ($p < 0.01$) on day 42 and IgM ($p = 0.018$) on day 21 at the level of 500 mg/kg CHC was higher than in the negative control. On day 21 and day 42, serum IgA tended to increase linearly with increased level of CHC from 250 mg/kg to 1,000 mg/kg ($p = 0.079$ and $p = 0.091$, respectively). The concentrations of serum IgA (day 21 and day 42) and IgM (day 21) showed quadratic ($p < 0.01$) responses to increased CHC levels. Serum IgG ($p > 0.05$) was not affected by CHC on both day 21 and day 42. Immune responses in the 500 mg/kg CHC group were similar to those observed in the three positive controls. Compared with the three positive controls, supplementation of CHC at 500 mg/kg increased serum concentrations of IgA ($p < 0.01$) both on day 21 and day 42.

Concentrations of serum IL-1β (day 21 and day 42) and IFN-γ (day 21) linearly ($p < 0.01$) decreased with increasing concentrations of CHC and responded quadratically ($p < 0.05$) on both day 21 and day 42. Dietary supplementation of 500 mg/kg CHC decreased levels of IL-1β ($p < 0.01$) and IFN-γ ($p < 0.05$) in the serum of broilers compared with NCON. Broilers fed 500, 750, and 1,000 mg/kg CHC displayed reduced serum concentrations of IL-1β ($p < 0.01$) and IFN-γ ($p < 0.01$) on day 21 compared with NCON. Similarly, 500 and 750 mg/kg CHC groups had lower concentrations of IL-1β ($p < 0.01$) and IFN-γ ($p = 0.017$) on day 42 comparing with NCON. Supplementation with 500 mg/kg CHC was more efficient in reducing serum IL-1β ($p < 0.01$) than AMP and Probio on day 21, and more efficient ($p < 0.01$) than positive controls on day 42. Supplementation with 500 mg/kg of CHC reduced IFN-γ ($p < 0.01$) more than AMP and Probio on day 21, while there was no difference between CHC and positive controls on day 42 ($p > 0.05$).

Intestinal Immunological Parameters

Supplementation with 500 mg/kg CHC increased concentrations of SIgA in the mucosa of the duodenum and jejunum ($p < 0.05$) on day 42 compared to that of NCON. CHC at 750 mg/kg increased jejunum mucosa SIgA ($p < 0.05$) concentration compared with NCON (**Figure 1**). Duodenum and jejunum mucosa SIgA concentrations were higher ($p < 0.05$) in the broilers fed 500 mg/kg CHC compared with those in positive controls.

Concentrations of IL-1β in the mucosa of jejunum and ileum in the treatments of 500, 750, and 1,000 mg/kg CHC were lower ($p < 0.05$) than that in NCON on day 42. No significant differences were observed in the mucosa of duodenal IL-1β and in the mucosa of duodenal, jejunal, and ileal TNF-α concentrations among all treatments ($p > 0.05$). Broilers fed with 750 mg/kg CHC had lower levels of IL-1β ($p < 0.05$) in ileum mucosa when compared with the Probio group. Moreover, there was no difference between CHC and positive controls in the mucosa of the duodenum and jejunum ($p > 0.05$). Furthermore, the level of TNF-α ($p > 0.05$) in the mucosa of duodenum, jejunum, and ileum did not differ among CHC and positive controls.

Microbial Diversity and Composition in Digesta

After denoising, sequences (4,253,546) from the 75 samples (with an average of 52,513 sequences per sample), representing

TABLE 3 | Effects of CHC on serum immunological parameters in broilers ($n = 6$)[1].

Item	AMP	Probio	MMT	CHC, mg/kg					SEM[2]	*P* value		
				0	250	500	750	1000		ANOVA[3]	Linear[4]	Quadratic[5]
21 day												
IgA, g/L	1.07^{bc}	1.08^{bc}	0.97^{bc}	0.77^{c}	0.98^{bc}	1.44^{a}	1.11^{ab}	0.92^{bc}	0.074	<0.01	0.079	<0.01
IgG, g/L	5.74	7.23	6.41	5.94	6.39	8.04	6.93	6.35	0.664	0.292	0.54	0.08
IgM, g/L	0.69^{b}	0.68^{b}	0.78^{ab}	0.69^{b}	0.81^{ab}	1.06^{a}	0.84^{ab}	0.70^{ab}	0.082	0.018	0.757	<0.01
IL-1β, pg/ml	32.08^{a}	29.27^{abc}	26.28^{bcd}	29.37^{ab}	27.60^{bcd}	25.22^{d}	26.35^{cd}	25.69^{cd}	0.803	<0.01	<0.01	0.045
IFN-γ, pg/ml	38.43^{ab}	39.00^{ab}	27.79^{cd}	43.85^{a}	28.30^{cd}	24.09^{d}	33.18^{bc}	32.90^{bc}	1.616	<0.01	<0.01	<0.01
42 day												
IgA, g/L	1.04^{bc}	1.00^{c}	1.10^{bc}	1.09^{bc}	1.15^{bc}	1.37^{a}	1.19^{b}	1.19^{b}	0.088	<0.01	0.091	<0.01
IgG, g/L	7.62	6.95	7.79	7.58	9.48	9.65	9.18	8.2	0.908	0.318	0.736	0.066
IgM, g/L	0.93	0.84	0.99	0.94	0.82	0.97	1.02	0.82	0.113	0.824	0.899	0.384
IL-1β, pg/ml	30.20^{abc}	31.71^{ab}	30.81^{abc}	32.88^{a}	28.62^{bcd}	25.92^{d}	28.47^{bcd}	30.05^{abc}	0.707	<0.01	0.033	<0.01
IFN-γ, pg/ml	39.00^{ab}	34.61^{b}	38.43^{ab}	40.45^{a}	34.73^{b}	34.19^{b}	34.04^{b}	39.99^{a}	1.622	0.017	0.803	<0.01

[1]Experimental diet were corn-soybean meal basal diets (NCON) and basal diets supplemented with 250, 500, 750, or 1,000 mg/kg CHC. Three additional diets containing 200 mg/kg antibacterial peptide (AMP), 200 mg/kg calsporin (Probio), or basal diets with 500 mg/kg montmorillonite (MMT) were set as the positive controls. [2]Values listed for means and pooled SEM of all data are actual data. [3]Statistical significance was determined using one-way ANOVA among all groups (including the three positive controls). [4,5]Linear and quadratic effects of added CHC levels, except for the positive controls (AMP, Probio, MMT). [a−d]Means within rows with different letter superscripts differ ($p < 0.05$). IgA immunoglobulin A, IgG immunoglobulin G, IgM immunoglobulin M, IL-1β interleukin-1β, IFN-γ interferon γ.

19,335 amplicon sequence variants (ASVs), were revealed for the subsequent analyses. Parse curves and species accumulation curves showed that the sampling of each group provided sufficient sequences to reflect the diversity and abundance of bacteria (**Supplementary Figure 1**). α-Diversity used to measure the distribution of species abundances in a given sample can be indicators of community richness (Sobs, Chao 1, and ACE indexes) and diversity (Shannon index) (Ai et al., 2017; Ji et al., 2017). The α-diversity of the microbiota in the ileum, cecum, and colon were analyzed (**Figure 2** and **Supplementary Figure 2**). In the ileum, CHC treatment decreased community abundance represented by lower Sobs, Chao 1, Ace, and Shannon compared with NCON ($p < 0.05$). The positive controls of AMP and MMT also decreased the community abundance represented by lower Sobs than NCON ($p < 0.05$). At this point of decreasing the richness of bacteria, the characteristic of CHC showed more similarity to MMT in that both of them showed similar Sobs, Chao 1, Ace, and Shannon. Shannon index represents the richness and evenness of the bacterial community. The positive control of probiotics (Probio) showed no significant difference from NCON in changing α-diversity of ileal microbiota. There was no statistically significant difference in α-diversity among the five groups in cecum and colon (**Supplementary Figure 2**). Principal coordinates analysis (PCoA) based on the ASVs showed a clear difference in community composition among groups (NCON, AMP, Probio, MMT, and CHC) in the ileal, cecal, and colonic microbiota of broilers (**Figure 3**).

At the phylum level, Firmicutes, Bacteroidetes, and Proteobacteria were predominant phyla in the ileal, cecal, and colonic microbiota in all five groups (**Figure 4**). In the ileum and cecum, the abundances of Firmicutes in CHC treatment and three positive controls were higher than that of NCON, while the abundances of Bacteroidetes in these groups were lower than that of NCON. In the colon, the abundances of Firmicutes and Bacteroidetes were not differently distributed.

A heatmap exhibited similarities and differences in bacterial communities at the genus level (**Figure 5**). *Lactobacillus*, *Romboutsia*, and *Candidatus_Arthromitus* were the dominant genera in the ileum, whereas *Lactobacillus*, *Romboutsia*, and *Barnesiella* were the dominant genera in the cecum and colon. The abundances of *Lactobacillales* in the CHC treatment were lower ($p < 0.05$) than those of the AMP and MMT treatments in the ileum. The abundances of *Romboutsia* in the ileum in the CHC group were higher ($p < 0.05$) than those of the three positive controls. The abundances of *Candidatus_Arthromitus* in the ileum in CHC-fed broilers were lower ($p < 0.05$) than those of Probio treatment and higher ($p < 0.05$) than those of NCON treatment. In the ileum, the abundances of *Alistipes* and *Faecalibacterium* in CHC treatment were similar to the three positive controls, which were all lower than that of NCON. CHC treatment had lower abundances of *Alistipes* in the ileum of broilers ($p < 0.05$) relative to those in the AMP and Probio treatments. The absorption effect of CHC on *Candidatus_Arthromitus*, *Alistipes*, and *Faecalibacterium* was similar to that of MMT. In the cecum and colon, there were no significant differences in the abundances of these dominant genera across treatments.

At the genus level, in the ileum compared with NCON, CHC treatment had significantly lower ($p < 0.05$) abundances of the pathogenic bacteria *Alistipes* (**Figure 5**). The abundance of pathogenic bacteria (*Alistipes*) in CHC treatment was lower ($p < 0.05$) than those of the AMP and Probio treatments. Compared with MMT, CHC treatment had a higher ($p < 0.05$) abundance of *Romboutsia* compared to NCON, AMP, Probio, and MMT. In addition, compared with NCON, CHC had a higher ($p < 0.05$) abundance of *Candidatus_Arthromitus*. However, CHC had no effect on the abundance of *Lactobacillus* which were

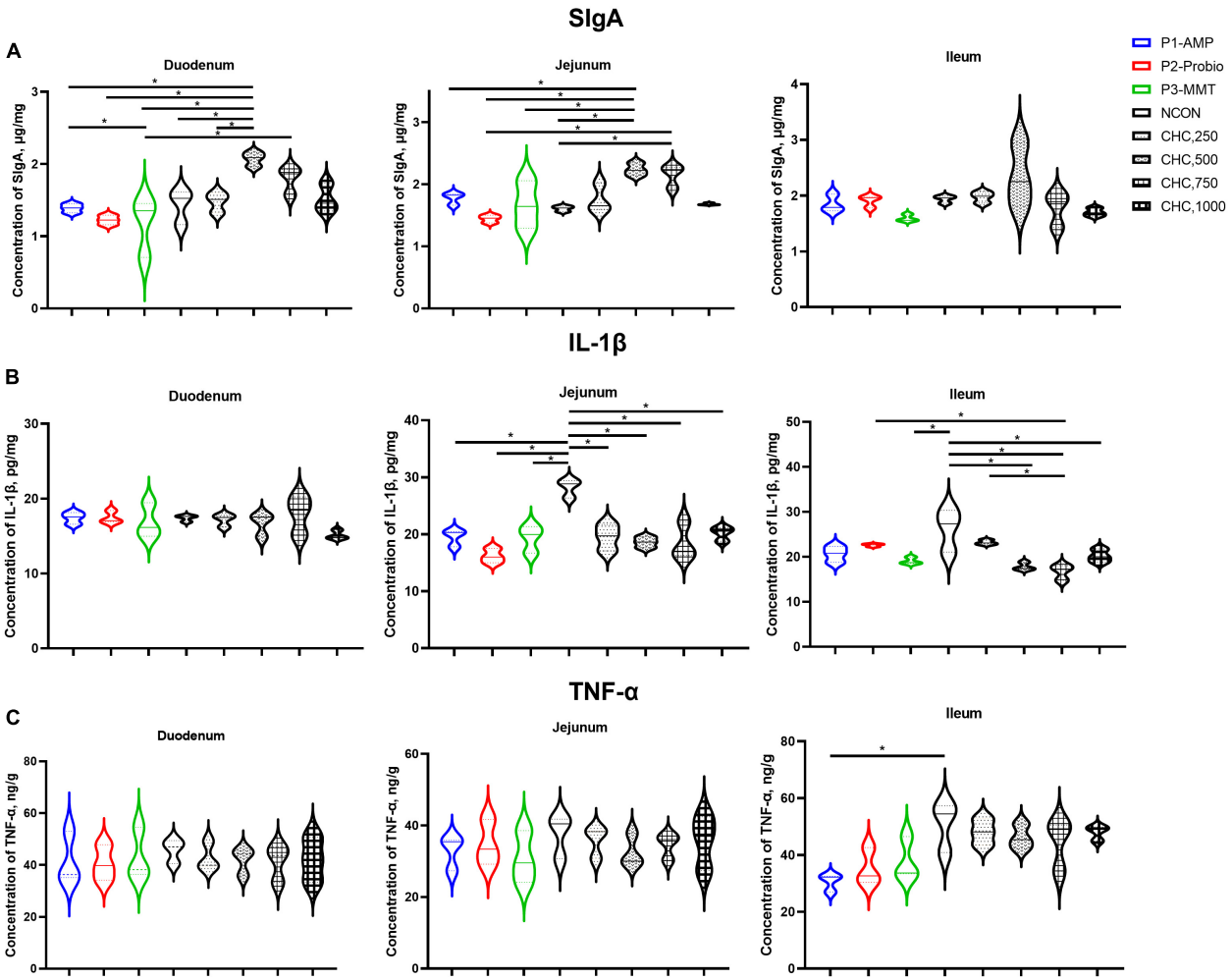

FIGURE 1 | Effects of CHC on intestinal mucosa secretory immunoglobulin A (SIgA), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) levels in broilers ($n = 6$). **(A)** The concentration of secretory immunoglobulin A (SIgA) in the mucosa of duodenum, jejunum, and ileum. **(B)** The concentration of interleukin-1β (IL-1β) in the mucosa of duodenum, jejunum, and ileum. **(C)** The concentration of tumor necrosis factor-α (TNF-α) in the mucosa of duodenum, jejunum, and ileum. The "*" indicates a significant difference between the two treatments ($p < 0.05$). Values are expressed as means (the solid line between two dashed lines) ± SEM. Experimental diets were corn-soybean meal basal diets (NCON) and basal diets supplemented with 250, 500, 750, or 1,000 mg/kg CHC. Three additional diets containing 200 mg/kg antibacterial peptide (AMP), 200 mg/kg calsporin (Probio), or basal diets with 500 mg/kg montmorillonite (MMT) were set as the positive controls.

lactic acid-producing bacteria that were beneficial for gut health. Compared to NCON, Probio treatment had lower ($p < 0.05$) abundances of *Alistipes*; and higher ($p < 0.05$) abundance of *Candidatus_Arthromitus*.

Differences in the composition of intestinal microbiota at the genus level were further explored by the LEfSe analysis (**Figure 6**). In the ileum, we found that 35 bacterial genera including gram-negative bacteria (such as *Barnesiella*, *Alistipes*, *unclassified_f__Lachnospiraceae*) and gram-positive bacteria (such as *UCG-005*, *[Ruminococcus]_torques_group*, *Faecalibacterium*) were enriched in the NCON group. The AMP group was characterized by a higher relative abundance of gram-negative bacteria (*Christensenellaceae_R-7_group*). In addition, gram-positive bacteria (*Romboutsia*), gram-negative bacteria (*Candidatus_Arthromitus*), and gram-positive bacteria (*Lactobacillus* and *Bacillus*) were enriched in CHC, Probio, and MMT groups, respectively.

In the cecum, gram-negative bacteria (*Alistipes*, *Barnesiella*, *norank_f__Desulfovibrionaceae*, *Parasutterella*, *Butyricimonas*, *Intestinimonas*, and *norank_f__Oscillospiraceae*) and gram-positive bacteria (*Colidextribacter* and *Lactobacillus*) in the NCON group had the highest richness among the five treatment groups. We also found gram-positive bacteria (*Blautia*), gram-negative bacteria (*Barnesiellaceae*, *unclassified_f__Lachnospiraceae*, *Bilophila*), and not classified bacteria (*CHKCI001*, *norank_f__norank_o__RF39*, and *Marvinbryantia*) were enriched in the AMP group. Furthermore, gram-positive bacteria (*Romboutsia* and *Turicibacter*) were particularly abundant in response to CHC, while only *Lactobacillus* (gram-positive bacteria)

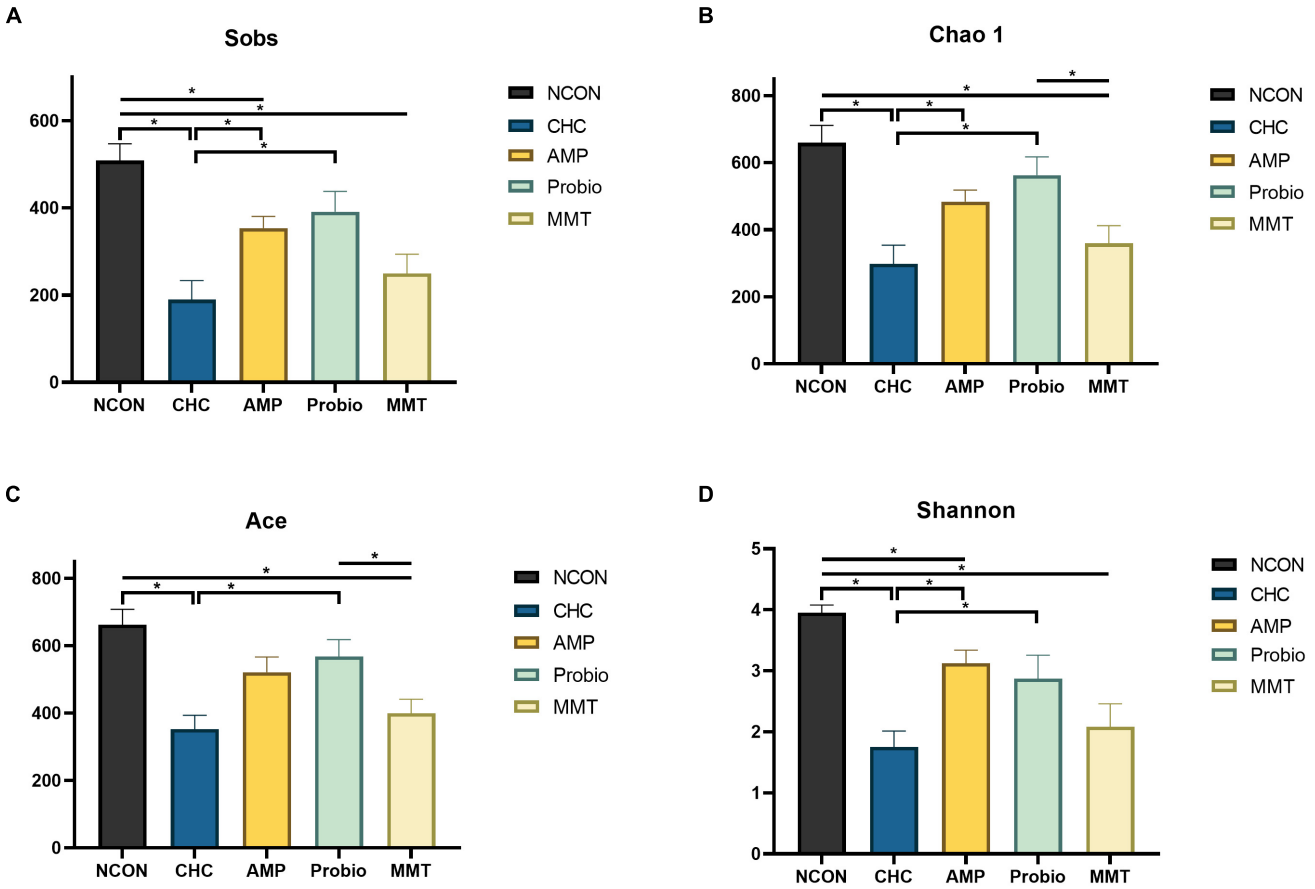

FIGURE 2 | Effects of CHC on α-diversity of ileal microbiota in broilers on day 42. **(A)** Represents Sobs index. **(B)** Represents Chao 1 estimator. **(C)** Represents ACE estimator. **(D)** Represents Shannon index. Four indexes analyzed the α diversity of broilers from different directions. NCON is the corn-soybean meal basal diet group. AMP is a 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group. The Kruskal-Wallis test was used for comparing multiple groups, and Mann–Whitney *U* test was used for two groups. The "*" indicates $p < 0.05$.

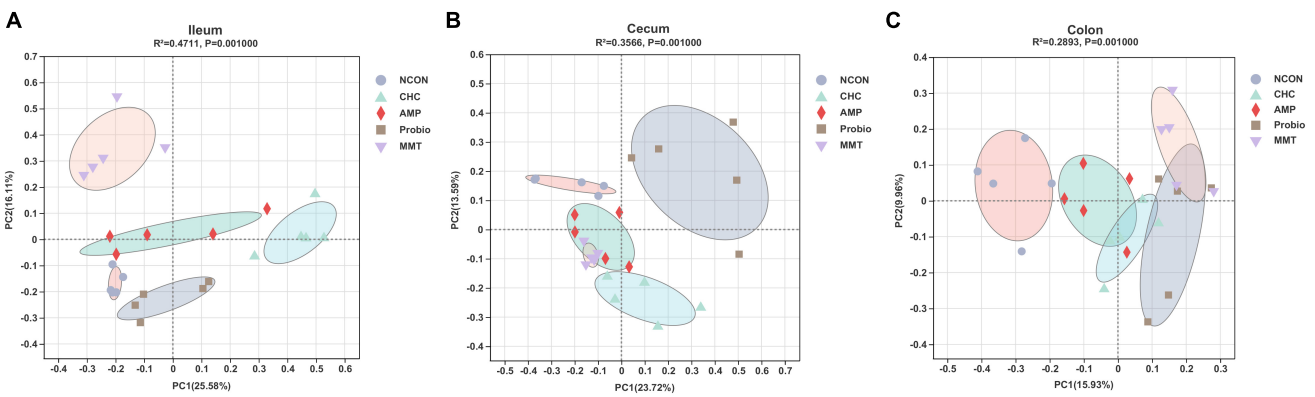

FIGURE 3 | Principal coordinates analysis (PCoA) of bacterial communities in the intestinal digesta of broilers at the ASV level (based on the Bray–Curtis distance). **(A)** PCoA of ileal microbiota composition. **(B)** PCoA of cecal microbiota composition. **(C)** PCoA of colonic microbiota composition. Each symbol represents five intestinal microbiotas in one treatment, and distance between symbols reflects relative dissimilarities in community structures. NCON is a corn-soybean meal basal diets group. AMP is a 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group.

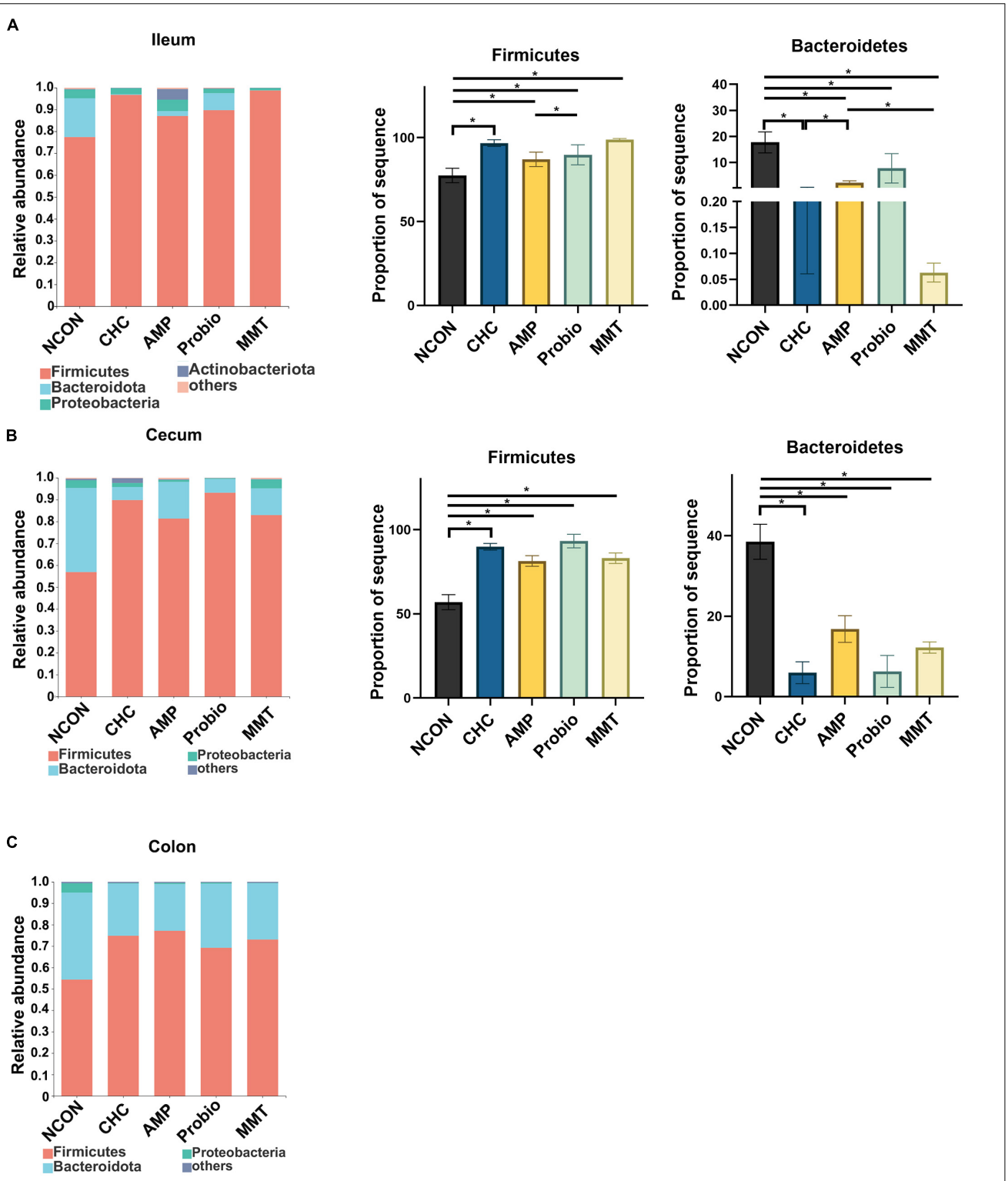

FIGURE 4 | Effects of dietary treatment on ileal, cecal, and colonic microbiota composition at the phylum level. **(A)** Ileal microbiota composition at the phylum level, and alterations of the abundance of bacterial phyla found in the ileum of broilers. **(B)** Cecal microbiota composition at the phylum level, and alterations of the abundance of bacterial phyla found in the cecum of broilers. **(C)** Colonic microbiota composition at the phylum level in broilers. NCON is a corn-soybean meal basal diets group. AMP is 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group. *P* values calculated using the ANCOM test and were adjusted by false discovery rate (FDR) correction according to the Benjamini-Hochberg procedure, and the level of significance was set at FDR < 0.10; * presented FDR < 0.10. Firmicutes (ileum), FDR = 0.031; Bacteroidetes (ileum), FDR = 0.022; Firmicutes (cecum), FDR = 0.014; Bacteroidetes (cecum), FDR = 0.014.

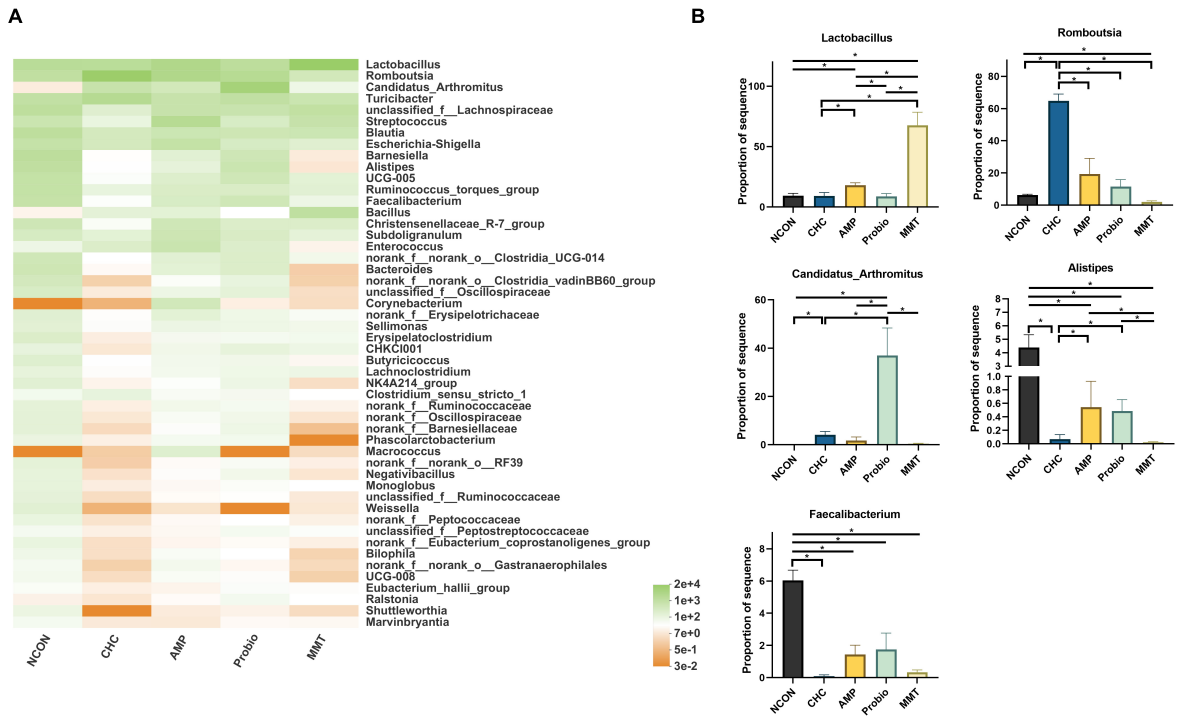

FIGURE 5 | Effects of dietary treatment on intestinal microbiota composition in the ileum at the genus level. **(A)** Ileal bacterial community heatmap at the genus level in broilers. **(B)** Significant alterations of the abundance of bacterial genera found in the ileum of broilers. NCON is a corn-soybean meal basal diets group. AMP is 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group. *P* values calculated using the ANCOM test and were adjusted by false discovery rate (FDR) correction according to the Benjamini-Hochberg procedure, and the level of significance was set at FDR < 0.10; * presented FDR < 0.10. *Lactobacillus*, FDR = 0.060; *Romboutsia*, FDR = 0.061; *Candidatus_Arthromitus*, FDR = 0.077; *Alistipes*, FDR = 0.094; *Faecalibacterium*, FDR = 0.096.

was enriched in the Probio group. The distribution of gram-negative bacteria (*Christensenellaceae_R-7_group*), gram-positive bacteria (*norank_f__Erysipelotrichaceae*), and *Family_XIII_AD3011_group* in group MMT were richer than those in other groups.

In the colon, the NCON group showed enrichment of gram-negative bacteria (*Alistipes*, *Parasutterella*, and *Anaerotruncus*) and gram-positive bacteria (*Butyricicoccus*, *Adlercreutzia*, and *Colidextribacter)*. Furthermore, the CHC group was characterized by a higher relative abundance of gram-positive bacteria (*norank_f__Clostridiales_vadinBB60_group*, *Ruminococcaceae_UCG-010*, and *Senegalimassilia*) and not classified bacteria (*CHKCI002*) than that in other groups.

Associations Between Intestinal Microbiota, Inflammatory, and Immune Factors

A Spearman correlation analysis was carried out to determine if any relationship existed among serum inflammatory traits, serum immunological markers, and intestinal microbiota (**Figure 7**).

In the ileum, the levels of serum IgA were negatively associated with *Subdoligranulum* and *Candidatus_Arthromitus* ($p < 0.05$). In addition, the levels of serum IL-1β and TNF-α were positively associated with the abundance of *Erysipelatoclostridium*, while negatively associated with the abundance of *Enterococcus* and *Macrococcus*. Levels of ileum mucosa SIgA and IL-1β were strongly associated with a series of bacteria (e.g., *Unclassified_f__Lachnospiraceae*, *Barnesiella*, *Alistipes*, *Faecalibacterium*, and *Ruminococcus_torques_group*) which indicated an active immune response induced by ileal bacteria.

In the cecum, the immune globulins (higher levels of IgA, IgG, and IgM indicating greater immune functions) exhibited a positive association with the abundance of *Lachnoclostridium*, *NK4A214_group*, *norank_f__Eubacterium_coprostanoligenes_group*, *Christensenellaceae_R-7_group*, *Subdoligranulum*, *Ruminococcaceae_UCG-005*, *Streptococcus*, *unclassified_f__Lachnospiraceae*, *Blautia*, and *Barnesiella*. In addition, correlation analysis revealed that *norank_f__Erysipelotrichaceae*, *norank_f__Oscillospiraceae*, *norank_f__Ruminococcaceae*, *unclassified_f__Oscillospiraceae*, and *Alistipes* had a strong positive association with the level of inflammatory factors (IL-1β and TNF-α) in the serum, while the bacteria *Romboutsia* and *Lactobacillus* had a strong negative association with the serum level of inflammatory factors (IL-1β and TNF-α).

In the colon, the abundance of *norank_f__norank_o__RF39*, *Sellimonas*, *Christensenellaceae_R-7_group*, *Subdoligranulum*, *Blautia*, and *Turicibacter* were positively associated with immune

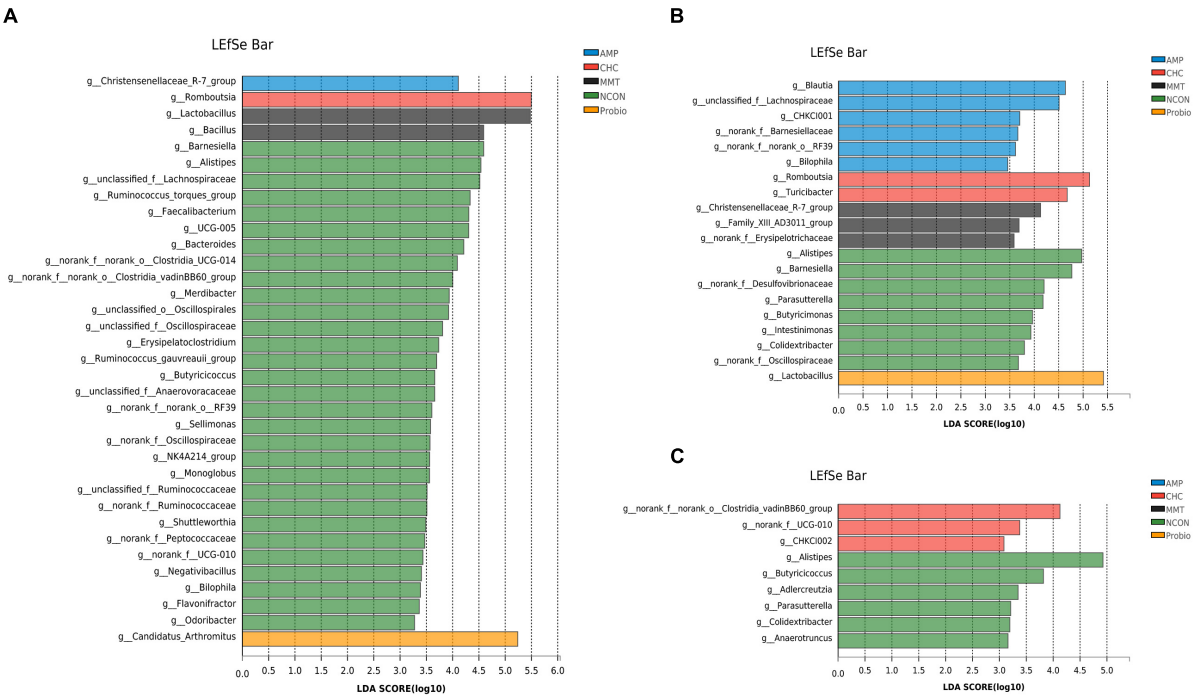

FIGURE 6 | LEfSe analysis shows the most differentially abundant taxa among treatment groups. **(A)** Difference in the abundance of species among five groups in the ileum. **(B)** Difference in abundance of species among five groups in the cecum. **(C)** Difference in abundance of species among five groups in the colon. LDA score > 3.5 is considered to be statistically significant. NCON is a corn-soybean meal basal diets group. AMP is 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group.

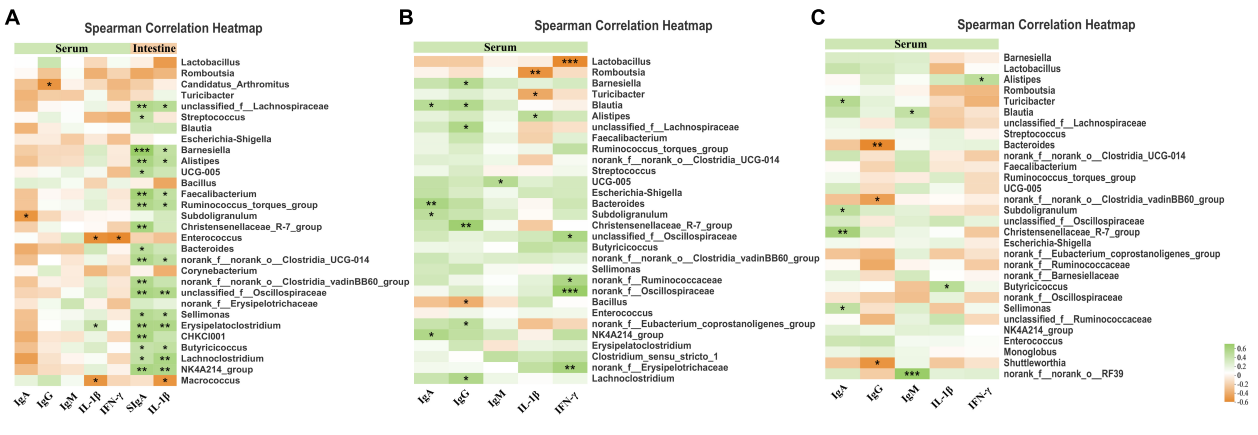

FIGURE 7 | The Spearman correlation among intestinal microbiota, anti-inflammatory, and immune parameters. **(A)** Heatmap of Spearman' s correlation between ileal microbiota, anti-inflammatory, and immune parameters. **(B)** Heatmap of Spearman' s correlation between cecal microbiota, anti-inflammatory and immune parameters. **(C)** Heatmap of Spearman's correlation between Colonic microbiota, anti-inflammatory, and immune parameters. The correlation was analyzed based on the relative abundance of 30 phylotypes at the genus level. Cells are colored based on the correlation coefficient between the significantly altered bacteria and anti-inflammatory or immune parameters. The green suggests a positive correlation, and the yellow suggests a negative correlation. The "*" indicates $0.01 < p \leq 0.05$, "**" indicates $0.001 < p \leq 0.01$, and "***" indicates $p \leq 0.001$.

globulins (IgA and IgM), while the abundance of *Shuttleworthia*, *norank_f__Clostridiales_vadinBB60_group*, and *Bacteroides* were negatively associated with the levels of IgG. Lower levels of IL-1β and IFN-γ displayed a negative association with the abundance of *Butyricicoccus* and *Alistipes*.

To better understand the microbiota dynamic changes in broilers, potential functions of microbiota in the ileum, cecum, and colon were predicted by the PICRUSt algorithm (**Supplementary Table 1**). In the ileum, the functions related to energy production and conversion; transport and metabolism of

amino acid, lipid and inorganic ion transport and metabolism; transcription, replication, recombination, and repair; secondary metabolites biosynthesis, transport, and catabolism; and signal transduction mechanisms in CHC group showed an increase ($p < 0.05$) compared with MMT group. There was no difference in the functions in the cecum and colon among treatment groups ($p > 0.05$).

DISCUSSION

Feed contamination with microbic or toxic agents can deteriorate the structure of the gastrointestinal tract and cause liver injury, which decreases the growth performance of animals. A practical approach is to use mycotoxin absorbents to minimize the negative effect of mycotoxins in feed. Another approach is the use of antibiotics to reduce susceptibility to infectious disease and improve growth performance, which has led to the widespread use of antibiotics in animal feed as growth promoters and medicated feed additives (Chattopadhyay, 2014). However, continuous usage of antibiotics may contribute to the emergence of a reservoir of drug-resistant bacteria, which may then transfer their resistance to pathogenic bacteria in both animals and humans (Bartlett et al., 2013). As a result, much research has focused on the development of antibiotic alternatives to maintain or improve animal performance and health.

Active charcoal is one of these absorbents, which is produced from charcoal in the presence of activation regents. Active charcoal is highly porous and has a high specific surface area that can effectively absorb toxins to physically trap molecules (Brooks et al., 2017; Mohammad et al., 2020). Montmorillonite (MMT) is another example of clay absorbents that are beneficial to broiler growth performance, feed efficiency, and gut morphology (Ma and Guo, 2008) due to its absorption capacity of feed mycotoxins (Abbès et al., 2012; Yang et al., 2014; Saleemi et al., 2020). Loading MMT with copper (Ma and Guo, 2008), calcium (Lillehoj et al., 2016), zinc oxide (Hu et al., 2013), or antibiotics (Qu et al., 2014) imparts additional functions to MMT from these ligands such as increasing the activities of endogenous digestive enzymes, improving intestinal morphology and acting as an antibacterial agent. Modification of active charcoal with antibacterial agents can enhance the activity of the antibacterial by up to 72% compared with the application of pure antibiotics (Burchacka et al., 2021).

Chinese herbal medicine resources are rich in China. Chinese herbal medicine additives contain a variety of trace elements and bioactive compounds, with antibacterial, anticancer, and anti-inflammatory properties (Guo, 2021). Chinese herbals have been widely used in human medicine, animal feed, food, and cosmetic industries (Lyu et al., 2017; Luo et al., 2019; Shi, 2019; Shen, 2021). Resources of some Chinese herbs like *P. chinensis*, *P. oleracea L.*, *A. argyi Folium*, and *P. multifida Poir* are rich because of easy growing independent of areas, temperature, and humidity. Activated charcoal was a highly porous form of carbon that could trap chemicals efficiently (Brooks et al., 2017; Mohammad et al., 2020). CHC which couples active charcoal with Chinese herbs (*P. chinensis*, *P. oleracea L.*, *A. argyi Folium*, and *P. multifida Poir*) may have dual functions in both absorptions like clays and antibacterial activity. Compared with MMT, CHC had 30-fold more surface area, seven-fold greater absorptive capacity, and five-fold greater pore distribution. A previous *in vitro* study revealed that CHC was able to absorb mycotoxins including deoxynivalenol, zearalenone, and fumonisin B1 (Wang et al., 2019).

In the current study in broilers, CHC supplementation (optimal dose of 500 mg/kg CHC), improved growth performance and tended to improve feed conversion rate. To reveal CHC functional mechanisms, three positive controls were selected: AMP prebiotics, Probio (*Bacillus subtilis*) representing probiotics, and MMT representing absorbents. Results showed that the treatments of three positive controls had no significant difference from NCON on growth performance. One of the reasons was the treatment doses of these positive controls were not optimized. Environmental challenges like pathogens and temperatures can also contribute to the various effects of prebiotics and probiotics on broiler growth performance (Santoso et al., 1999; Xia et al., 2004; Biggs et al., 2007; Sohail et al., 2012; Abudabos et al., 2020).

Charcoal-herb extractum complex improved both cellular immune and humoral immune responses as indicated by serum IL-1β, IFN-γ, and IgA concentrations. IL-1β and IFN-γ are important inflammatory cytokines that play crucial roles in the pathogenesis of a range of inflammatory and autoimmune diseases (Rožman and Švajger, 2018; Cheng et al., 2019). The observed stimulation of immune responses was attributed to the ligands of Chinese herbs. Chinese herbs contain numerous active compounds related to anti-inflammatory and antibacterial functions (Guan et al., 2006; Liu et al., 2013; Iranshahy et al., 2017; Cao et al., 2020). Another important role of CHC was to modulate bacterial composition in the intestinal tract. First, CHC, like other positive control treatments (AMP, Probio, and Absorb), lowered the diversity of species abundance in the ileum. Decreasing the richness of bacteria caused by CHC was similar to responses attributable to MMT (MMT). In the cecum and colon, CHC and MMT showed no significant difference, which indicated that the absorptive functions of CHC and MMT acted in the foregut rather than the hindgut. Second, CHC inhibited the abundance of *Alistipes* which is harmful bacteria related to causing intestinal inflammation in the ileum (Schirmer et al., 2019; Valido et al., 2022). This result suggested that although CHC had advantages in surface area and pore size, the function of absorption may also rely on other properties such as hydrophobic character, distances between porous sheets, and electrical conductivity (Nadziakiewicza et al., 2019).

Charcoal-herb extractum complex had no beneficial effect on the abundance of *Lactobacillus*, which was the lactic acid-producing bacteria. These characteristics of CHC on the abundance of specific pathogenic bacteria were likely represented by the composition of Chinese herbs. In other words, changing the composition of extractum of Chinese herbs may change the inhibition or production of specific bacteria. Compared with CHC, MMT favored the abundance of *Lactobacillus* in the ileum. MMT was beneficial to the survival of strains in the gastrointestinal tract (Nadziakiewicza et al., 2019),

and combination administration of *Lactobacillus* with MMT protected rats from mytoxin-induced immune disorders (Ben Salah-Abbès et al., 2016). Compared with CHC, AMP, and MMT, interestingly, the addition of Probio (*Subtilis bacillus*) did not cause significant alteration of *Lactobacillus* abundance in the gastrointestinal tract. However, the addition of Probio decreased the abundance of harmful bacteria *Alistipes*, and enhanced the abundance of beneficial bacteria *Candidatus_Arthromitus*. This indicated that as with other probiotics, *S. bacillus* expressed antibacterial properties by producing bacteriostatic substances such as organic acids, bacteriocins, and antibiotics (Rolfe, 2000).

Charcoal-herb extractum complex increased the abundance of Firmicutes and decreased Bacteroidetes in the ileum and cecum. Firmicutes were dominant in the ileum, cecum, and cecum of broilers (Rychlik, 2020). Firmicutes play a crucial role in host health, including regulating host immunity and maintaining intestinal barrier integrity (Louis and Flint, 2017). Meanwhile, Bacteroidetes have the ability to release LPS, which then leads to heightened inflammatory responses (Ortega-Hernández et al., 2020). Thus, a decreased proportion of Bacteroidetes is related to lower inflammatory factors. A higher Firmicutes/Bacteroidetes ratio is associated with enhanced lower inflammation and reduced infection risk in humans (Mariat et al., 2009) and pigs (Molist et al., 2012; Hu et al., 2020). Therefore, we speculate that CHC supplementation exhibits immune and anti-inflammatory effects closely associated with the increased abundance of Firmicutes and decreased abundance of Bacteroidetes in ileum and cecum of broilers.

We also conducted a correlation analysis on immune responses and intestinal bacteria. In the cecum, the abundance of *Romboutsia* was associated negatively with serum levels of IL-1β. *Lactobacillus* abundance was associated negatively with IFN-γ in the cecum. IL-1β is secreted by the sentinel cells (macrophage and monocytes) of the innate immune system (Granowitz et al., 1992) or other cell types like epithelial, endothelial, and fibroblasts (Hoffmann et al., 2005; Choi et al., 2008). IFN-γ is produced by NK cells, T and B cells (Harris et al., 2000). These negative associations suggest that *Romboutsia*, and *Lactobacillus* promote positive immune responses. *Lactobacillus* strains were proved to alleviate inflammation by inhibition of IL-1β expression (Yun et al., 2020) *in vivo*, and activated macrophages are multi-faceted *in vitro* (Rocha-Ramírez et al., 2017). *Romboutsia* was important bacteria that was associated with the health status of the gastrointestinal tract (Ricaboni et al., 2016). The drastic reduction of *Romboutsia* in intestinal mucosa may represent a potential microbial indicator of intestinal dysbiosis (Mangifesta et al., 2018).

We also found a strong positive association between a series of bacteria and ileum mucosal SIgA and ileum mucosal IL-1β. SIgA is involved in the mucosal immune response that can protect against various pathogens by neutralization, inhibition of adherence, and agglutination. SIgA does not activate the complement pathway, thus, it is more anti- than proinflammatory (Russell et al., 2015). *Alistipes* were highly relevant in diseases, such as liver fibrosis (Rau et al., 2018), colorectal cancer (Moschen et al., 2016), cardiovascular disease (Zuo et al., 2019), and mood disorders (Bangsgaard Bendtsen et al., 2012). *Faecalibacterium* in the intestine was significantly enriched in patients with tuberculosis (Maji et al., 2018) or Crohn's disease (Hansen et al., 2012). These results indicated that the bacteria like *Alistipes* and *Faecalibacterium* could locally stimulate the secretion of inflammatory factors by intestinal cells, and increase mucosal immune response.

CONCLUSION

In summary, CHC supplementation (optimal dose of 500 mg/kg) increased growth performance and tended to increase feed conversion rate in broilers. It had a beneficial effect on intestinal microbial composition and enhanced the immune status of broilers. CHC supplementation increased the abundance of Firmicutes in the ileum and cecum, while decreasing the abundance of Bacteroidetes in ileum and cecum. CHC markedly reduced the abundances of pathogenic bacteria *Alistipes*, while enriching the abundances of beneficial bacteria (*Romboutsia* and *Lactobacillus*) in the ileum. The bacteria (*Faecalibacterium* and *Alistipes*) were strongly negatively associated with the levels of mucosa SIgA and IL-1β in the ileum. CHC decreased the level of the inflammatory cytokines IL-1β and IFN-γ in serum, and improved immune status (represented by the levels of IgA and IgM in serum, and SIgA in the mucosa of duodenum and jejunum). CHC demonstrated dual functions of absorption like clay and antibacterial like antibacterial peptides.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee of China Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LW and BD designed and wrote the manuscript. YZ and XG critically edited the text. LW, LG, and BD finalized the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.856634/full#supplementary-material

Supplementary Figure 1 | Dilution curve and rank abundance curve. **(A)** Rarefaction curves for total amplicon sequence variants (ASV), showing the sequencing depth with increasing sequencing depth. **(B)** Rank Abundance curve, showing the richness and evenness of the observed species (97% identity) based on the 16S rDNA gene.

Supplementary Figure 2 | Effects of CHC on α-diversity of cecal and colonic microbiota in broilers on day 42. Bar graphs show changes in indexes of **(A)** Sobs (upper left panel), Chao 1 (upper right panel), ACE (bottom left panel), and Shannon (bottom right panel) in cecum, **(B)** Sobs (upper left panel), Chao 1 (upper right panel), ACE (bottom left panel), and Shannon (bottom right panel) in colon. Four indexes analyzed the α diversity of broilers from different directions. NCON is a corn-soybean meal basal diets group. AMP is 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group.

Supplementary Figure 3 | Effects of dietary treatment on intestinal microbiota composition at the genus level in the cecum and colon. **(A)** Cecal bacterial community heatmap in broilers. **(B)** Colonic bacterial community heatmap in broilers. NCON is a corn-soybean meal basal diets group. AMP is 200 mg/kg antibacterial peptide supplemented group. Probio is 200 mg/kg calsporin supplemented group. MMT is 500 mg/kg montmorillonite supplemented group. CHC is 500 mg/kg activated charcoal-herb extractum complex supplemented group.

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Synergy of Dietary Quercetin and Vitamin E Improves Cecal Microbiota and Its Metabolite Profile in Aged Breeder Hens

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In the present study, the synergistic effects of quercetin (Q) and vitamin E (E) on cecal microbiota composition and function, as well as the microbial metabolic profile in aged breeder hens were investigated. A total of 400 (65 weeks old) Tianfu breeder hens were randomly allotted to four experimental groups (four replicates per group). The birds were fed diets containing quercetin at 0.4 g/kg, vitamin E (0.2 g/kg), quercetin and vitamin E (QE; 0.4 g/kg and 0.2 g/kg), and a basal diet for a period of 10 wks. After the 10 week experimental period, the cecal contents of 8 aged breeder hens per group were sampled aseptically and subjected to high-throughput 16S rRNA gene sequencing and untargeted metabolomic analysis. The results showed that the relative abundances of phyla *Bacteroidota*, *Firmicutes,* and *Actinobacteriota* were the most prominent among all the dietary groups. Compared to the control group, the relative abundance of the families *Bifidobacteriaceae*, *Lachnospiraceae, Tannerellaceae*, *Mathonobacteriaceae, Barnesiellaceae,* and *Prevotellaceae* were enriched in the QE group; and *Bacteroidaceae, Desulfovibrionaceae, Peptotostretococcaceae,* and *Fusobacteriaceae* were enriched in the Q group, whereas those of *Lactobacillaceae, Veillonellaceae, Ruminococcaceae, Akkermansiaceae,* and *Rikenellaceae* were enriched in the E group compared to the control group. Untargeted metabolomics analyses revealed that Q, E, and QE modified the abundance of several metabolites in prominent pathways including ubiquinone and other terpenoid–quinone biosynthesis, regulation of actin cytoskeleton, insulin secretion, pancreatic secretion, nicotine addiction, and metabolism of xenobiotics by cytochrome P450. Furthermore, key cecal microbiota, significantly correlated with important metabolites, *for example,* (*S*)-equol positively correlated with *Alistipes* and *Chlamydia* in E_vs_C, and negatively correlated with *Olsenella*, *Paraprevotella,* and *Mucispirillum* but, a contrary trend was observed with *Parabacteroides* in QE_vs_C. This study establishes that the synergy of quercetin and vitamin E alters the cecal microbial composition and metabolite profile in aged breeder hens, which lays a foundation for chicken improvement programs.

Keywords: aged breeder hens, gut microbiota, metabolite, dietary quercetin, vitamin E

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INTRODUCTION

The gut microbiota plays important biological roles in metabolism, nutrition, physiology, and immunoregulation in both animals and humans (O'Hara and Shanahan, 2006; Konturek et al., 2015). There is a close correlation between the age of the host and its microbiota, thus, during aging, the diversity and richness of the gut microbiota increase (Claesson et al., 2012; Scholtens et al., 2012; Lim et al., 2015), whereas, in old age the composition becomes less diverse and more dynamic (Bischoff, 2016). Furthermore, the intestinal structure and immune barrier integrity are closely correlated with the gut microbiota; hence, it is important to promote gut microbiota composition to improve gut health (Grivennikov et al., 2012). In addition, the physiological performance of broiler breeder hens mostly declines at approximately 65–70 weeks of age (Liu et al., 2018).

Diet is an influential factor that affects the composition, diversity, and function of the gut microbiota in both animals and humans (David et al., 2014; Sonnenburg and Bäckhed, 2016; Zmora et al., 2019). Therefore, age-related dietary changes can affect the composition and function of gut microbiota (Clements and Carding, 2018). Moreover, the use of antibiotics in both animals and humans generates problems, such as antibiotic resistance (Laxminarayan et al., 2013), and reduced diversity of the gut microbiota (Sonnenburg and Sonnenburg, 2019). Hence, developing dietary interventions or possible therapeutic approaches (alternatives to antibiotics) to counteract age-related dysbiosis in aged animals could improve performance (Ghosh et al., 2020). Furthermore, microbial fermentation of dietary supplements produces vital metabolites, such as short-chain fatty acids (SCFAs) of biological significance (Zhu et al., 2019; Chen et al., 2020; Uyanga et al., 2021). Therefore, a negative change in gut microbiota composition and metabolite profile can cause several metabolic and physiological disorders in animals (Sekirov et al., 2010).

The physiological performance of broiler breeder hens mostly declines at approximately 65–70 weeks of age due to age-related degeneration of the small intestine morphological structures, such as villi height, crypt depth, bacterial composition, and organ dysfunction (Liu et al., 2018; Yang et al., 2020).

Vitamin E, a fat-soluble nutrient, has potent antioxidant function with several other biological effects, such as stress alleviation, protection of cells against apoptosis, anti-inflammatory function, and immunoregulation (Jiang, 2014; Wang et al., 2021; Amevor et al., 2022b). Vitamin E intake has been reported to enhance gut microbiota composition and improve gut health in chickens (Choi et al., 2020; Wang et al., 2021); however, there are insufficient data to demonstrate the correlation between α-tocopherol (vitamin E) and gut microbiota.

Quercetin, a known flavonoid and prebiotic, exhibits anti-microbial, anti-inflammatory, anti-aging, anti-oxidative, and immunoregulatory effects. Previous studies have indicated that quercetin and other flavonoids alter the gut environment by regulating the gut microbiota composition and its metabolites, as well as intestinal barrier function (Etxeberria et al., 2015; Singh et al., 2019; Dong et al., 2020; Shu et al., 2020).

However, no studies have investigated the effects of the dietary combination of quercetin and vitamin E on the cecal microbiota and its metabolite profile in breeder chickens. Therefore, in the present study, the cecal microbial composition and its metabolites in aged breeder hens fed a combination of quercetin and vitamin E were investigated using 16S rRNA gene sequencing and untargeted metabolomics analysis. Therefore, comparing the differences in the cecal microbiota composition and its potential biochemical metabolites might help us understand how the dietary combination of quercetin and vitamin E alter the gut metabolism, health, and performance of aged breeder hens.

Abbreviations: DAMs, Differentially accumulated metabolites; E, Vitamin E; HPLC, High-Performance Liquid Chromatography; KEGG, Kyoto Encyclopedia Genes and Genomes; OTUs, Operational taxonomic units; Q, Quercetin; PLS-DA, Partial least squares discriminant analysis.

MATERIALS AND METHODS

Experimental Animals, Diets, and Treatments

A total of 400 (65 weeks old) Tianfu broiler breeder hens were randomly allotted to four dietary groups (n = 100 hens each), with 4 replicates per group (n = 25 hens each). Four experimental diets were prepared, and each of the groups was fed one of the following four diets: Basal diet (Control) only designated C; Q diet, the basal diet supplemented with dietary quercetin (0.4 g/kg); E diet, the basal diet supplemented with dietary vitamin E (0.2 g/kg); and QE diet, the basal diet supplemented with the combination of dietary quercetin (0.4 g/kg) and vitamin E (0.2 g/kg). The composition and nutritional values of the basal diet for the experiment have been described in our previous study (Amevor et al., 2021a).

Quercetin and vitamin E were supplied by Shaanxi Huike Plant Development Co., Ltd., China (Xian, Shaanxi, China). The purity of the quercetin was determined by high-performance liquid chromatography (purity 95%). The experimental hens were kept in an individual wire cages with the following dimensions: width: 48.8 cm, depth: 38.1 cm, and height: 38.1 cm, under controlled lighting of 16 l:8 D, temperature of 22 ± 1°C and optimal ventilation during the experimental period. The chickens had access to free flow water *ad libitum*. The chickens were managed for a period of 10 weeks. During the experimental period, data on the feed intake and body weight were recorded.

Sample Collection

At the end of the experimental period (10 weeks), two hens per replicate were randomly selected and euthanized for sample collection. From each of the selected hens, cecal contents were collected aseptically, and to normalize any dissimilarity of the gut microbiota, three samples per each replicate (0.5 g per sample) were mixed and collected (32 cecal samples) into sterilized tubes, and immediately frozen in liquid nitrogen and later stored at −80°C for subsequent microbial DNA extraction.

DNA Extraction, V4 Region of 16S rDNA Amplicon Pyrosequencing, and Bioinformatics Analysis

Extraction of Microbial DNA

Total genomic DNA was extracted from the cecal samples using CTAB method, and the DNA concentration and purity were monitored on 1% agarose gels. The DNA was then diluted to 1 ng/μL using sterile water.

16S rRNA Amplicon Pyrosequencing

16S rRNA genes of the distinct region (V4/16S) were amplified using specific primers (515F: GTGCCAGCMGCCGCGGTAA - 806R: GGACTACHVGGGTWTCTAAT). All PCR were carried out with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 2 μM of forward and reverse primers, and 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, and finally at 72°C for 5 min. The same volumes of the 1x loading buffer (Catalog No. 10482055; containing SYB green) were mixed with the PCR products, and then electrophoresis was conducted on 2% agarose gel. PCR products were mixed at an equidensity ratio. Thereafter, the mixture of the PCR products was purified using a Qiagen Gel Extraction Kit (Catalog. No. 28704; Qiagen, Germany) according to the manufacturer's instructions. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kits (Catalog No. FC-121-3,001; Illumina, United States) following the manufacturer's instructions. Furthermore, the quality of the library was assessed using a Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2,100 system. Thereafter, the library was sequenced on an Illumina NovaSeq platform at NovoGene Co., Ltd. (Beijing, China) and 250 bp paired-end reads were generated.

Sequence Processing and Bioinformatics Analysis

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. The paired-end reads were merged using FLASH (V1.2.7; Magoč and Salzberg, 2011) to obtain raw tags. Quality filtering of the raw tags was performed under specific favorable filtering conditions to obtain high-quality clean tags (Bokulich et al., 2013) according to the Quantitative Insights Into Microbial Ecology (QIIME, Version 1.9.1) quality controlled process (Caporaso et al., 2010). The tags were compared with the reference database (Silva database, using the UCHIME Algorithm (Edgar et al., 2011) to detect the chimera sequences, after, the chimera sequences were removed (Haas et al., 2011) to obtain the Effective Tags.

For operational taxonomic units (OTUs) production, sequence analysis was performed using Uparse software (Uparse v7.0.1001; Edgar, 2013). The sequences with ≥97% similarity, were assigned to the same OTUs. The representative sequences for each OTU were screened for further annotation.

Moreover, to study the phylogenetic relationship of the different OTUs, and the differences among the dominant species in different cecal samples per group, multiple sequence alignments were conducted using the MUSCLE software (Version 3.8.31; Edgar, 2004). Thereafter, the OTU abundance information was normalized using a standard sequence number corresponding to the cecal samples with the least sequences. Subsequent analyses of alpha and beta diversities were performed based on the normalized output data obtained.

Alpha diversity indices including Observed-species, Chao1, Shannon, Simpson, ACE, and Good-coverage were calculated with QIIME software (Version 1.9.1) and displayed with R software (Version 2.15.3). In addition, Beta diversity on both weighted and unweighted unifrac was calculated using the QIIME software (Version 1.9.1). Moreover, cluster analysis was followed by principal component analysis (PCA) to reduce the dimensions of the original variables using the *FactoMineR* package and ggplot2 package in R software (Version 2.15.3). Principal Coordinate Analysis (PCA) was performed to obtain the principal coordinates, and visualize complex, multidimensional data. Thereafter, a distance matrix of the weighted or unweighted unifrac among the samples was transformed to a new set of orthogonal axes, by which the maximum variation factor was demonstrated by the first principal coordinate, and the second maximum variation factor was demonstrated by the second principal coordinate. PCA analysis was performed using the WGCNA package, stat packages, and ggplot2 package in R software (Version 2.15.3).

Furthermore, unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed using QIIME software (Version 1.9.1). Tax4Fun was functionally predicted by the nearest neighbor method based on the minimum 16S rRNA sequence similarity following the procedure outlined by Huang and Liao (2021). Briefly, the 16S rRNA gene sequence of the prokaryotic whole genome was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2012) using the BLASTN algorithm with a bitscore >1,500 to establish a correlation matrix, and map the KEGG database prokaryotic whole-genome function information annotated by the ultrafast protein classification tool to generate metabolic functional annotations (Meinicke, 2015).

Untargeted Metabolomics Profiling of the Cecum Content

Metabolites Extraction Tissue Sample

The cecal samples were prepared and the supernatant was injected into a liquid-mass spectrometry (LC–MS/MS) system for analysis (Want et al., 2012; Yuan et al., 2012). Furthermore, the ultra-high-performance liquid Chromatography (UHPLC)-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive™ HF mass spectrometer (Thermo Fisher, Germany) at Novogene Co. Ltd. (Beijing, China). The cecal samples were then injected onto a Hypersil Gold column (100 × 2.1 mm, 1.9 μm) using a 17 min linear gradient at a flow rate of 0.2 ml/min. The eluents for the positive polarity mode

were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol), and the solvent gradient was set as follows: 2% B, 1.5 min; 2–100% B, 12.0 min; 100% B, 14.0 min; 100–2% B, 14.1 min; and 2% B, 17 min. Then, the Q ExactiveTM HF mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 40 arb, and aux gasflow rate of 10 arb.

The raw data files generated by UHPLC–MS/MS were processed using Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite.

Statistical Analysis

Statistical analyses were performed using statistical R software (Version 2.15.3). Metabolites were annotated using the KEGG database. Principal component analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) were performed using metaX. Univariate analysis (t-test) was used to calculate statistical significance (p-value). Metabolites with VIP > 1 and $p < 0.05$ and fold change (FC) ≥ 2 or FC ≤ 0.5 were considered differentially accumulated metabolites (DAMs) in pairwise comparisons among the treatments (Q, E, QE, and C). The functional annotation of the DAMs was conducted in the KEGG database (Kanehisa et al., 2012), and pathways with $p < 0.05$ were declared significantly enriched pathways (Nguyen et al., 2021).

Pearson's correlation coefficients were computed and visualized between the selected differential bacterial genera and differential metabolite expression using the *ggplot2* package in R statistical software with a significance threshold set at $p < 0.05$.

RESULTS

Impacts of Dietary Q, E, and Q + E on Feed Intake and Body Weight of Aged Hens

Figure 1 represents the feed intake and body weight of the aged hens. It was observed that during the 10-week experimental period, Q + E significantly increased the average daily feed intake per hen as compared with the other dietary groups. Moreover, vitamin E also significantly increased feed intake as compared to the control and quercetin dietary groups (**Figure 1A**, $P < 0.05$). Furthermore, Q + E groups significantly increased the body weight as compared with the control and vitamin E groups (**Figure 1B**, $P < 0.05$).

Diversity and Structure of the Cecal Microbiota

The composition and diversity of the cecal microbiota were profiled in the cecum of 8 aged breeder hens per group (C, Q, E, and QE). From this, an average read ± standard deviation of 102316.29 ± 1613.62, 103707.13 ± 1040.30, 103329.50 ± 1662.03, and 104619.38 ± 1371.48, were obtained from the 4 dietary treatments, respectively (**Supplementary Table S1**). These corresponded to 1,403 distinct operational taxonomic units (OTUs) at the 97% identity level were obtained from all samples; however, after rare OTUs <0.005% of the total OTUs were removed, an average of 924 OTUs were retained for further downstream analyses (**Supplementary Table S2**).

The curves for the observed OTUs and species rank (**Figures 2A,B**) approached a plateau, suggesting that the sequencing depth was adequate for the coverage of all OTUs present in the cecal samples. Alpha diversity indices, such as Shannon, Simpson, Chao1, and abundance-based coverage estimator (ACE; **Figures 2C–F**) did not differ statistically among the 4 dietary groups. These results corroborate the results of principal component analysis (PCA) that, 32 cecal samples showed no difference in the community phylotype structure (**Supplementary Figure S1**), indicating that the aged breeder chickens had similar microbial communities in the cecum among the treatments. On the other hand, the first two principal components could explain 43.99% variation in the bacterial diversity in the cecum.

In contrast, the weighted unifrac distance between OTUs clustered the 4 dietary treatments into 2 major clusters: Cluster I with only the QE group, while Cluster II consisted of C, Q, and E groups (**Supplementary Figure S2**). At the phylum level, the top 11 groups of the cecal microbiota were largely dominated by *Bacteroidota*, *Firmicutes,* and *Actinobacteriota* (**Supplementary Figure S2**). At the family level, **Figure 3** shows a contrasting pattern of abundance of the various microbiota identified among the 4 dietary groups, suggesting that the inclusion of either vitamin E, quercetin, or a combination of quercetin and vitamin E (quercetin + vitamin E) alters the family level in the cecum in contrast to the control group. For instance, the relative abundance of the families *Bifidobacteriaceae*, *Lachnospiraceae, Tannerellaceae*, *Mathonobacteriaceae, Barnesiellaceae,* and *Prevotellaceae* was enriched in the cecal microbiota of the QE group; that of *Bacteroidaceae, Desulfovibrionaceae, Peptotostretococcaceae,* and *Fusobacteriaceae* was enriched in the cecal microbiota of the Q group, whereas, the relative abundance of the families *Lactobacillaceae, Veillonellaceae, Ruminococcaceae, Akkermansiaceae,* and *Rikenellaceae* was enriched in the cecal microbiota of the E group, compared with the control group (**Figure 3**).

Similarly, the top 35 KEGG pathway enrichment analyses of the families (**Figure 4**) showed trends similar to those of the family levels presented in **Figure 3**. Among the 35 topmost pathways, carbohydrate metabolism increased in abundance in the C group compared to the Q, E, and QE groups (**Figure 4**; **Supplementary Table S3**). In addition, the control group had the highest family enrichment in the metabolism of terpenoids and polyketides, metabolism of other amino acids, nervous system, cellular community prokaryotes, membrane transport, replication and repair, transcription, endocrine and metabolic diseases, translation, nucleotide metabolism, aging, folding, sorting and degradation (**Figure 4**). These results suggest that the aged breeder hens in the control group spent more energy on maintenance other than reproduction and health relative to the birds fed with dietary Q, E, and QE.

In contrast, QE enhanced the abundance of genes involved in metabolism, signal transduction, the immune system, genetic information processing, cell motility, biosynthesis of other secondary metabolites, energy metabolism, infectious diseases,

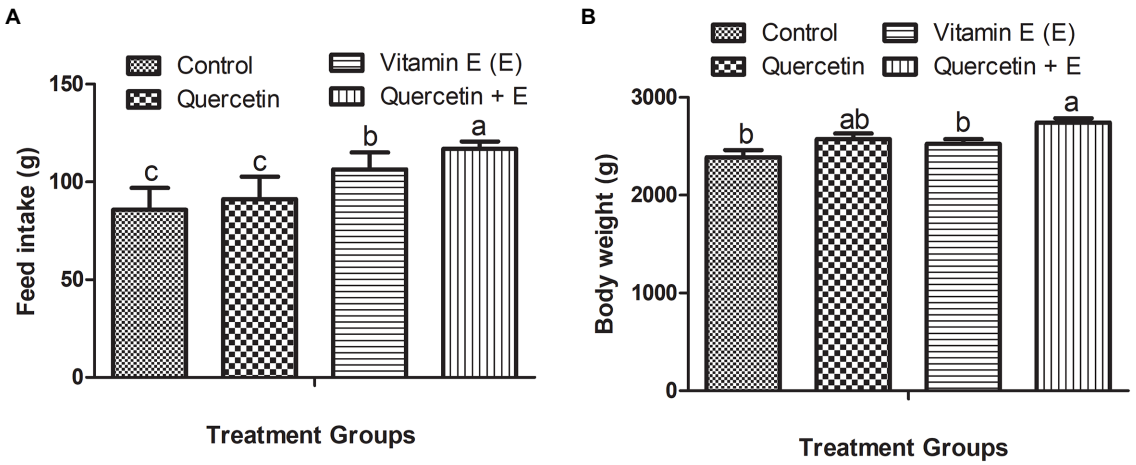

FIGURE 1 | Impacts of dietary quercetin, vitamin E, and the combination of quercetin and vitamin E on the feed intake and body weight of aged broiler breeder hens. Bars without the same letter differed significantly ($p < 0.05$). **(A)** Feed intake; **(B)** body weight.

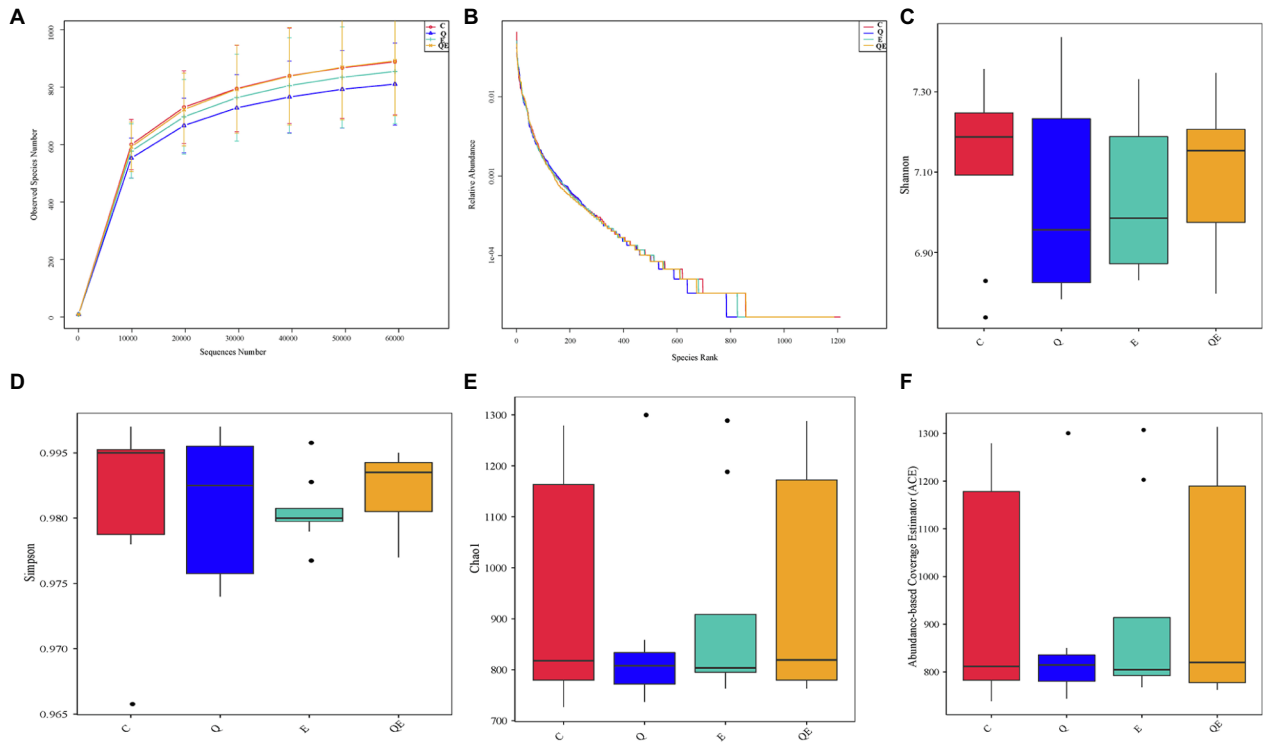

FIGURE 2 | Effects of dietary treatments [(control group (C), quercetin group (Q), vitamin E group (E) and combination of quercetin and vitamin E group (QE)] on cecal microbiota diversity and composition in aged breeder chickens. **(A)** Rarefaction curves, computed at the subsample size of 6,000 per sample, exhibit the effects of sequencing efforts on the observed number of OTUs at 97% sequence similarity. The error bars represent standard deviation of the eight replicates. **(B)** Abundance curve of species identified. **(C)** Shannon diversity index boxplot. **(D)** Simpson diversity index plot. **(E)** Chao1 diversity index boxplot. **(F)** Abundance-based coverage estimator (ACE) diversity index boxplot.

cell growth and death, amino acid metabolism, enzyme families, metabolism of cofactors and vitamins, lipid metabolism, transport and catabolism, glycan biosynthesis and metabolism, signaling molecules and interaction, cellular processes and signaling, and drug resistance (**Figure 4**; **Supplementary Table S3**).

Effects of Dietary Quercetin, Vitamin E, and Combination of Quercetin and Vitamin E on Cecal Metabolites

To profile and select differential metabolites for biomarker development in our attempt to make aged breeder chickens

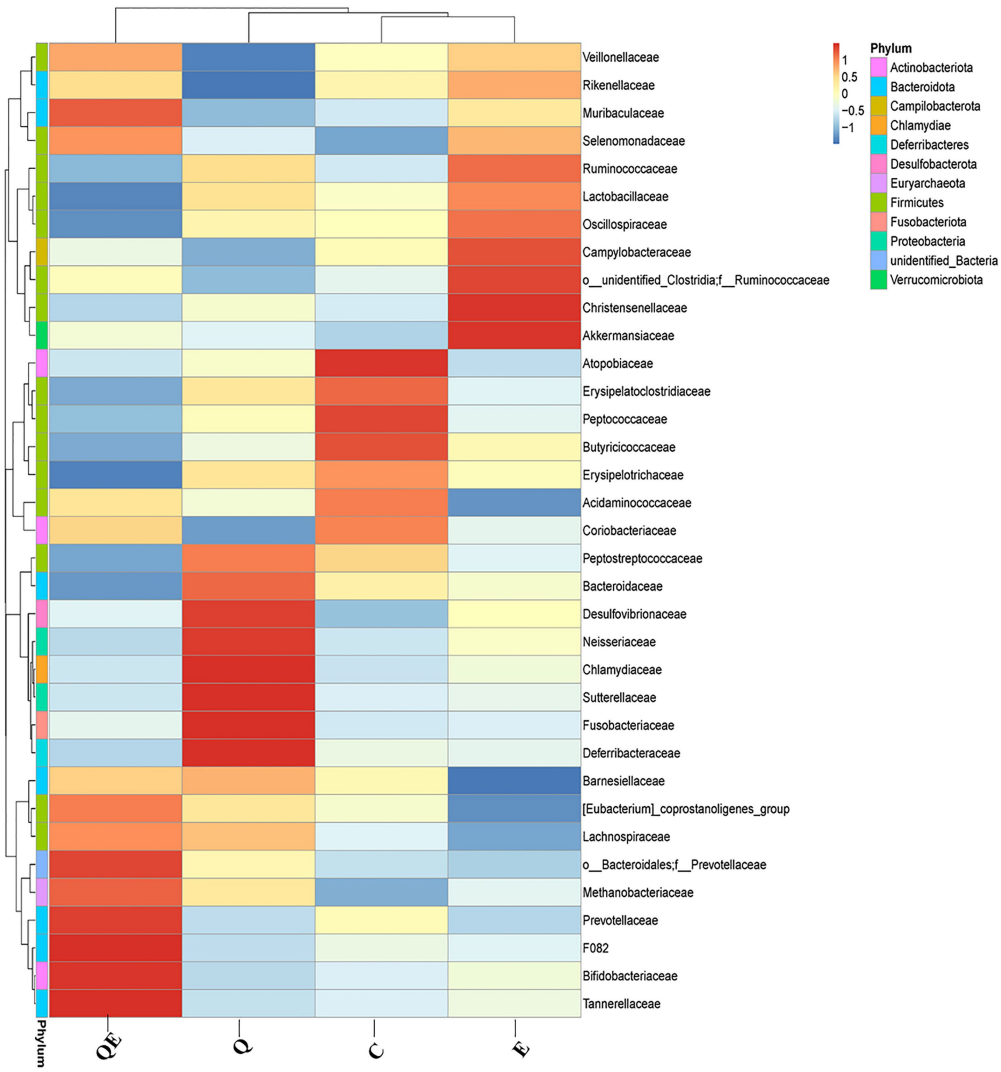

FIGURE 3 | Cecal microbiota composition and diversity at the phylum and family levels among the aged breeder chickens fed with quercetin (Q), vitamin E (E), and QE compared with the control group (C) by phylogenetic relationship and weighted unifrac distance between OTUs. The legend for color intensity of family and phylum are given on the right-hand side of the heatmap clustering figure.

productive, healthy, and prolific, we further conducted non-targeted metabolome profiling of the cecum contents in the 32 chickens used in this study *via* UHPLC combined with LC–MS/MS technology. We detected 1,896 metabolites comprising 1,210 and 686 metabolites at the positive and negative nodes, respectively (**Supplementary Tables S4A,B**). Owing to the large volume of data, we performed further analysis on the 1,210 metabolites detected in the positive node.

To screen for differentially accumulated metabolites (DAMs) among the groups, we applied partial least squares-discriminant analysis (PLS-DA; **Supplementary Figure S3**) together with the variable importance of the projection VIP > 1 and $p < 0.05$ and fold change (FC) ≥ 2 or FC ≤ 0.5. A total of 58, 56, and 55 DAMs were detected in E_vs_C, Q_vs_C, and QE_vs_C, respectively (**Figures 5A,B**; **Supplementary Table S5**). The supplementation of vitamin E or quercetin + vitamin E largely induced the upregulation of more metabolites relative to the control group (**Figure 5A**; **Supplementary Table S5**). For example, DL-Carnitine accumulated 6.23, 3.48, and 2.64 times higher in the cecum of aged breeder chickens in the E, QE, and Q groups, respectively than that in the control group (**Supplementary Table S5**). This compound is well known to improve broiler growth, productivity, carcass characteristics, and immunity by facilitating fatty acid β-oxidation, and decreasing esterification reactions and triacylglycerol storage in adipose tissue (Ghoreyshi et al., 2019).

Another typical compound, cuminaldehyde, was reported to exhibit beneficial effects against various diseases and improve egg production, egg quality, hatchability traits, and blood serum mineral content in laying quails (Şimsek et al., 2015; Goel et al., 2020), and this compound increased in accumulation in the E, Q, and QE groups (thus, 1.90, 1.60, and 1.83 times

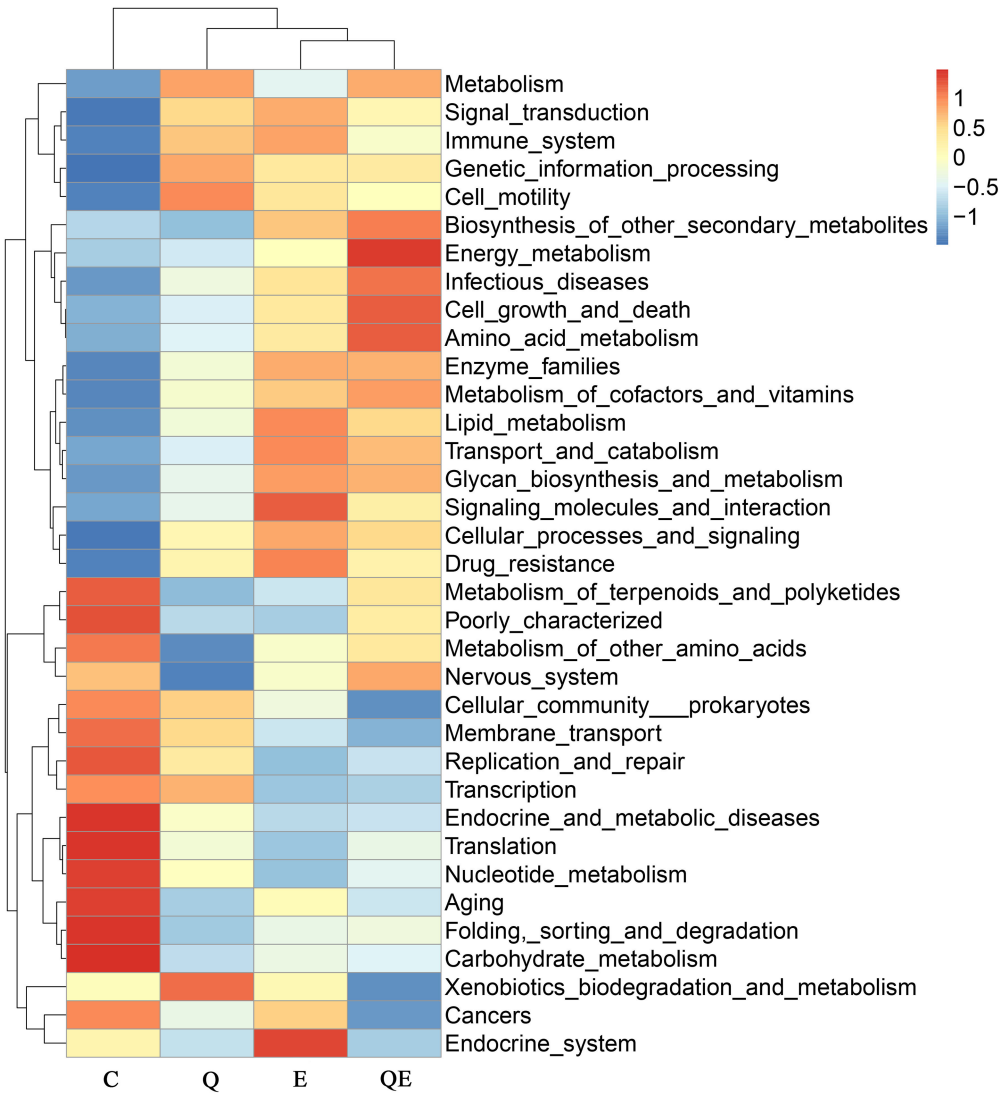

FIGURE 4 | Heatmap clustering of topmost 35 Kyoto Encyclopedia Genes and Genomes of the genus microbiota identified in the cecum of the aged breeder chickens fed with dietary quercetin (Q), vitamin E (E), and QE compared with the control group (C).

higher) than that of the C group (**Figure 6**; **Supplementary Table S5**).

Among the DAMs detected, 13 were detected concurrently among the 3 pairwise groups (**Figure 5b,6**), suggesting that either of the 4 groups had some metabolites in common, although there were several metabolites that were unique to some pairwise groups (**Figure 5B**). This was more evident from the clustering of the 58, 56, and 55 DAMs in E_vs_C, Q_vs_C, and QE_vs_C, respectively (**Figure 6**).

We further subjected the DAMs detected in the pairwise groups to KEGG pathway enrichment analyses resulting in 45 unique enriched pathways (**Supplementary Figure S4**; **Supplementary Table S6**). Surprisingly, no pathway was significantly enriched in E_vs_C ($p > 0.05$), but in the case of Q_vs_C and QE_vs_C, 5 pathways (ubiquinone and other terpenoid–quinone biosynthesis, regulation of actin cytoskeleton, insulin secretion, pancreatic secretion, and nicotine addiction) and 1 pathway (metabolism of xenobiotics by cytochrome P450) were significantly enriched ($p < 0.05$). We focused on these pathways to identify the metabolites and their ion intensities which are presented in the histogram (**Figure 7**). We found that Phylloquinone accumulated higher in the E and QE groups than in the C group (**Figure 7A**). These results suggest a modulating role of this compound in determining the performance of aged breeder chickens.

In addition, E and QE reduced the abundance of acetylcholine (**Figure 7B**); however, the accumulation of two main metabolites including 3-Succinoylpyridine and 1-Naphthol, that are involved in the metabolism of xenobiotics by cytochrome P450, was increased in the E or QE group compared to the C group (**Figure 7C**).

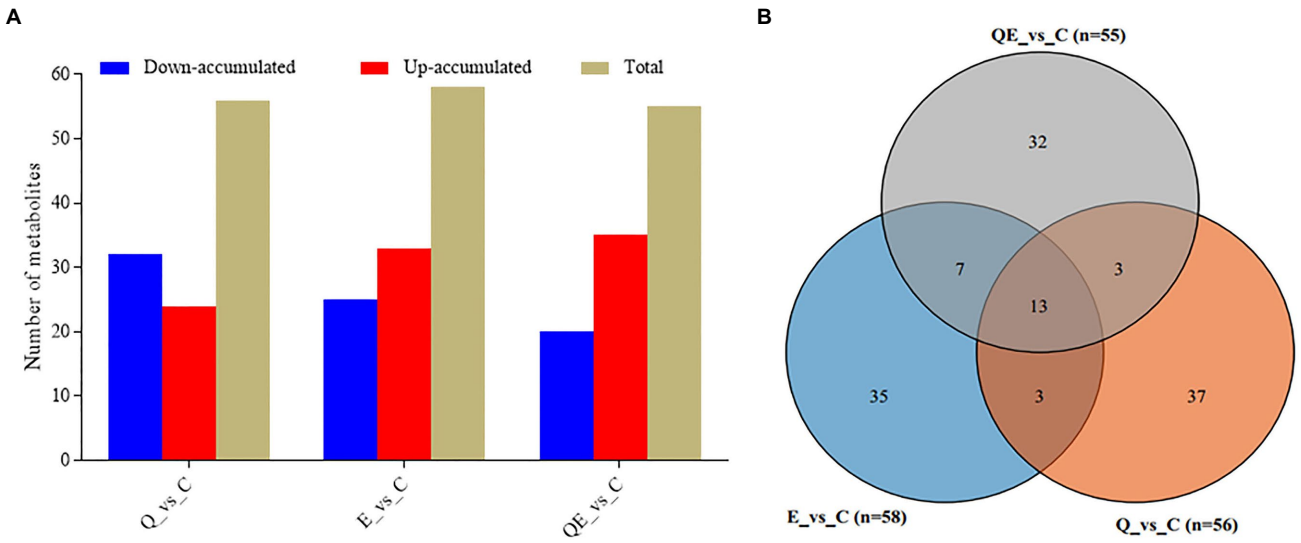

FIGURE 5 | Differentially accumulated metabolites (DAMs) detected in the cecum of the aged breeder chickens fed with dietary quercetin (Q), vitamin E (E), and QE compared with the control group (C). **(A)** Total number of DAMs and their accumulation levels. **(B)** Venn diagram of DAMs detected in the C group against either Q, E, or QE group. The partial least square discriminant analysis of log2 fold change ≥1 and variable importance in projection ≥1 were used to screen for DAMs.

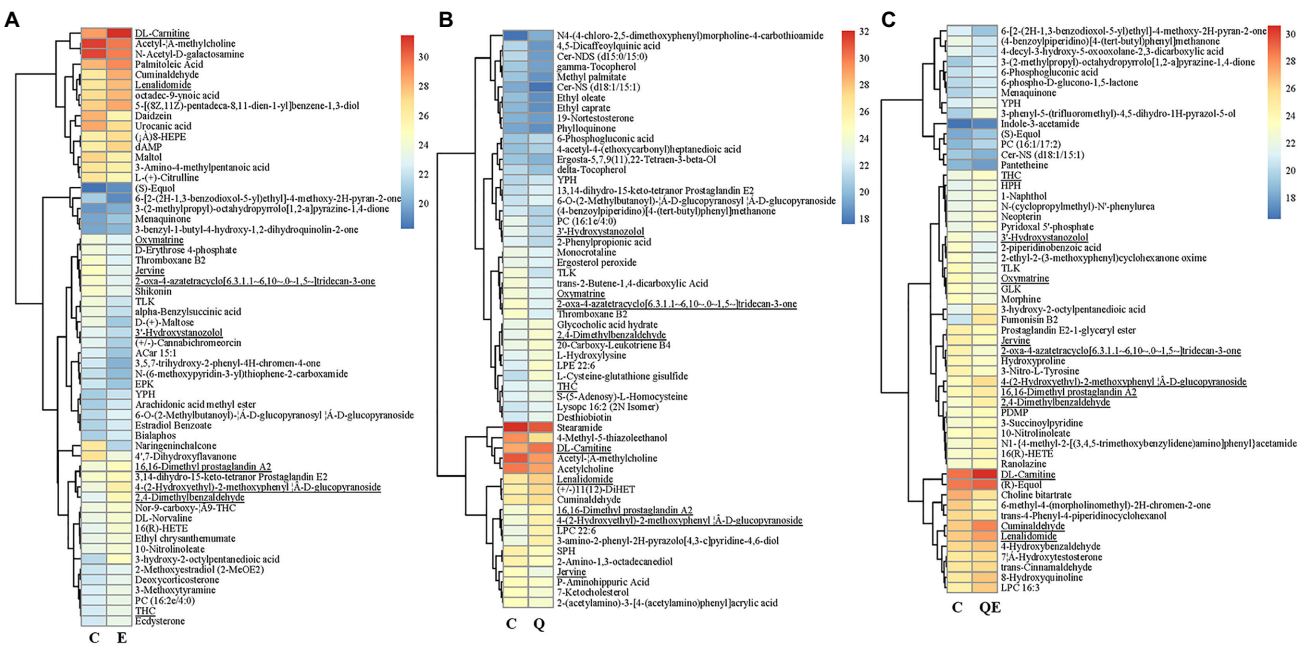

FIGURE 6 | Heatmap clustering of differentially accumulated metabolites (DAMs) and their transformed ion intensities (log2) detected in the cecum of the aged breeder chickens fed with quercetin (Q), vitamin E (E), or QE compared with the control group in pairwise comparison (C). **(A)** E_vs_C. b. Q_vs_C. c. QE_vs_C. The partial least square discriminant analysis of log2 fold change ≥1 and variable importance in projection ≥1 were used to screen for DAMs. The underline compounds in **(A–C)** represent those detected concurrently among the three pairwise groups. Deoxyadenosine monophosphate (dAMP).

The correlation analysis of differential metabolites among the dietary groups was performed. The purpose of the differential metabolite correlation analysis is to check the consistency of each metabolite and metabolite change trends among the dietary groups and to analyze the correlation between each metabolite by calculating Pearson's correlation coefficient between all metabolites. We observed a synergistic or mutually exclusive relationship between different metabolites [**Figure 8** (A) E_vs_C. (B) Q_vs_C. (C) QE_vs_C. (D) Q_vs_E. (E) QE_vs_Q. (F) QE_vs_E]. **Figure 8** showed the top 20 differential metabolites

between the dietary groups. For example, certain type of metabolites have the same trend of change, which was positively correlated, whereas others have negative correlation. Thus, when the linear relationship between the two metabolites is enhanced, it tends to 1 for positive correlation and −1 for negative correlation. Several important differential metabolites, such as ((s)-Equol, DL-carnitine, cuminaldehyde, and Estradiol Benzoate), (DL-carnitine, gamma-Tocopherol, etc.), ((s)-Equol, cuminaldehyde, DL-carnitine, etc.), (gamma-Tocopherol, Arachidonic acid methyl ester, etc.), (gamma-Tocopherol, cuminaldehyde, phylloquinone, 2-phenylpropionic acid, Lysope, Arachidonic acid methyl ester, etc.), and (Lysope, Daidzein, Propanoic acid, D-(+)-Proline, Dihydroxyflavanone, etc.) were highly expressed between the E_vs_C, Q_vs_C, QE_vs_C, Q_vs_E, QE_vs_Q, and QE_vs_E groups, respectively.

Furthermore, we performed Pearson's correlation analysis between selected differential metabolites and differential microbiota to identify the metabolites that could be used to increase or decrease the abundance of some microbiota at the genus level in the cecum of aged breeder hens (**Figures 9A–F**). *Alistipes* which has been reported as one of the dominant species in the cecum of birds (Pandit et al., 2018; Rychlik, 2020) was positively correlated with 10 metabolites in E_vs_C, and 4 metabolites in QE_vs_C (**Figures 9A,C**). One major metabolite detected in E_vs_C and QE_vs_C, (*S*)-equol was positively correlated with *Chlamydia* and *Alistipes* in E_vs_C (**Figure 9A**) and negatively correlated with *Olsenella*, *Paraprevotella,* and *Mucispirillum,* but contrary trends were observed for *Parabacteroides* in QE_vs_C (**Figure 9C**). In addition, it was observed that *Oribacterium* was negatively correlated with Aflatoxin M1, whereas it was positively correlated with 3-Methoxytyramine, 2-Deoxyadenosine, N-acetyl-L-histidine, and Menaquinone in Q_vs_E (**Figure 9D**). Moreover, 3-Hydroxy-2-octylpentanedioic acid positively correlated with six differential bacterial genera *Frateuria*, *Rhodanobacter*, *Candidatu-koribacter*, *Pseudolabrys*, *Phenylobacterium*, and *Methanocorpusculum*.

DISCUSSION

In animals, the gut is home to several microbiota closely associated with metabolism, reproduction, and health. However, during the late stages of life, microbiota composition becomes less diverse and dynamic (Bischoff, 2016). Many studies have reported that diets alter the composition, diversity, and function of gut microbiota, as well as its metabolites in animals (Sonnenburg and Bäckhed, 2016; Zmora et al., 2019). For instance, flavonoids, vitamins, and amino acids have been reported to improve the gut microbiota composition and health of animals (Etxeberria et al., 2015; Choi et al., 2020; Dong et al., 2020; Shu et al., 2020; Uyanga et al., 2021; Wang et al., 2021). In our previous study, we found that dietary supplementation with quercetin and vitamin E improved egg production, feed efficiency, egg quality, immunity, follicle development, liver function, and reproductive organ function in aged laying hens (Amevor et al., 2021a, b), which was consistent with other studies (Liu et al., 2014; Abedi et al., 2017; Dalia et al., 2018; Yang et al., 2021).

Dietary quercetin and vitamin E may promote performance in aged breeder hens by altering the gut microbiota, thereby improving nutrient absorption in the small intestine, stimulating the production and activity of digestive enzymes, and stimulating digestive and physiological metabolism (Dong et al., 2020; Uyanga et al., 2021; Wang et al., 2021; Amevor et al., 2022a). It was observed in this present study that the supplementation of the combination of dietary quercetin and vitamin E improved the palatability of the diet, hence, resulted in improving the feed intake. The results of this study showed that microbial composition revealed no significant differences in the community phylotype structure and alpha diversity of the cecal microbiota among the dietary groups. These findings were consistent with the study by Dong et al. (2020), who reported that dietary quercetin supplementation in broiler chickens fed with oxidized oil showed no significant differences among the alpha diversity indices

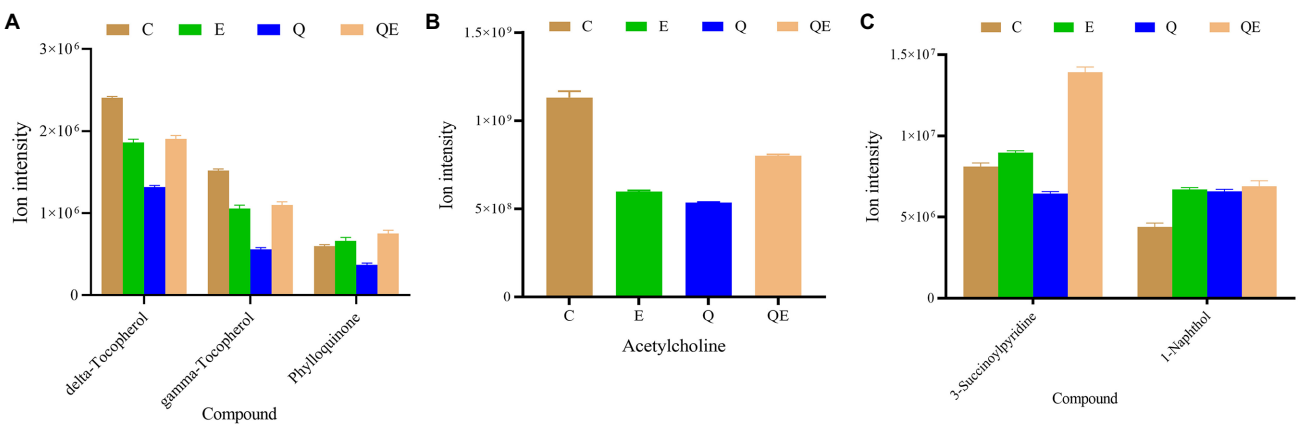

FIGURE 7 | Ion intensities of metabolites involved in differentially expressed pathways by Kyoto Encyclopedia of Genes and Genomes among the aged breeder chickens (control group (C), quercetin group (Q), vitamin E group (E), and QE). **(A)** Ubiquinone and other terpenoid–quinone biosynthesis (delta-Tocopherol, gamma-Tocopherol, and Phylloquinone). **(B)** Regulation of actin cytoskeleton, Insulin secretion, Pancreatic secretion, and Nicotine addiction (Acetylcholine). **(C)** Metabolism of xenobiotics by cytochrome P450 (3-Succinoylpyridine and 1-Naphthol).

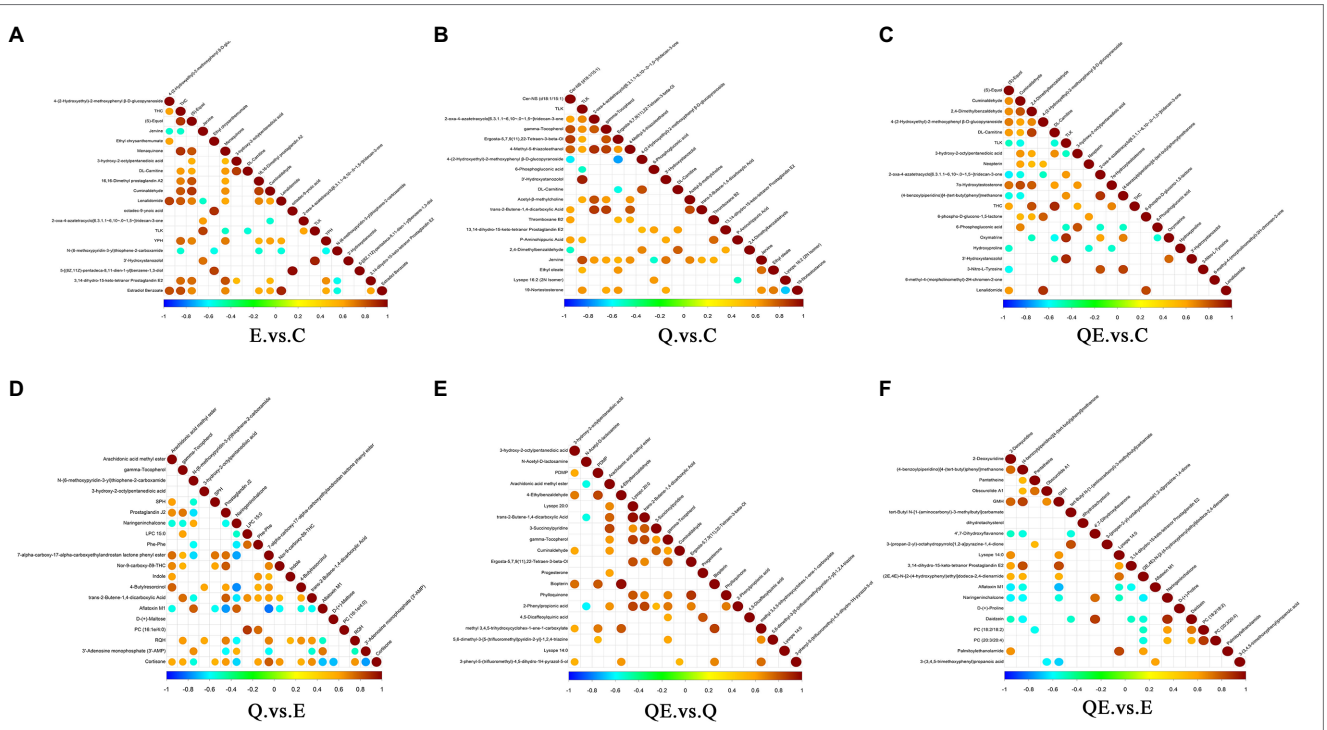

FIGURE 8 | Interaction of differential metabolites among the dietary groups. The highest correlation is 1, which is a complete positive correlation (red), and the lowest correlation is −1, which is a complete negative correlation (blue). The part without color indicates $p > 0.05$. The figure shows the Correlations of the top 20 differential metabolites sorted by value of p from small to large. The upper frame is positive ion mode, the lower frame is negative ion mode. **(A)** E_vs_C. **(B)** Q_vs_C. **(C)** QE_vs_C. **(D)** Q_vs_E. **(E)** QE_vs_Q. **(F)** QE_vs_E.

(Dong et al., 2020), as well as the study by Chen et al. (2020), who reported that plant essential oils supplementation had no effect on cecal microbial alpha diversity in broiler chickens. Moreover, we observed more variation in the cecal microbiota in the aged hens among the groups, which was consistent with previous studies in Cynomolgus monkeys and humans, indicating that, the gut microbiota of adult groups of animals or humans shows more variation than that in the younger group (Lan et al., 2013; Duan et al., 2019). In addition, Pourabedin et al. (2015) reported that overall microbial diversity was not affected by dietary treatment with either xylo-oligosaccharides or virginiamycin in chickens. This finding suggest that the diversity of the gut microbiota may change dynamically in aged chickens.

In the present study, *Bacteroidota*, *Firmicutes,* and *Actinobacteriota* were dominant among the groups at the phylum level. This finding was consistent with previous studies in chickens (Pourabedin et al., 2015; Huang et al., 2018; Pandit et al., 2018; Khan et al., 2020), and the results of Chen et al. (2020), who reported a decreased relative abundance of the phylum *Firmicutes*, but an increased relative abundance of *Bacteroidota* in birds fed with plant essential oils. The differences observed between the present study and previous studies may be attributed to differences in chicken breeds, age of chickens, feed composition, and feeding patterns (Zeng et al., 2015).

QE and Q increased the abundance of *Firmicutes* ([*Eubacterium*]*_coprostanoligenes_group* and *Lachnospiraceae*) compared with the control group, which is consistent with the findings of Zhao et al. (2021) who found that quercetin reversed monosodium glutamate-induced abdominal obesity as a result of hypothalamic damage which causes health problems by elevating the abundance of *Firmicutes*. Additionally, QE decreased the abundance of *Ruminicoccaceae,* whereas, QE/Q/E reduced the abundance of *Erysipelatociostridiaceae, Peptococcaceae, Butyricicoccaceae*, *Erysipeltrichaceae,* and *Acidaminococcaceae.* This result was consistent with the work by Lan et al. (2021), who reported that quercetin partially abrogates gut microbiota disorders by increasing *Lactobacillus* abundance and decreasing *Ruminicoccaceae* abundance. Variations in the abundance of some prominent families may be the basis for the enhanced reproductive performance, immunity, and gut function observed in our earlier studies (Amevor et al., 2021a,b).

Two probiotic strains *Bifidobacterium breve* JM1192 and *B. infantis* BL2416 have been reported to improve body weight gain and prevent the deleterious effects and mortality induced by *Salmonella typhimurium* infection in chicks through different mechanisms, such as competitive exclusion and promotion of cytokine release (El-Sharkawy et al., 2020); therefore, the increased abundance of *Bifidobacteriaceae* in the QE dietary treatment group is not surprising. Supplementation with QE increased the relative abundance of *Lachnospiraceae,* which is involved in butyrate production, and has the capability to form conjugated linoleic acid from linoleic acid (Neyrinck et al., 2012). In addition, *Lachnospiraceae* was reported to play important roles in

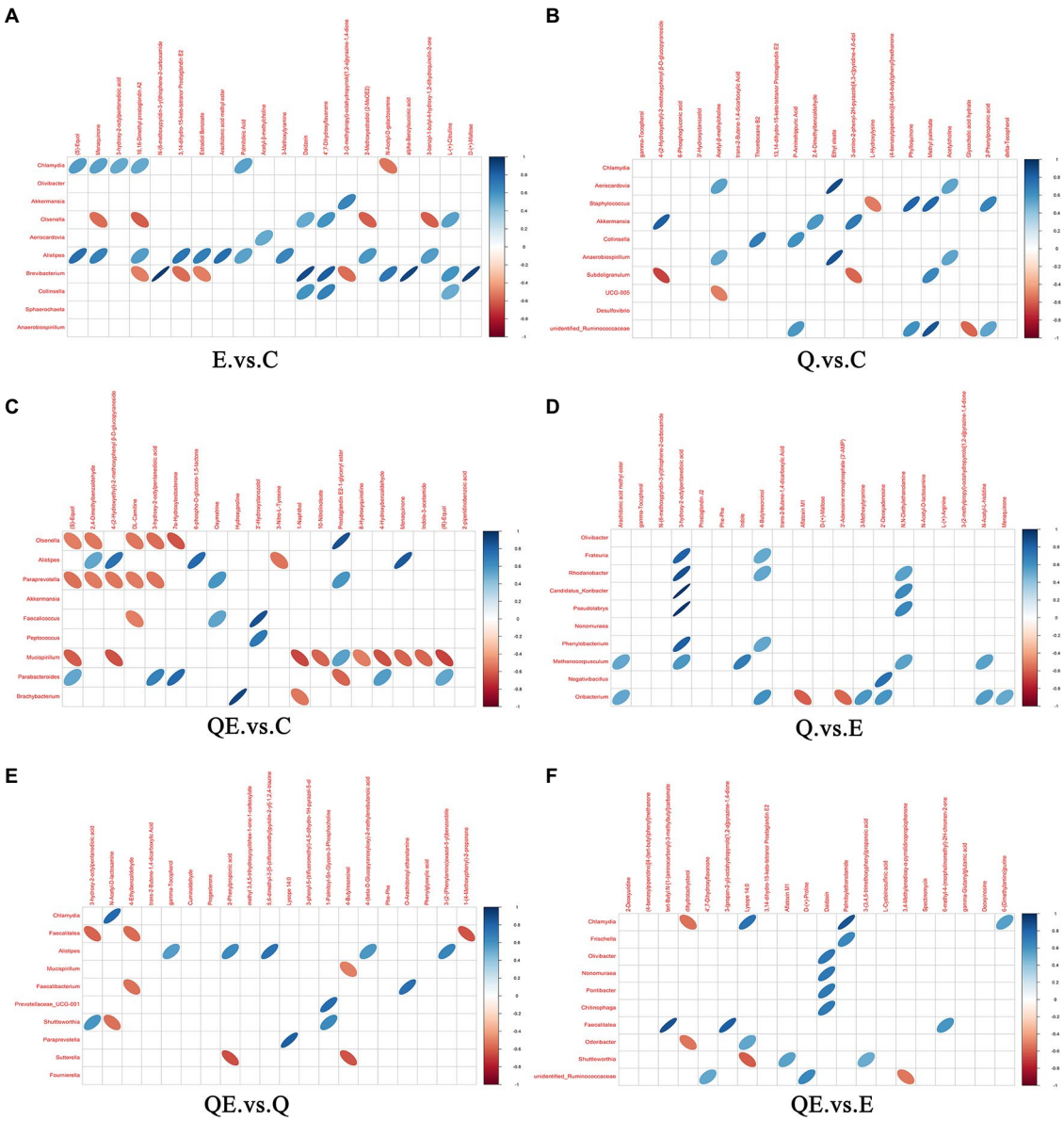

FIGURE 9 | Correlation between microbiota and metabolites in the cecum of aged breeder hens fed quercetin (Q) or vitamin E (E) or QE compared with the control group (C) in a pairwise comparison. **(A)** E_vs_C. **(B)** Q_vs_C. **(C)** QE_vs_C. **(D)** Q_vs_E. **(E)** QE_vs_Q. **(F)** QE_vs_E. The upper frame represents the negative ion mode, and the lower frame represent the positive ion mode. Blue means positive correlation, red means negative correlation, and blank means not statistically significant ($p > 0.05$). Intersection of microbiota and metabolites with oval shape object indicates significant correlation coefficient ($p < 0.05$) and those with no oval object (shape) indicate no significant correlation ($p > 0.05$).

dietary glycan foraging, beneficial metabolite production, and immunity, as well as promoting neurodevelopment in animals (Oliphant et al., 2021). Moreover, *Rikenellaceae,* which is enhanced by vitamin E supplementation in this study, is responsible for producing organic acids, such as propionic and succinic acids by the fermentation of glucose, melibiose, mannose, and lactose, to form iso-methyl branched-chain fatty acids or long-chain saturated acids (Graf, 2014). The biological roles of these bacteria promote animal performance (Neyrinck et al., 2012; Graf, 2014).

Among the numerous pathways enriched according to our KEGG functional analysis, the microbiota in the cecum of the QE dietary treatment group increased the biosynthesis of several metabolic compounds, including amino acids, lipids, and glycans compared with the control group, most of which were identified in the study by Huang et al. (2018).

In addition, nucleotides are semi-essential nutrients, and under conditions of rapid growth, stress, and disease lead to insufficient synthesis based on the capacity of the animal (Kruger and Werf, 2018). Interestingly, the inclusion of either

Q, E, or QE in the diet of aged breeder hens was uniquely enriched in nucleotide metabolism; thus, inclusion of feed supplements (Q, E, or QE) may have enhanced the synthesis of nucleotides. Animals with enhanced nucleotide synthesis are reported to have improved productivity in terms of average daily gain and feed conversion ratio, improved immune cells and antibodies, reduced impact of pathogenic infections, improved gut development and integrity, and improved meat quality (Kruger and Werf, 2018; Wu et al., 2018). In addition, the activities of reproductive hormones, such as estrogen and progesterone, which are involved in follicle development in chickens are coordinated by signal transduction (Brady et al., 2021; Chen et al., 2021) which is increased in enrichment among the Q, E, and QE groups compared to the control group.

Biomarkers are biological characteristics that are objectively quantified and evaluated as indicators of normal or abnormal biological processes, pathological processes, or pharmacological responses to therapeutic intervention (Stowasser, 2016). In recent years, with advancements in technology, several biomarkers have been developed and deployed in the poultry industry (Ducatelle et al., 2018; Baxter et al., 2019). For instance, D-lactate is a metabolite produced by intestinal bacteria and has been deployed as a serum biomarker of intestinal permeability in chickens (Lei et al., 2013). With this in mind, we profiled metabolites in the cecum of 32 aged breeder hens fed with dietary C, Q, E, or QE.

The inclusion of QE and vitamin E increased the abundance of essential metabolites, such as (*S*)-equol, which has been reported to enhance chicken performance. For example, (*S*)-equol, a major gut metabolite of daidzein (isoflavone-derived metabolite), has shown antioxidant, estrogenic, unique anti-androgenic, and anti-cancer activities, as well as cardiovascular protective properties (Li et al., 2018). Strikingly, QE dietary treatment exclusively increased the abundance of 8-Hydroxyquinoline 2.76 fold change higher to that in the control group. This compound has been reported to have a wide range of antimicrobial properties, including antibacterial, antiviral, and anti-parasitic effects (Prachayasittikul et al., 2013). This compound and others, such as Ranolazine, 3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol, Fumonisin B2, and many more presented in **Supplementary Table S5C**, may be the basis for improved performance, organ characteristics, and egg quality in QE diet-treated aged breeder hens (Prachayasittikul et al., 2013; Tardieu et al., 2019).

In contrast, QE decreased the accumulation of metabolites, such as pantothenic acid, Morphine, Prostaglandin E2-1-glyceryl ester, Hydroxyproline, Choline bitartrate, and 3-Nitro-L-Tyrosine. Among these metabolites, 3-Nitro-L-Tyrosine, a derivative of Tyrosine which is involved in Tryptophan metabolism and is further catabolized to the neurotransmitters adrenalin, noradrenalin and dopamine is associated with modulation of feather pecking in chickens (Birkl et al., 2019), suggesting that the synergy of QE may influence egg production, egg quality, body conformation, reproductive organ function, gut functioning, and immunity in chickens.

To identify the biosynthetic pathways, we further searched for differentially enriched pathways *via* KEGG analysis among the three pairwise groups obtained from E, Q, QE, and C. One such prominent pathway was ubiquinone and other terpenoid–quinone biosynthesis pathways with delta- and gamma-tocopherol, and Phylloquinone. Surprisingly, delta- and gamma-tocopherol have been implicated in enhancing egg yolk quality and health status (Hansen et al., 2015; Skřivan et al., 2020). These two pathways were decreased in the Q treatment group, suggesting that inclusion of only quercetin may affect the bioavailability of vitamin E derivatives, thus, there is a need to use quercetin together with a vitamin E in diet supplementation. Moreover, the bioavailability of vitamin K derivative (Phylloquinone) as a result of supplementation with either vitamin E or QE was evidenced in the present study. The supplementation of E and QE promoted the abundance of 3-Succinoylpyridine and 1-Naphthol, which were reported to be involved in the metabolism of xenobiotics by cytochrome P450. Moreover, cytochrome P450 is implicated as the key enzyme for the metabolism or biotransformation of T-2 toxin to 3'OH-T-2 in chickens. This mechanism promotes chicken performance (Shang et al., 2013). These findings are consistent with those reported by Liu et al. (2021), who stated that dietary yeast culture supplementation, improves the biosynthesis of glutathione metabolism, lipopolysaccharide proteins, and ubiquinone and other terpenoid–quinone metabolic pathways which are prominent pathways associated with immune function, egg production, and reproductive efficiency in aged breeder hens (Liu et al., 2021). Therefore, delta- and gamma-tocopherol, Phylloquinone, Acetylcholine, 3-Succinoylpyridine, and 1-Naphthol were validated and targeted for biosynthetic metabolic pathway engineering to improve the performance, gut health, and egg quality of aged breeder chickens.

The bacterial genera most commonly used as probiotics include *Bacillus, Lactobacillus, Enterococcus, Bifidobacterium,* and *Streptococcus* and which influence intestinal function and performance. For instance, they modulate the host immune system, provide energy through microbial metabolites in host microbiota cross talk *via* short-chain fatty acid production, and influence the intestinal structure, integrity and function (Khan et al., 2020). For example, (*S*)-equol showed contrasting correlations with different microbiota in the current study, confirming that there is cross-talk between microbiota and metabolites in the cecum of aged breeder chickens. Therefore, the results presented in this study can be validated and used to discover biomarkers and their use to facilitate layer breeding and genetic improvement programs.

CONCLUSION

In general, dietary supplementation with a combination of quercetin and vitamin E, elevated the relative abundance of the genera *Bifidobacteriaceae*, *Lachnospiraceae, Tannerellaceae*, *Mathonobacteriaceae, Barnesiellaceae,* and *Prevotellaceae* and decreased the abundance of the genera *Chlamydiaceae* and *Campylobacteraceae* in the cecum of the aged hens compared to the other groups. Many key metabolites (DL-Camitine, Cuminaldehyde, Lenalidomide, and (R)-Equol) and prominent KEGG pathways (Phylloquinone, 3-Succinoylpyridine, and 1-Naphthol, etc.) responsible for chicken productivity were enriched in the cecum of hens in the QE group. In addition,

many microbiota and metabolites were significantly correlated. The findings from this study provide valuable information for understanding the mechanisms by which QE promotes productivity in aged breeder hens by altering the gut microbiota and metabolite profile, providing information on developing natural, effective, and safe dietary alternatives to antibiotics, and finally providing information on biomarker discovery and validation that facilitate layer breeding and genetic improvement programs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA789878.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Sichuan Agricultural University, Chengdu, China, under permit number 2019502005 (Chengdu, China).

AUTHOR CONTRIBUTIONS

FA, XZ, and GS designed and conceived this study. FA, ZC, JF, and ZN conducted the experiments. FA, ZC, XiaxD, DX, XDe, WS, YW, XC, JF, JH, and YT collected the samples and performed the analysis of samples. FA, XZ, GS, ZC, BK, DL, YW, and YZ analyzed the data. FA, XZ, GS, CZ, and XiaxD wrote the manuscript. FA, XZ, QZ, DL, YW, BK, XiaoD, and FA revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.851459/full#supplementary-material

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REVIEW
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Gut Microbiota: A Novel Therapeutic Target for Parkinson's Disease

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Parkinson's disease (PD) is the second most common neurodegenerative disease characterized by motor dysfunction. Growing evidence has demonstrated that gut dysbiosis is involved in the occurrence, development and progression of PD. Numerous clinical trials have identified the characteristics of the changed gut microbiota profiles, and preclinical studies in PD animal models have indicated that gut dysbiosis can influence the progression and onset of PD *via* increasing intestinal permeability, aggravating neuroinflammation, aggregating abnormal levels of α-synuclein fibrils, increasing oxidative stress, and decreasing neurotransmitter production. The gut microbiota can be considered promising diagnostic and therapeutic targets for PD, which can be regulated by probiotics, psychobiotics, prebiotics, synbiotics, postbiotics, fecal microbiota transplantation, diet modifications, and Chinese medicine. This review summarizes the recent studies in PD-associated gut microbiota profiles and functions, the potential roles, and mechanisms of gut microbiota in PD, and gut microbiota-targeted interventions for PD. Deciphering the underlying roles and mechanisms of the PD-associated gut microbiota will help interpret the pathogenesis of PD from new perspectives and elucidate novel therapeutic strategies for PD.

Keywords: α-synuclein, diet, gut microbiota, microbiota-targeted therapy, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive, and disabling neurodegenerative disorder that affects the middle-aged and elderly population. It has been ranked second neurodegenerative disorders worldwide just after Alzheimer's disease (AD) (1). Since its description over 200 years ago by James Parkinson, PD has shown the fastest rising prevalence of all neurodegenerative diseases worldwide (2, 3). As reported in the Global Burden of Disease Study 2016, the prevalence and incidence of PD are observed in 1–2% of the population aged >60 years, which have not changed significantly in industrialized countries including the United States and UK, between 1990 and 2016 (2, 3). However, the global burden of PD has more than doubled to over 6 million as a

result of increasing numbers of older people (4, 5), with potential contributions from environmental factors and longer disease duration. With the remarkable evolution of China's socioeconomic conditions and increases in population during the past few decades, the total number of patients with PD has correspondingly increased, which poses a significant challenge in a rapidly aging population (6). The increased proportion of elderly people, longer life expectancy, improved medical facilities, and growing industrialization in China have contributed to a further increase in PD prevalence, which is associated with high treatment and direct healthcare costs. To date, the estimated total number of patients with PD in China could be as high as 3.62 million. PD affects not only the quality of life (QOL) of aging populations but also their life spans, requiring a greater level of care and attention. From 1999 to 2019, the mortality from PD increased from 5.4 (95% confidence interval [CI]: 5.3–5.5) per 100,000 population in 1999 to 8.8 (95% CI: 8.7–8.9) per 100,000 population in 2019 in the United States (7). Currently, the pathogenesis, diagnosis, prognosis, and management of PD are the important aspects of the "one body, two wings" scheme of the China Brain Project.

Generally, PD is characterized by cardinal motor symptoms including tremor, bradykinesia, rigidity, and postural instability; a diverse range of non-motor disorders such as rapid eye movement sleep disorder, anosmia, constipation, depression, cognitive impairment, and dysautonomia; and dysfunction of the autonomic nervous system and enteric nervous system, including hyperhidrosis, dysuria, and orthostatic hypotension (8). PD is referred to as the complex disease in clinical practice (9) and its clinical features impact the QOL of PD patients. Currently, the clinical diagnosis of PD is based on aforementioned typical motor symptoms and novel diagnostic biomarkers such as imaging markers, fluid and tissue α-synuclein markers have been developed (5). However, multimorbidity of non-motor disorders acts synergistically to heighten the risk of adverse outcomes for patients with PD, which usually appear 20 years before the onset of motor symptoms (10). To date, an accurate diagnosis of PD remains challenging and the characterization of the earliest stages of the disease is ongoing.

Li et al. found that the five highest frequencies of non-motor complications in PD patients are sleep disorders, depression, lower urinary tract symptoms, Alzheimer's disease, and constipation in the early stage of PD, making both diagnosis and treatment difficult (10). Pathologically, two major hallmarks of PD are the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and the formation of Lewy bodies and Lewy neurites. The main toxic component of Lewy bodies is the protein α-synuclein which plays a pivotal role in PD pathogenesis. There are several key molecular pathogenic mechanisms—including genetic factors, α-synuclein aggregation, impairment of protein clearance, mitochondrial dysfunction, ferroptosis, neuroinflammation, and oxidative stress—involved in PD pathogenesis (11–13). The exact PD pathogenesis is yet to be completely elucidated owing to the multifactorial nature of the disease, leading to the lack of disease-modifying treatment strategies. Current pharmacological and non-pharmacological treatments such as dopamine replacement therapy with levodopa are mainly focused on relieving the symptoms but have multiple side effects (14). Although highly efficacious symptomatic treatments were successfully developed and adopted in clinical practice for PD, these therapies are ineffective in restoring dopaminergic neurons and stopping or slowing disease progression. Given that the 10-year prognosis for death or life-limiting disability for those who are diagnosed as PD is upwards of 80% (9), there is a desperate need for curative treatments that go beyond symptom management. The ability to stop, prevent, or mitigate progression remains an urgent unmet challenge in PD research at present. Many novel anti-Parkinson's therapeutic agents such as anti-apoptotic agents, monoamine oxidase inhibitors, microRNAs, and viral vector gene therapy have shown general success in preclinical studies across different animal models (15, 16), but little benefit has been reported in human intervention studies or clinical trials. Thus, a better understanding of the PD pathogenesis underlying these symptoms and identification of new therapeutic targets are needed to develop effective therapeutic approaches for PD.

ALTERED MICROBIOTA PROFILES AND FUNCTIONS IN PD

The strong connection between the gastrointestinal environment status and central nervous system (CNS) function—the brain–gut axis—has received increasing attention in the scientific and medical communities. The normal bidirectional communications in the gut–brain axis play vital roles in maintaining homeostasis of the gastrointestinal tract and brain; disruption of this complex relationship has been shown to be associated with the pathogenesis of several neurological disorders including PD (17). Traditionally, the cross-talks along the gut–brain axis have been viewed primarily as mediated by neuro-hormonal factors or inflammatory mediators (18). Recent research has identified another key factor that could influence both gut and brain function: the gut microbiota (17, 19).

Altered Gut Microbiota Characteristics in PD

The gut microbiota—which is also referred to as the second brain, forgotten organ, individual's identity card, and host's fingerprint—may affect brain activity through the microbiota–gut–brain axis under both physiological and pathological conditions (20, 21). With the development of omics techniques, the gut microbiota is considered as one of the important factors in regulating the gut–brain interactions. There is growing acceptance that the gut can influence CNS function and vice versa giving rise to the communication pathway—the microbiota–gut–brain axis. Recent breakthroughs have been made in understanding the intestinal origin of PD. Evidence indicates that gut dysbiosis might trigger and/or exacerbate the progression of PD (17, 22–24), which has opened up new avenues to explore the PD pathogenesis and its novel treatment

approaches. Many studies have highlighted that the gut microbiota can regulate the gut–brain axis through endocrine, immune, and direct neural mechanisms, thus supporting the hypothesis that the pathological process of PD spreads from gut to brain (25, 26). This notion is supported by pathophysiologic evidence: the major component of Lewy bodies, α-synuclein, has been found to be accumulated abnormally in the enteric nervous system (ENS) (27). Holmqvist et al. showed that injection of human α-synuclein fibrils into the gut tissue of healthy rodents is sufficient to induce aggregated α-synuclein pathology within the vagus nerve and brainstem, which provided the first direct experimental evidence that α-synuclein can propagate from the gut to the brain (28). Recently, another study also demonstrated gut-to-brain spread of pathological α-synuclein fibrils following their injection into the mouse muscularis layer of the pylori and duodenum, but not when the mice were subjected to truncal vagotomy following α-synuclein fibril injection (29).

Cersosimo and Benarroch also found that gastrointestinal dysfunctions including constipation, bloating, nausea, dysphagia, sialorrhea, vomiting, and gastroparesis are common in PD (i.e., seen in over 80% PD cases) (30) and can begin decades before the onset of motor symptoms. Adams-Carr et al. demonstrated that people with constipation are at a higher risk of developing PD than those without and that constipation can predate diagnosis of PD by over a decade (31). These gastrointestinal symptoms are strongly associated with α-synuclein-related neurodegenerative changes in the ENS (32). The deposition of α-synuclein is not only found in the gastrointestinal tract of PD patients with gastrointestinal symptoms (33) but also in the gastrointestinal tract of patients before they present with PD-related motor symptoms (34). This indicated that α-synuclein pathology is likely initiated in the ENS first, and then spreads to the CNS via the vagus nerve (35). These findings strengthened Braak's hypothesis in that PD pathology may originate in the periphery and gradually propagate to the brain, where it eventually leads to clinically confirmed PD. Therefore, the concept of "gut-originality" is now widely accepted in present PD pathogenesis research and recent accumulating evidence has shown that gut microbiota may play a key role in the progression of PD.

In the past decade, many case-control studies in different regions including Western countries and China have investigated the overall structure of gut microbiota in PD patients, and explored the correlations between gut microbiota and PD clinical characteristics. Elucidating the alterations of the gut microbiota will provide a foundation to improve our understanding of the PD pathogenesis and support the potentially microbiota-modifying therapeutics. Human α-synuclein overexpressing (ASO) mice that received fecal microbiota transplantation from PD patients showed increased severity of manifestations than those animals that received fecal microbiota from healthy individuals, which provided direct evidence that dysbiosis of the gut microbiota may play a causal role in the development of PD.

In fact, the alterations of the gut microbiota in PD patients have been repeatedly demonstrated. A recent study also found significant microbiota differences in the PD appendix (36). However, the structure and composition of the gut microbiota are affected by various exogenous factors such as age, sex, body mass index, race/ethnicity, geography, diets, drugs, and life styles, which must be taken into account when evaluating the varying results of different studies. These external factors contributed to variations in diversity and composition of the gut microbiota, making it difficult to compare the gut microbiome composition across different studies and achieve a unanimous conclusion. Nevertheless, those case-control comparative studies can still provide some clues to explore the roles of the gut microbiota in the development of PD.

Gut microbiota dysbiosis was observed in patients with prodromal and/or clinically established PD when compared with well-controlled subjects. Using culture-independent high-throughput sequencing techniques, the overall structure and composition of the PD-associated gut microbiota have been investigated, and characteristics of the altered microbiota profiles in PD patients have been identified. While certain findings were replicated across several studies, various contradictory findings were reported. Generally, bacterial diversity is the most validated metagenomic marker of gastrointestinal health and metabolic disorders. Many previous studies have observed decreased bacterial diversity in PD patients, but higher α-diversity in PD patients (23, 37). One study also showed that β-diversity (between samples) differed between PD patients and controls (38). The loss of bacterial diversity, mainly measured with α-diversity indices such as Shannon and Simpson, has been linked to the clinical features of PD. A recent study performed by Heinzel et al. found that constipation, possible rapid eye movement sleep behavior disorder (RBD), physical inactivity, smoking, urate levels, and subthreshold parkinsonism might be particularly linked to the prodromal microbiome in PD. Constipation, physical inactivity, and occupational solvent exposure showed associations with bacterial α-diversity, while sex, physical inactivity, possible RBD, constipation, and smoking were associated with β-diversity. Age and urate-lowering medication were associated with both α- and β-diversity (39). However, Plassais et al's investigation showed that the gut microbiome α-diversity is not a biomarker of PD (40). Regardless of these inconsistent results, the changed overall structure of the gut microbiota and its associations with PD clinical features implies that microbial dysbiosis may be a contributor to the etiopathogenesis of PD, even in its early stage.

As far as the altered composition of PD-associated gut microbiota was concerned, no consensus has emerged from existing human studies of PD and gut microbiota regarding which bacterial taxa are most relevant to PD. One meta-analysis of 15 case-control studies observed that Prevotellaceae, Lachnospiraceae, and Faecalibacterium were decreased significantly in patients with PD compared to healthy controls, while Ruminococcaceae, Bifidobacteriaceae, Christensenellaceae, and Verrucomicrobiaceae were enriched in patients with PD (41). Another recent meta-analysis of 10 relevant studies found an abundance of Megasphaera and Akkermansia, and reduced

Roseburia in PD patients (42). To date, the most consistently shown PD-related changes of gut microbial composition include an increase in the relative abundances of Verrucomicrobiaceae and Akkermansia and a decrease in Prevotellaceae and Prevotella (38).

Prevotella, a highly specific prodromal marker of PD, has been associated with RBD (43) and with progressive PD motor symptoms over 2 years (44). A previous study showed that constipation is lowest and subthreshold parkinsonism is least frequent in individuals with the Prevotella-enriched enterotype (39). In addition, constipation severity is significantly correlated with the decrease of Blautia and Faecalibacterium, short chain fatty acids (SCFAs)-producing taxa that can exert positive effects on the intestinal mucosa (45) and which are decreased in PD. In contrast to PD, SCFA-producing bacteria in one study were not decreased in idiopathic RBD, which suggested that a decrease of SCFA-producing bacteria may be a prerequisite for the development of PD (46). Another specialized mucin-degrading Verrucomicrobiaceae genus, Akkermansia, is a potential next-generation microbe that has anti-inflammatory properties and is responsible for good health (47, 48). Akkermansia is associated with enhancement of wound healing, augmented antitumor responses, protection against obesity, and induced intestinal adaptive immune responses during homeostasis (49–52). However, Akkermansia cannot always be considered a potentially beneficial bacterium, because several studies have consistently reported a greater abundance of Akkermansia in the fecal samples of PD and AD patients than that in healthy controls (53–56).

Amorim Neto et al. observed that A. muciniphila (typical strain) induced mitochondrial calcium overload and α-synuclein aggregation in an enteroendocrine cell line (57). The PD-associated gut dysbiosis, such as increased Akkermansia and decreased SCFAs-producing bacteria, can increase the intestinal permeability and intestinal inflammation, which subsequently facilitates exposure of the intestinal neural plexus to toxins such as lipopolysaccharide (LPS) and pesticides; this can lead to abnormal aggregation of α-synuclein fibrils and generation of Lewy bodies (22). Choi et al. demonstrated that oral administration of Proteus mirabilis, increased markedly in PD mouse models, was sufficient to provoke selective death of dopamine neurons and motor deficits in mice, accompanied by neuroinflammation and accumulation of aggregates of α-synuclein in both the colon and brain (58). One gastric pathogen, Helicobacter pylori, was found to be associated with increased severity of motor dysfunction, decreased dopamine levels in the brain, and decreased levodopa absorption as well as autoimmune and inflammatory reactions (59).

Although the above case-control studies identified several key functional bacteria in the PD-associated gut microbiota, these cross-sectional gut microbiome studies could still not establish a causal relationship between variations in the gut microbiome and PD. Moreover, little is still known about whether such alterations precede disease onset and how they relate to risk and prodromal markers of PD. This knowledge gap greatly restricts the application of those key intestinal functional bacteria in the non-invasive diagnosis and microbiome-targeted treatment for PD.

Recently, a longitudinal study found that microbiota differences (e.g., in Roseburia, Prevotella, and Bifidobacterium) detected at baseline can be replicated at a follow-up timepoint 2 years later, and that there might be changes in gut microbiota composition in patients with faster disease progression (44). In addition, these altered key functional bacteria or biomarkers are correlated with non-motor symptoms, disease duration, treatment options, and even cognitive impairment (23), which suggest that gut microbiota can participate actively in the process of PD. Prospective long-term longitudinal microbiome studies are needed to monitor disease progression and characterize alterations in the taxonomic composition of the microbiome that lead to, or might even define, the disease state. However, exactly how the gut microbiota may impact PD-related symptoms remains unclear.

Altered Gut Microbiota Functions in PD

While the present taxonomic changes of the gut microbiota in PD only provide information regarding correlations but not causation, a recent study has found casual associations between several gut bacteria such as Lentisphaera, Eubacterium hallii, Anaerostipes, and Clostridium sensu stricto 1 and PD through a Mendelian randomization approach (60). However, a correlation is not necessarily equal to causation, as just define how one variable change relatively to another. In fact, disentangling cause and effect is difficult. Given that microbiota functions are conserved across taxonomic groups, they are much more informative than taxonomic data, since it is what the microorganisms do that we care about and not who they are.

Notably, microbial functioning analysis is more productive than a purely taxonomic approach to understanding the gut microbiome in PD. Recently, metatranscriptomics, metaproteomics, and metabolomics have been used to explore the functionality of the microbiota and, therefore, provides some strong insights into microbial activities in the gut and their associations with health and diseases. Different to metagenomics or 16S rRNA gene sequencing, the fecal metabolome provides a functional readout of microbial activity and can be used as an intermediate phenotype mediating host-microbiome interactions (61). Bacterial metabolites mirror the altered gut microbiota composition in patients with PD. Tan et al. found that fecal metabolites in PD were significantly different from that in healthy controls, with the largest effect size seen in an NMR-based metabolome (62).

Differentially abundant fecal metabolites including bioactive molecules with putative neuroprotective effects (e.g., SCFAs, ubiquinones, and salicylate) and other compounds (e.g., trimethylamine N-oxide, ceramides, and sphingosine) are increasingly implicated in neurodegeneration. SCFAs such as acetate, butyrate, and succinate are produced owing to the fermentation process of gut bacteria. Notably, accumulating evidence highlights the important functions of SCFAs in many neurological diseases such as multiple sclerosis, AD, and PD (46, 56, 63–65). Reduced levels of SCFAs were found in PD patients, which increased the incidence of endotoxin and neurotoxin, both

potentially associated with the development of PD. In PD patients, low SCFAs are significantly associated with poor cognition and low BMI; lower butyrate levels correlate with worse postural instability-gait disorder scores (62). In addition, SCFAs can promote gastrointestinal motility and regulate the function of the enteric nervous system, which is why a reduction of SCFAs might contribute to the development of gastrointestinal motility disorders such as constipation in PD (64). Fecal SCFAs were found to be inversely correlated with several PD-related clinical variables such as the Non-Motor Symptoms Scale score, the Rome III constipation/defecation subscore, stool consistency associated with constipation on the Victoria Bowel Performance Scale, and the Geriatric Depression Scale-15 (66). Microbial SCFAs play both direct and indirect key roles in microbiota–gut–brain axis signal communication via regulation of the gut epithelial barrier and blood–brain barrier integrity, neuronal survival, inflammatory cascades, and endocrine signaling (67).

A reduction of SCFAs-producing bacteria leading to SCFAs deficiency may result in detrimental effects in PD patients, including gut leakiness, increased colonic inflammation, increased risk of α-synuclein deposition in the gastrointestinal tract, and microglial activation in the brain (68, 69). SCFAs were recently reported to have protective properties against dopamine and tyrosine hydroxylase depletion in the substantia nigra (70). Liu et al. also found that butyrate exerts protective effects against PD in mice via stimulation of glucagon like peptide-1 (71). This finding suggested that alterations to SCFAs may play a role in the pathophysiology of PD and increase of SCFAs can retard the development of PD.

Apart from altered SCFAs, microbial dysbiosis can also affect lipid metabolism, including an upregulation of bacteria responsible for secondary bile acid synthesis. Bile acids are produced in the liver from cholesterol and then metabolized by gut microbiota-derived enzymes into secondary bile acids (72). Li et al. found that microbially derived toxic bile acids such as deoxycholic acid and lithocholic acid are heightened in PD (36). The increases of deoxycholic acid and lithocholic acid can propel the accumulation of pathological α-synuclein aggregates, which can potentially propagate from the gut to the brain through retrograde transport (73). However, Castrocaldas et al. demonstrated that tauroursodeoxycholic acid can rescue mitochondrial function and prevent 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced dopaminergic cell death in different animal models of PD (74). Ursodeoxycholic acid is another secondary bile acid that has neuroprotective effects (75) and is currently being tested in clinical trials for PD (76), which can prevent the damaging effects of deoxycholic acid and lithocholic acid. In a chronic PD mouse model, pretreatment with tauroursodeoxycholic acid can protect against dopaminergic neuronal damage, prevent microglial and astroglial activation, as well as the dopamine and 3-4-dihydroxyphenulacetic acid reductions caused by MPTP. Pretreatment with tauroursodeoxycholic acid can prevent protein oxidation and autophagy, in addition to inhibiting α-synuclein aggregation (77). This finding suggests that fecal biliary abnormalities may also play a crucial role in PD pathogenesis, and targeting microbial-derived secondary bile acids may be a new avenue for the earlier PD diagnosis and alleviation of PD symptoms.

Other microbiota-derived metabolites, including proteolytic metabolism products (e.g. p-cresol sulfate) and tryptophan catabolites (e.g. kynurenine and indolelactic acid), have shown significant alterations in PD, which are also crucial metabolic events underlying PD. Other metabolites derived from gut microbiota-based metabolism of dietary polyphenols—e.g., 3,4-dihydroxybenzoic acid (3,4-diHBA), 3-hydroxyphenylacetic acid (3-HPPA), and 3-hydroxybenzoic acid (3-HBA) — have shown an in vitro ability to inhibit α-synuclein oligomerization and mediate aggregated α-synuclein-induced neurotoxicity (78). Chung et al. found that plasma levels of trimethylamine N-oxide (TMAO), a gut microbiota-derived metabolite, are associated with faster increases in levodopa-equivalent dose and tend to increase the risk for PD-dementia conversion, and can therefore be considered as a biomarker in early PD (79). Microbiota-derived metabolite studies can provide comprehensive biochemical underpinnings to unravel the underlying mechanisms of PD pathogenesis, offer biomarkers that reflect PD pathological processes, and possibly substantially improve therapeutic strategies against PD. Thus, alterations of the gut microbiota and its associated microbiota functions might modulate the microbiota–gut–brain axis, and play crucial roles in the pathogeneses of PD, which provide novel insights into PD pathogenesis and microbiome-targeted therapeutic options for PD.

MECHANISMS OF GUT MICROBIOTA IN PD

As mentioned above, dysbiotic gut microbiota are understood to play crucial roles in the occurrence and development of PD by increased intestinal permeability, aggravated neuroinflammation, abnormal aggregation of α-synuclein fibrils, oxidative stress, and decreased neurotransmitter production (**Figure 1**).

Increased Intestinal Permeability

Increased intestinal permeability, also known as "leaky gut," has been detected in patients with PD—even in cases with minimal or no gastrointestinal manifestations (80). Pathogenetically, the gastrointestinal tract is proposed to be the initial site of pathological changes in PD. During dysbiosis, the destruction of intestinal epithelial barrier integrity can influence the initial step of the following cascade of neurodegeneration in PD. Accumulating evidence has emphasized the important role of the intestinal barrier and intestinal permeability on health and disease. Forsyth et al. found that increased intestinal permeability in PD patients, which is a feature of intestinal barrier function, strongly correlated with markers of increased exposure to endotoxin, and with a marker indicating increased oxidative stress burden in the intestine, which together may be responsible for the abnormal accumulation of α-synuclein in enteric neurons

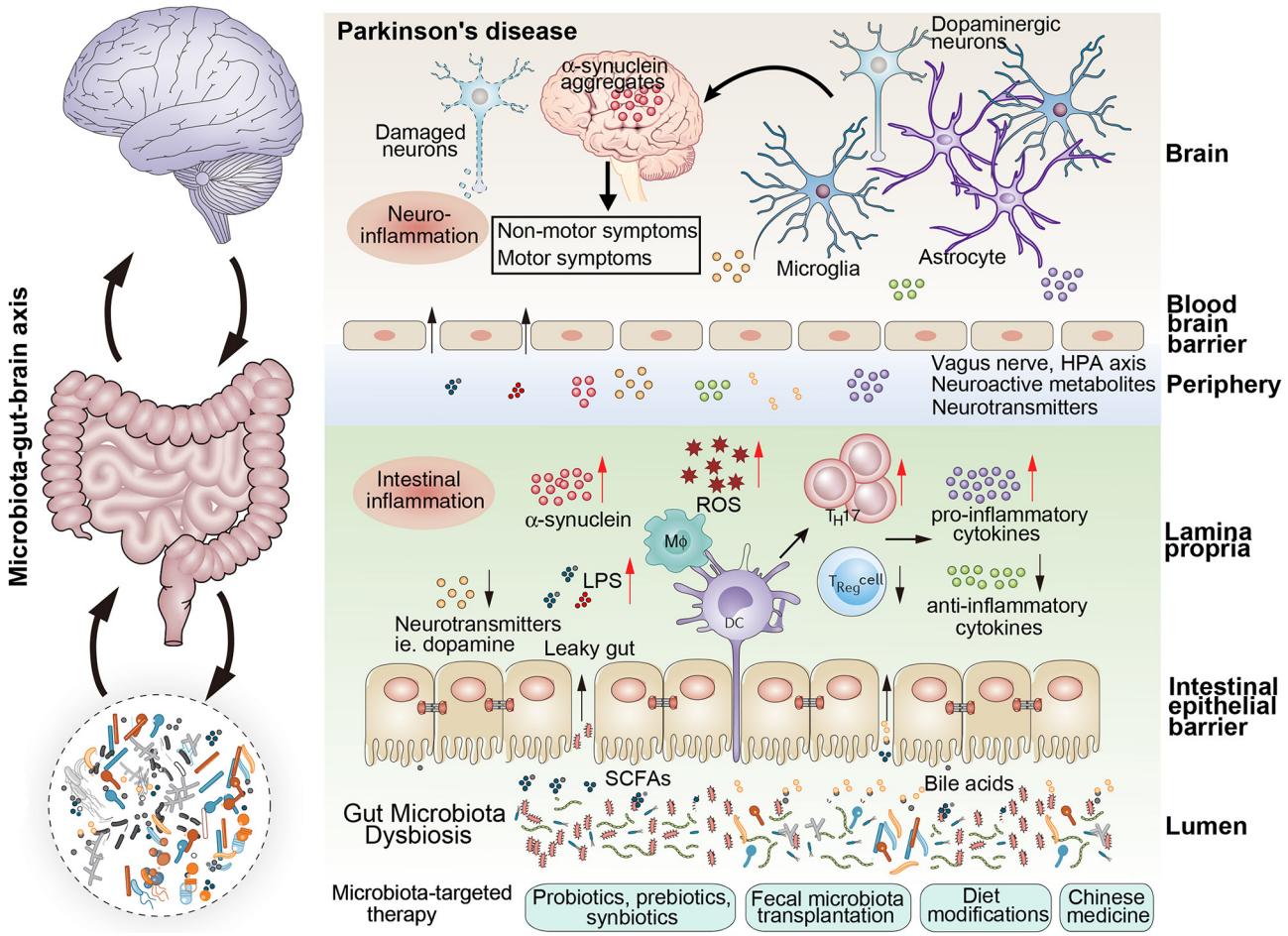

FIGURE 1 | The schematic representation of the gut microbiota dysbiosis associated with parkinson's disease. Microbiota dysbiosis plays vital roles in the occurrence and developemnt of parkinson's disease. Dysbiotic gut microbiota was associated with the increased intestinal permeability, aggravated neuroinflammation, abnormal aggregation of α-synuclein fibrils, oxidative stress, and decreased neurotransmitter production, which played vital roles in the ocurrence and development of Parkinson's disease. Several microbota-targeted therapies can be used to modify the composition of the gut microbiota to revert a dysbiotic condition: probiotics, psychobiotics, prebiotics, synbiotics, postbiotics, fecal microbiota transplantation, dietary modifications and Chinese medicines.

(81). Fecal zonulin and α-1-antitrypsin, markers of intestinal barrier permeability, have been found to be increased in PD patients (82, 83). In addition, reduction of tight junction proteins (TJs) expression in PD patients has been linked with increased intestinal permeability. The expression of TJs such as occludin, but not of zona occludens 1 (ZO-1) decreased significantly in lysates of sigmoid/descending colon biopsies of PD patients (84). Perez-Pardo et al. reported a reduction of ZO-1 immunolabeling in sigmoid colon biopsies from PD (85). However, the elevated fecal markers were not associated with other clinical features of PD, which suggested that a PD subpopulation should be considered when enrolling PD cohorts.

Several animal studies have provided causal evidence that intestinal impairments can trigger PD-related pathology. Kelly et al. demonstrated that increased intestinal permeability correlated with the presence of α-synuclein aggregates in a mouse model of PD (86). Another previous study found that increased intestinal permeability triggered the aggregation of α-synuclein in enteric neurons and dopaminergic neurons of the substantia nigra, ultimately leading to neuronal degeneration in aged α-synuclein transgenic mice (87). Further, in ASO mice, LPS injection reduced intestinal barrier integrity and expedited the onset of motor symptoms (88). Bhattarai et al. found that chronic administration of rotenone results in disrupted colonic epithelial permeability and development of motor symptoms only in conventionally raised mice but not in germ-free mice. Those results implied that gut microbiota are not only required but may in fact be the mediators of the effect of environmental toxins on intestinal permeability and the development of motor symptoms (24). Polymannuronic acid can greatly increase the integrity of intestinal barrier and blood–brain barrier as indicated by increased expressions of TJs in both mice colon and substantia nigra pars compacta in PD mice model, which can improve motor functions by preventing dopaminergic neuronal

loss (89). The important roles of intestinal permeability have been highlighted in PD initiation, which paves the way for novel PD therapy targeting the restoration of the intestinal barrier. Indeed, future studies are still needed to determine whether increased intestinal permeability plays a causative role in PD initiation or is a consequence of PD pathogenesis.

Inflammation

Inflammation, the hallmark of PD, has been linked to the development of non-motor symptoms in PD, which greatly impact patients' QOL and can often precede motor symptoms. Mounting evidence has documented increased levels of a variety of inflammatory molecules in the brain and cerebrospinal fluid as well as blood from PD patients such as IL-1β, IL-2, IL-6, IL-10, TNF-α, CCL5, and CRP (90, 91). Several studies have found that calprotectin, a fecal marker of intestinal inflammation, was significantly elevated in PD patients compared to age-matched controls (83, 92). The level of fecal calprotectin correlates well with macroscopic and histological inflammation as detected by colonoscopy and biopsies, respectively. Another previous study observed increased fecal calprotectin levels in patients with inflammatory bowel disease (IBD) (93), and epidemiological data indicate a link between IBD and PD (94, 95). The detection of the fecal calprotectin may be a useful tool to detect the signs of gut immune system activation present in PD patients, possibly in the early stage of the disease (92). Klingberg et al. found that increased gut dysbiosis was associated with elevated fecal calprotectin (96). In fact, intestinal inflammation in PD is related to gut dysbiosis and gut permeability such that it can contribute to disease pathogenesis. During gut dysbiosis, the metabolites or components of pathogenic bacteria may cause inflammation, while the metabolic changes in the symbiotic bacteria can regulate inflammation, which result in PD pathology together.

Recent investigations have suggested that gut dysbiosis is linked to aberrant immune responses, which not only increases intestinal inflammation but also increases microglial activation and neuroinflammation (97, 98), which are central events in the pathogenesis of PD (99). As mentioned above, intestinal barrier deficiency can increase systemic exposure to inflammatory microbial products such as LPS, inducing intestinal inflammation, microglial activation, neuroinflammation, and oxidative stress and in turn neuronal pathological α-synuclein aggregates. LPS is a potential influence on brain glia originating in the gut microbiota, while depletion of gut bacteria reduces microglia activation (100). Hasegawa et al. found that plasma level of the LPS-binding protein was decreased in PD patients (101), suggesting more LPS reaches the CNS. Evidence from animal models has shown that LPS may not only enhance the inflammatory response in the CNS of PD patients but also accelerate the neurodegenerative process by the effects on α-synuclein (102). α-synuclein-induced microglial activation (neuroinflammation) would potentiate further α-synuclein aggregation and propagation specifically in the nigrostriatal dopaminergic neurodegeneration, thus contributing to PD progression (100). The abundance of LPS-producing Bacteroides was found to be correlated with the plasma levels of TNF-α in Taiwanese patients with PD (91). Lin et al. found that the relative abundances of Verrucomicrobia and Bacteroides correlated with elevated TNF-α and IFN-γ in patients with PD, suggesting the development of a systemic sub-inflammatory status associated with gut dysbiosis (91). Sampson et al. revealed that gut dysbiosis or specific gut pathogens can promote neurodegeneration and motor deficits by activating neuroinflammation in an ASO mouse model (100), that can be used as potential therapeutic targets for PD treatment. In addition, decreased SCFAs also downregulates regulatory T cells and upregulates Th17 cells, which fails to suppress neuronal inflammation and ultimately leads to neurodegeneration (22, 103). Singh et al. also found that increased inflammation and decreased innate lymphoid cells (ILCs) in DJ-1−/− mice, which can be regulated by intestinal bacteria such as Alistipes and Rikenella (104).

Microbiota-modifying therapies may provide benefits to PD patients, but studies are lacking. Strong epidemiological evidence has suggested that smokers and coffee drinkers have a reduced risk of PD, possibly by shifting the composition of gut microbiota toward improved barrier function and decreased release of pro-inflammatory cytokines from the gut into the bloodstream, which likely reduces neuroinflammation (105). In one study, IBD patients who received anti-TNF therapy had a 78% reduction in the incidence of PD, suggesting that suppressing peripheral inflammation can protect against PD. In a rotenone-induced PD mouse model, fecal microbiota transplantation (FMT) could suppress inflammation mediated by the LPS-TLR4 signaling pathway both in the gut and brain (106). Sun et al. also found that FMT can correct the gut microbiota dysbiosis, reduce the activation of microglia and astrocytes in the substantia nigra, suppress neuroinflammation, and reduce TLR4/TNF-α signaling in the gut and brain (107). Another study found that acupuncture can protect dopaminergic neurons and enhance motor function, which may be associated with the regulation of gut dysbiosis and thus the inhibition of neuroinflammation in PD mice (108). Hence, further studies aimed to modify gut dysbiosis to suppress intestinal inflammation and neuroinflammation can potentially attenuate the neurodegenerative process in PD.

α-Synuclein Pathology

Inflammation and α-synuclein misfolding are both key pathological mechanisms underlying α-synucleinopathies such as PD (109). Aggregation of α-synuclein is central to the pathogenesis of PD. α-synuclein consists of 140 amino acids, and the gene encoding it—synuclein α (SNCA)—comprises five exons and is located on chromosome 4q21.3-q22 (110). α-synuclein exhibits characteristics of prion-like protein during PD pathogenesis, with the misfolded α-synuclein turning the endogenous physiological protein into a pathogenic protein (26). Alterations in α-synuclein dosage lead to familial PD, while α-synuclein also contributes to fibrilization of amyloid-β and tau, two key proteins in AD, which suggest a central role for α-synuclein toxicity in neurodegeneration (111). The misfolding of α-synuclein into aggregates within nerve cells may contribute to neurodegeneration. Besides α-synuclein aggregation in PD

patients, a previous study also found that α-synuclein fibrils are frequently observed in the brain even in healthy elderly subjects without motor or cognitive impairment, and sometimes in the intestinal neural plexus (22). The presence of aggregated α-synuclein in sigmoid colon biopsies may have 100% sensitivity and specificity for patients later developing pathologically advanced PD (112). The drugs designed to treat or prevent PD are focused on the prevention or elimination of α-synuclein aggregation; however, no successful cases have yet been reported yet.

The interaction between α-synuclein aggregation and the gut microbiota in PD is receiving increasing attention. Recent evidence suggests that α-synuclein aggregation may begin in the gut and gradually travel to the brain along the vagus nerve, with altered gut microbiota being a potential trigger for α-synuclein misfolding (29, 113, 114). Gut dysbiosis cause misfolding and abnormal aggregation of α–synuclein in the intestine, which can be transported from the ENS to CNS. Several reports have observed that gut microbiota play an important role in regulating misfolded and abnormal aggregation of α-synuclein and the gut–brain axis (115). Generally, the gut dysbiosis in PD can affect the gastrointestinal mucosal barrier, leading to the translocation of bacteria or their products such as LPS. These bacterial products can increase oxidative stress and intestinal inflammation, which in turn increases the intestinal permeability, and increases the ability of α-synuclein to communicate with the ENS.

LPS, bacterial products from gram-negative bacteria that are increased in people with PD, can increase the nitration and oligomerization of α-synuclein by increasing the level of inducible nitric oxide synthase. In addition, LPS can downregulate TJs such as occludin in the intestinal epithelial cells and upregulate TNF-α, which activates macrophages and promotes the expression of α-synuclein, as observed in a mouse model (116). In rodent models, LPS can increase intestinal permeability and α-synuclein expression in the large intestine, which provides direct evidence between gut dysbiosis and α-synuclein pathology (86, 88). Other bacterial metabolites such as SCFAs, especially butyrate, serve an important role in maintaining intestinal integrity, and a lack of SCFAs can increase intestinal permeability. The decreased SCFA-producing bacteria in PD patients can lead to disruption of the intestinal mucosal barrier, and this serves as a prerequisite for entry of α-synuclein into the ENS to maintain excessive α-synuclein expression or even promote its misfolding (117). Therefore, the altered gut microbiota profiles and their bacterial metabolites can influence α-synuclein expression, misfolding, and transportation in the early stage of PD, which can be used as potential targets to antagonize α-synuclein pathology.

Various studies have targeted α-synuclein directly at different stages of its synthesis and action as a potential therapeutic intervention. In vitro bacterial treatments and in vivo FMT also support the role of the gut microbiome in α-synuclein aggregation, gastrointestinal inflammation, and motor symptom development (88, 107). A previous study observed that ASO mice that received healthy gut microbiota showed improved motor deficits by down-regulating α-synuclein expression. Zhong et al. found that FMT can alleviate physical impairment, decrease fecal SCFAs and the expression of α-synuclein, and inhibit the activation of microglia in a mouse model of PD (118). Clinical observations found that probiotics can reduce the production of α-synuclein aggregates by inhibiting the reactive oxygen species level and finally alleviating the condition of patients with PD (119). Recently, Wang et al. demonstrated that Lactobacillus plantarum DP189 can reduce α-synuclein accumulation in the substantia nigra of the PD mouse model via inhibiting NLRP3 inflammasome, which can resist the development of PD (120, 121). Furthermore, previous studies have reported that coffee contains possible neuroprotective compounds such as caffeine and neuroprotective polyphenols (122, 123). Coffee can ameliorate motor deficits and dopaminergic neuronal loss, and reduce α-synuclein aggregation in MPTP-induced mice via regulating gut microbiota (124). Coffee can increase the level of anti-inflammatory Bifidobacteria and decrease the levels of Clostridium spp. and Escherichia coli that invade the gut mucosa in PD (125). These observations suggested that reducing α-synuclein expression, aggregation, or propagation by targeting gut microbiota modulation may represent a potential novel therapeutic option for PD treatment.

Oxidative Stress

Oxidative stress plays a vital role in the degeneration of dopaminergic neurons in PD. An imbalance between reactive oxygen species (reflecting oxidative stress status) and antioxidant activity promotes inflammatory conditions in PD, which can create a vicious cycle and worsen neuronal cell death. Oxidative stress is considered the common underlying mechanism for cellular insult and apoptosis of dopaminergic neurons, regardless of whether PD is genetic or idiopathic (126). Thus, therapies targeting the suppression of oxidative stress may delay or reduce the severity of PD (127). It is increasingly accepted that oxidative stress may be aggravated by concomitant PD-associated gut dysbiosis (128). Generally, healthy gut microbiota have an immense antioxidative and anti-inflammatory role, while gut dysbiosis can manifest low-grade inflammation, cellular degeneration, and an imbalance of cellular energy followed by an increasing oxidative stress state (129). An unbalanced cycle of oxidative stress caused by gut microbiota dysbiosis may have the effect of gradually promoting specific phenotype of PD.

Oxidative stress may activate enteric neurons and glial cells, contributing to accumulation and misfolding of α-synuclein in the ENS (130). An α-synuclein accumulation in the intestine can transfer to the brain, leading to microglia activation, causing an increase in oxidative stress that exacerbates neuroinflammation (68). As mentioned above, the increased intestinal permeability in PD can increase enteric and systemic exposure to LPS and other bacterial products, resulting in increased intestinal oxidative stress (131). Nishiwaki et al. reported that Akkermansia may increase intestinal permeability and expose the intestinal neural plexus to oxidative stress, which can lead to abnormal aggregation of prion-like α-synuclein fibrils in the intestine (46).

Recently, probiotics and prebiotics that can modulate the gut microbiota have been used to regulate oxidative stress in PD patients. Tsao et al. demonstrated that Lactobacillus salivarius AP-32 lowered oxidative stress and inflammation, increased serum antioxidant activity, altered the fecal microbiota composition, and increased the levels of SCFAs in fecal samples in a PD-like model (127). Wang et al. found that another strain of Lactobacillus, L. plantarum DP189, can delay the neurodegeneration caused by the accumulation of α-synuclein in the substantia nigra of PD mice via suppressing oxidative stress, repressing proinflammatory response, and modulating gut microbiota (120). Marsova et al. found that Lactobacillus can reduce the level of oxidative stress (reactive oxygen species) in the PD nematode model by regulating the Nrf2/ARE pathway (132). These probiotic supplements directly and indirectly suppress oxidative stress by upregulating antioxidant pathways and by increasing antioxidant capacity, which can influence PD pathology.

Neurotransmitters

The gut microbiota produces many neurotransmitters found in the human brain such as dopamine, serotonin, gamma-aminobutyric acid, and norepinephrine (133–135) and have been shown to influence various neurodegenerative disorders including PD. These neurotransmitters are involved in brain functions such as emotion, movement, learning, and memory actively. Generally, the gut microbiota directly produces neurotransmitters by encoding genes for specific enzymes or regulates the host biosynthesis of neurotransmitters with bacterial metabolites (136). In addition, dietary tryptophan can be metabolized by gut microbiota, which can produce various metabolites such as kynurenic acid and quinolinic acid. Several studies found that kynurenic acid is neuroprotective owing to its anti-inflammatory properties in the intestinal lumen (137, 138), while quinolinic acid is a blood–brain barrier modulator (139). Previous studies suggested that a healthy gut microbiota is associated with balanced neurotransmitter levels in the host. Studies have demonstrated that the absence or deprivation of gut bacteria in both germ-free and antibiotic-treated mice can influence the concentrations of neurotransmitters and their precursors in the gut and blood, and alter the expression of neurotransmitter receptors within the brain (140, 141). An imbalance in neurotransmitters eventually affects the pathogenesis of neurological and psychological disorders.

Dopamine, a major neurotransmitter, plays a critical role in vital functions such as cognition, motivation, and voluntary motor movements both peripherally and centrally. Optimal dopamine bioavailability is essential for normal brain functioning and protection against the development of neurological diseases (142). Levodopa is widely used in the treatment of PD, which can cross the blood–brain barrier and can be transformed into dopamine in the brain, thereby alleviating the symptoms of PD (143). However, the bioavailability of levodopa, required to ensure sufficient amounts of dopamine will reach the brain (144). Growing evidence has indicated that the gut microbiota can influence drug pharmacokinetics directly or indirectly and correspondingly bioavailability, efficacy, or adverse effects (145, 146). The microbiota, and in particular microbiome-encoded enzymes, can alter the absorption, distribution, metabolism, and elimination of drugs to consequently enhance or dampen clinical response and adverse effects. van Kessel et al. found that bacterial tyrosine decarboxylases (TDC) can efficiently convert levodopa to dopamine. In situ levels of levodopa were compromised by the relative abundance of gut bacterial TDC in the patients with PD. They also found that the higher bacterial TDC at the site of levodopa absorption—the proximal small intestine—influenced the levels of levodopa in the plasma significantly (147).

The gut microbes belonging to the genera of Bacteroides, Prevotella, Bifidobacterium, Lactobacillus, Enterococcus, Clostridium, and Ruminococcus can regulate dopaminergic activity (142). van Kessel et al. also demonstrated that the TDC genes (tdc) can be found in more than 50 Enterococcus strains (mainly E. faecalis and E. faecium) and several Staphylococcus and Lactobacillus species, while lower plasma levels of levodopa in rats treated with levodopa/carbidopa correlated with the level of bacterial tdc in the jejunum (147). They also found important associations between gut bacterial tdc-gene abundance and anti-PD medication (147). In addition, reduced levodopa availability is found in H. pylori-positive PD patients, while eradication of H. pylori improves levodopa bioavailability and motor control. These findings may be explained by the observation that H. pylori can bind levodopa in vitro via surface adhesins (148). All these findings imply that the regulation of neurotransmitters with the specific bacteria from the gut microbiota will be a useful therapeutic strategy for PD treatment.

GUT MICROBIOTA AS A THERAPEUTIC TARGET FOR PD

As mentioned above, the gut microbiota is intimately connected to the occurrence, development and progression of PD, especially in the early stages. A better understanding of the microbiota–gut–brain axis in PD can provide an opportunity to monitor an individual's health by manipulating the gut microbiota composition. Several approaches like administration of probiotics, psychobiotics, prebiotics, synbiotics, postbiotics, FMT, dietary modifications and Chinese medicines have been tried to mitigate the dysbiosis-induced ill effects and alleviate PD progression (**Figure 1**).

Probiotics, Psychobiotics, Prebiotics, Synbiotics and Postbiotics

Probiotics are live microorganisms which when administered in sufficient amounts confer a health benefit on the host. Psychobiotics are a special class of probiotics that deliver mental health benefits to individuals (149). Psychobiotics are currently being investigated as direct and/or adjunctive therapies for psychiatric and neurodevelopmental disorders and possibly for neurodegenerative disease, and they may emerge as new therapeutic options in the clinical management of brain disorders. Prebiotics are mostly fibers that are non-digestible

food ingredients that beneficially affect the host's health by selectively stimulating the growth and/or activity of some genera such as Lactobacilli and Bifidobacteria (150). Synbiotics are the combination of prebiotics and probiotics, wherein the prebiotic component selectively favors a probiotic strain. Postbiotics are functional bioactive compounds, secreted by live bacteria or released after bacterial lysis, such as non-viable microbial cells, cell walls, lysates, fractions, secretions, components and metabolites (eg, SCFAs acetate, propionate, and butyrate) that endows healthiness to the host like live probiotic cells when received in adequate amount (151).

As mentioned earlier, lactic acid bacteria such as Bifidobacterium, Lactobacillus, and Streptococcus species, are by and large the commonly used probiotic strains in clinical practice. Previous study has found that the combination of L. acidophilus and B. infantis can alleviate the symptoms of bloating and abdominal pain in PD patients (152), while one probiotic mixture with four strains including B. bifidum, L. reuteri, L. acidophilus, and L. fermentum can decrease the movement disorders society-unified PD rating scale (MDS-UPDRS) scores (153). Lu et al. demonstrated that L. plantarum PS128 supplementation for 12 weeks with regular anti-parkinsonian medication improved the UPDRS motor score and QOL of PD patients (154). Other probiotic strains or mixtures can also help to relieve gastrointestinal dysfunctions such as constipation and defecation habits in PD patients (155–158). Of course, the precise mechanisms of probiotics against PD, although not yet clear, are most likely via multiple routes, i.e., oxidative stress, inflammatory and anti-inflammatory pathways, as well as apoptosis.

Several studies have provided important clues regarding the mechanism of action of probiotics in parkinsonism mouse models. Recently, L. plantarum DP189 was found to be an effective psychobiotic to reduce α-synuclein aggravation in MPTP-induced PD mice via regulating oxidative damage, inflammation, and gut microbiota dysfunction (120). One widely studied L. plantarum strain—PS128—can suppress glial cell hyperactivation, attenuate MPTP-induced oxidative stress and neuroinflammation, modulate the gut microbiota, promote intestinal motility and mucin production, and alleviate motor deficits and neurotoxicity in mouse models of PD (159–161). In germ-free mice, PS128 can increase the levels of both serotonin and dopamine in the striatum and improve anxiety-like behaviors (162). In a murine model of PD, selected lactic acid bacteria mixture including L. plantarum CRL 2130 (a riboflavin producer), S. thermophilus CRL 807 (an immunomodulatory strain), and S. thermophilus CRL 808 (a folate producer) improved motor behavior and neuroinflammation in PD (163). Another Lactobacillus strain, namely L. salivarius AP-32, could enhance the activity of host antioxidant enzymes via direct and indirect modes of action in a rat model of PD (127). Similarly, L.rhamnosus HA-114 treatment improved hippocampal-dependent cognition in a PD model (164).

Srivastav et al. found that a probiotic mixture containing LGG, B. animalis lactis, and L. acidophilus increases the butyrate level, and subsequently rescues the nigral dopaminergic neurons from MPTP and rotenone-induced neurotoxicity (165). Ishii et al. demonstrated that oral supplementation of probiotic B. breve strain A1 can improve facilitation of hippocampal memory extinction via restoration of aberrant higher induction of neuropsin in a PD mouse model (166). Beyond the commonly used probiotics, our previous study also found that probiotic Clostridium butyricum could improve motor deficits, dopaminergic neuron loss, synaptic dysfunction, and microglia activation in a PD mouse model via the gut microbiota–GLP-1 pathway (19). The probiotic metabolites such as butyrate, also referred to as postbiotics, exert protective effects against PD in mice via stimulation of colonic glucagon like peptide-1 secretion (71). Furthermore, Hsieh et al. showed that long-term administration of probiotics has neuroprotective effects on dopamine neurons and further attenuates the deterioration of motor dysfunctions in MitoPark PD mice (167). Indeed, the benefit of the probiotics or psychobiotics for PD treatment is eminent, which can be used as an adjunct therapeutic option for PD. However, the selected probiotic strains, adequate doses, and duration of administration influences the efficacy for PD treatment (168). Future studies, especially mechanistic explorations and randomized controlled trials (RCT), will likely provide adequate evidence for PD treatment, which will promote their future translational and clinical applications.

Prebiotics, mainly dietary fibers, have been used to mitigate various diseases such as gastrointestinal dysfunction and allergic disorders (169). To date, no studies have evaluated the effects of prebiotics in PD patients alone; however, the success of sodium oligomannate (GV-971) for mild-to-moderate AD, which targets the gut microbiota, has provided a promising avenue for mining prebiotics for PD (170, 171). The decreased SCFAs-producing bacteria in PD can be rectified by prebiotic fibers and in turn impact the regulation of inflammatory processes and intestinal barrier integrity. Of course, direct oral supplementation of butyrate leads to an increase in plasma concentration that may well result in direct actions in the brain (172). A study examining β-galacto-oligosaccharides (GOS), fructooligosaccharide (FOS), or placebo in a rat model reported alterations in pivotal receptors for synaptic plasticity and memory function (173). This finding suggested that prebiotics may play a role in the neurological preservation of the CNS. The combination of prebiotics with probiotics, i.e., synbiotics, may efficiently restore the eubiosis of gut microbiota and improve gastrointestinal functions, which can be beneficial for PD treatment.

Recently, Liu et al. found that polymannuronic acid plus L. rhamnosus GG as a novel synbiotic promoted their individual neuroprotection against PD, which could therefore be one of the ideal oral agents for PD therapy (174). An RCT found that the consumption of a fermented milk containing multiple probiotic strains and prebiotic fiber was superior to placebo in improving constipation in patients with PD (156). Recently, postbiotics have drawn attention because of their immunomodulatory, anti-inflammatory, anti-obesogenic, anti-proliferative, anti-hypertensive, hypocholesterolemic, and antioxidant activities (175), although the exact mechanisms have not been fully elucidated. Among these important postbiotics, SCFAs, were

found to be related with the occurrence and development of PD. Previous studies have found that the administration of one dominant SCFA butyrate in PD animal models was reported to ameliorate motor impairment and dopamine deficiency, and inhibit neuroinflammation (71, 176, 177). These findings prove that butyrate might act as a potential therapy for PD patients (178). Thus, the roles of probiotics, psychobiotics, prebiotics, synbiotics and postbiotics reveal their potential therapeutic value for management of PD. Further studies, especially well-designed RCTs, are needed in more populations to determine the optimum formulae, efficacy, treatment modalities, treatment duration, and side effects of these agents.

FMT

FMT has been regarded as a more comprehensive method to reconstruct the gut microbiota, by transplanting gut microbiota of healthy donors into patients' intestines (117). This therapy can be traced back to 1700 years when it was used for the first time, when a Chinese medical scientist named Ge Hong treated patients with severe diarrhea or gastroenteritis (179). Gut dysbiosis mediates the progression of PD, suggesting that the restoration of gut microbiota may be an emerging effective therapeutic option for PD. The exploration of FMT as an effective therapeutic strategy in PD treatment has garnered considerable attention. At present, the application of FMT for PD treatment is still at the initial stage, and only a few case studies and small-sample studies have found some clinical efficacy of FMT. Kuai et al. found that FMT can alleviate the PD patient's constipation symptoms and improve their motor and non-motor symptoms (117). One case study reported that FMT can significantly reduce the short-term constipation and tremor of lower limbs in patients with PD in China (180). Another case report showed that FMT via colonoscopy resulted in improvement of PD motor and non-motor symptoms at 6 months, including constipation (181). A large descriptive cohort study of 2010 patients who underwent FMT and received follow-up for more than 3 months found that FMT is a safe and effective method for the treatment of gastrointestinal dysfunction (182), and another study conducted by Li et al. reported similar results (183). Xue et al. showed that 15 PD patients who received FMT (10 patients via colonoscopy and five via nasointestinal route) reported improved sleep state, quality of life, anxiety and depression, and motor symptoms at 1 and 3 months of follow-up (184). However, the long-term curative effect of FMT for PD is still unstable, which might be associated with the donor's fecal microbiota composition, delivery route, and persistence of microbiota reconstruction after FMT. Animal experiments also suggest that FMT is helpful for treating PD. The possible mechanisms of FMT are manipulation of gut microbial composition, gut barrier fortification, pathogen suppression, and immunomodulation. Sun et al. found that FMT from normal mice donors into the PD recipients can mitigate gut microbial dysbiosis, which can increase the levels of Firmicutes and Clostridiales and reduce Proteobacteria, Turicibacterales, and Enterobacteriales (107). In addition, FMT can alleviate physical dysfunction, boost the levels of striatal serotonin and dopamine, and inhibit neuroinflammation in PD mice. Sampson et al. found that colonization of ASO mice with microbiota from PD-affected patients enhances motor dysfunction and increase microglia activation compared to microbiota transplants from healthy human donors (100). A recent study by Zhao et al. showed that FMT treatment can correct the gut microbiota dysbiosis and improve symptoms in a rotenone-induced PD mouse model, wherein suppression of inflammation mediated by the LPS-TLR4 signaling pathway both in the gut and the brain possibly plays a significant role (106). Thus, FMT has great potential as a therapeutic modality for PD in the future. However, certain adverse events of FMT such as infection and sepsis, transmission of enteric pathogens, bleeding, cytomegalovirus reactivation, and pneumonia should not be ignored (185). There is a need for high-quality clinical trials with larger sample sizes to gather enough clinical evidence so that FMT can qualify for wider clinical application in PD.

Diet Modifications

A growing body of epidemiological studies have reported that diet affects (positively or negatively) the onset of neurodegenerative disorders including PD. The amount, type, and balance of dietary macronutrients (carbohydrates, proteins, and fats); high consumption of vegetables, fruits, and omega-3 fatty acids; and healthy diet patterns such as the Mediterranean diet may have a great neuroprotective influence on PD (186). One systemic review of 64 studies found that the Mediterranean diet, high in fiber and polyphenols, is related to a lower risk of PD onset (187), while the Western diet, low in fiber, may correlate with an increased incidence of PD and exacerbates the severity of PD (188). O'Keefe et al. also demonstrated that westernized diet is associated with the morbidity and mortality of westernized diseases (189). Barichella et al. found that higher caloric intake of macronutrients and micronutrients correlate with worse PD-related symptoms (190); whereas, calorie restriction or dietary restriction activate key pathways that might be important for preventing or slowing down the progression of PD (191). Zapała et al. revealed significantly higher intake of margarine and red meat in the patients with PD relative to healthy controls (192). In contrast, the specific components of the Mediterranean diet such as fresh fruits and vegetables, nuts and other dried fruits, olive oil, wine, and spices are the reason of this positive effect (187). Consumption of flavonoid-rich foods and Polyunsaturated fatty acids can reduce the risk of developing PD. Thus, the types of diet may be one of the more critical triggers or therapy for PD.

Diet can directly or indirectly impact health via various mechanisms. Recent mounting evidence suggests that the effect of diet on brain health is not because of a diet-induced inflammatory response, rather because of the effect of the composition of the diet on the gut microbiome (193). Previous studies have found that diet may be the single greatest factor determining the structure and metabolic function of the gut microbiota (189, 194). Wu et al. demonstrated that long-term dietary patterns are linked to gut microbial enterotypes (194). An international review has provided irrefutable evidence from around the world that the human microbiome can be modified by dietary changes in children and adults (195). Generally, a healthy diet such as a Mediterranean diet can increase beneficial

gut bacteria, which can correct the gut dysbiosis in PD and alleviate parkinsonism symptoms. High dietary fiber or carbohydrate polymers in the Mediterranean diet can be utilized by the gut microbiota to release metabolites such as SCFAs that have a beneficial effect on PD as they increase the motility of the gastrointestinal tract by modulating ENS activity.

Ketogenic diet, characterized by low carbohydrate and high fat with an adequate protein, is receiving acceptance as a potential therapy for PD (196). Shaafi et al. observed a beneficial influence of ketogenic diet on the motor function in a PD rat model (197). Ketogenic diet causes an increase in SCFAs and a decrease in γ-glutamyl amino acid by altering specific microbial diversity. Ang et al. found that ketogenic diets can alter the gut microbiota resulting in decreased intestinal Th17 cells, which contribute to a reduction in gastrointestinal inflammation (196, 198). However, no study has yet explored the effects of ketogenic diet on gut microbiome in PD patients (199).Moreover, high dietary intake of ω-3 polyunsaturated fatty acids can influence the gut microbiota composition, which have anti-inflammatory properties that can reduce oxidative stress and therefore reduce α-synuclein accumulation (200). An animal study also demonstrated that supplementation of ω-3 fatty acids is associated with restoration of disturbed gut microbiota caused by early-life stress (201). The ω-3 fatty acids deficient-diet lead to increased fear-induced freezing behavior, decreased sociability, and increased depressive behavior in the offspring when they became adults. In addition, coffee and caffeine in the diet have also been consistently correlated with decreased risk of PD, which can counteract shifts in the Firmicutes/Bacteroides ratio, resulting from a Western diet (202). Other studies have mentioned that the beneficial effects of caffeine in reducing PD risk may be associated with the alterations of the gut microbiota (105, 203).

Calorie restriction and fasting-mimicking diet show some neuroprotective effects against PD via increasing β-hydroxybutyrate, fibroblast growth factor 21, and ghrelin levels, which may be a result of changes in the composition of the gut microbiome (204, 205). Cox et al. found that calorie restriction slows age-related microbiota changes (reducing the level of Bacteroides) in an AD model in female mice (206) and confers improved long-term rehabilitation of ischemic stroke via gut microbiota (207). Calorie restriction selectively enriches Lactobacillus-predominant microbial communities and suppresses the expression of core microbial genes related to LPS biosynthesis, in addition to reducing LPS levels and systemic inflammation. In one recent study, Zhou et al. showed that FMT from normal mice with fasting-mimicking diet treatment into antibiotic-pretreated PD mice recovered the latter's motor function, alleviated the loss of dopaminergic neurons in the substantia nigra pars compacta, and normalized dopamine and 5-hydroxytryptamine levels in the mice striatum, suggesting that its neuroprotective effects may be mediated by reshaping the gut microbiota to modulate microbial dysbiosis. The most striking findings from that study are that a fasting-mimicking diet can be a novel means of preventing and treating PD by promoting a favorable gut microbiota composition and metabolites, which suggests that gut microbiota is the mediator for the diet-microbiota–gut–brain axis (208). However, whether diet modifications can delay the progression of PD from the prodromal phase to the overt motor phase, and whether diet modifications can modify disease course, disease progression, and response to levodopa treatment in those who suffer from motor symptoms still need further exploration.

Although diet modifications or nutritional intervention cannot directly prevent or delay the progression of PD, it can influence both the microbiota–gut–brain axis by modifying the microbiota composition and the neuronal functions of the ENS and CNS to ameliorate the progression of PD pathogenesis (209). Clarifying these roles of dietary patterns in PD will be meaningful for future personalized-dietary interventions such as microbiota-directed diets in patients with PD.

Chinese Medicines

During the past few decades, Chinese medicine has gained wider and increasing acceptance among both the public and medical professionals owing to its therapeutic efficacy for many conditions including PD. With the advantages of low cost, high safety, and high biological activity, Chinese medicine has great advantages in the prevention and treatment of PD. Traditional Chinese medicines, mainly Chinese herbs, have shown potential clinical efficacy in attenuating the progression of PD. Clinical studies have shown that Chinese herb formulas as adjuncts improved both motor and non-motor symptoms simultaneously, and reduced the dose of dopaminergic drugs and occurrence of dyskinesia, which suggested the neuroprotective roles of these herbs for PD (210). Generally, the neuroprotective effects of Chinese herbs are highly depended on mixed formulas, not on a single active constituent. To date, several constituents of Chinese herbs including resveratrol, curcumin, and ginsenoside have reported neuroprotective function (211). Growing evidence suggests that Chinese herbs and herbal extracts may help the recovery of dopamine neurons and have a positive effect on ameliorating PD in animal models (212).

Chinese medicine can combat PD through multiple pathways such as anti-inflammatory anti-oxidant pathways, alleviate mitochondrial dysfunction, regulate autophagy, inhibit endoplasmic reticulum stress, and modulate gut microbiota. Among these pharmacological mechanisms, regulating the gut microbiota has emerged as a new avenue to understanding traditional Chinese medicines for PD treatment. The interactions between gut microbiota and Chinese medicines can influence the therapeutic outcomes for PD, as gut microbiota can metabolize Chinese medicines to produce new absorbable active small molecules which have active pharmacological effects, while Chinese medicines can regulate the composition of gut microbiota and its metabolites (213, 214). Although there are no large-scale cohort studies and mechanistic research on Chinese medicine, gut microbiota, and PD, Chinese medicine with multiple benefits endowed by the gut microbiota has made it a potential therapeutic approach for prevention and treatment of PD and other neurodegenerative diseases.

PERSPECTIVES

The gut microbiota is one of the important factors involved in maintaining host health and disease. Mounting evidence has suggested that gut dysbiosis is associated with the development and progression of PD. The acceptance of gut-origin hypothesis has highlighted the importance of gut microbiota in the prodromal stage of PD, which is often 10–20 years before the onset of motor symptoms. The dysbiotic gut microbiota (including altered microbial metabolites) may play crucial roles in the occurrence of PD via various mechanisms such as increased intestinal permeability, aggravated intestinal inflammation and neuroinflammation, abnormal aggregation of α-synuclein fibrils, imbalanced oxidative stress, and decreased neurotransmitters production. Although the cause-effect links between gut microbiota and PD remain unclear, emerging evidence from PD animal model studies support that the dysbiotic gut microbiota can aggravate PD pathology, while re-establishment of the gut microbiota can delay or correct the onset of PD. This suggested that the gut microbiota can be considered as a diagnostic tool and therapeutic target for PD. Thus, novel therapeutic options aimed at modifying the gut microbiota composition and modulation of microbiota–gut–brain axis using probiotics, psychobiotics, prebiotics, synbiotics, postbiotics, FMT, dietary modifications, and Chinese medicines can influence the initial step in the cascade of neurodegeneration in PD, representing a forward-looking approach for PD. However, there is still a long way to go before a cure for PD can be discovered. Future studies are essential to further elucidate the cause-effect relationship between gut microbiota and PD, improved PD therapeutic and diagnostic options, disease progression tracking, and patient stratification capabilities to deliver personalized treatment and optimize clinical trial designs.

AUTHOR CONTRIBUTIONS

ZL, MZ, YY, XL, XY, YC, LZ, and FC discussed the contents, wrote, reviewed, and edited the manuscript. The final manuscript was read and approved by all authors.

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Improvement Effect of *Bifidobacterium animalis* subsp. *lactis* MH-02 in Patients Receiving Resection of Colorectal Polyps: A Randomized, Double-Blind, Placebo-Controlled Trial

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Background: Postoperative symptoms, bowel dysfunction and recurrence are common problems after resection of colorectal polyps. We aimed to evaluate the efficacy of *Bifidobacterium* in the postoperative patients.

Methods: In this single-center, randomized, double-blind, placebo-controlled trial, adults (≥ 18 years) undergoing endoscopic resection of colorectal polyps were treated with probiotics (*Bifidobacterium animalis* subsp. *lactis* MH-02, 2×10^9 colony-forming units per packet) or placebo once daily for 7 days. The primary clinical endpoint was a reduction in the mean total postoperative symptoms score within 7 days postoperatively. Secondary clinical endpoints were the single symptom scores, time to recovery of bowel function, and changes in the intestinal microbiota. This study is registered with the number ChiCTR2100046687.

Results: A total of 100 individuals were included (48 in probiotic group and 52 in placebo group). No difference was seen in the mean scores between the two groups (0.29 vs. 0.43, P = 0.246). Colorectal polyps size (P = 0.008) and preoperative symptoms (P = 0.032) were influential factors for the primary endpoint. Besides, MH-02 alleviated difficult defecation (P = 0.045), and reduced the time to recovery of bowel function (P = 0.032). High-throughput analysis showed that MH-02 can help restore the diversity of intestinal microbiota, and increased the relative abundance of *Bifidobacterium*, *Roseburia*, *Gemmiger*, *Blautia* and *Ruminococcus*, while reduced the relative abundance of *Clostridium* at genus level (P < 0.05).

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Conclusion: In this prospective trial, MH-02 showed efficacy in patients with resection of colorectal polyps, particularly in the recovery of bowel function, and the changes in the intestinal microbiota may provide evidence for further exploration of the therapeutic mechanisms.

Keywords: *Bifidobacterium*, MH-02, colorectal polyps, postoperative symptoms, intestinal microbiota

INTRODUCTION

Colorectal polyps are a common intestinal disease characterized mainly by protruding masses on the mucosal surface of the colorectum (1). It occurred more often in people over the age of 40, and the prevalence in the Chinese population is as high as about 20% (2). Although colorectal polyps are considered as benign lesions, certain specific pathological types such as adenoma can develop into colorectal cancer (3). Endoscopic resection of colorectal polyps is an early preventive measure for colorectal cancer (4), but the procedure often results in the onset of postoperative complications such as bleeding, abdominal pain and bloating (5), accompanied by a high recurrence rate (6). Thus, there is an urgent need to find agents that reduce postoperative complications and recurrence of colorectal polyps.

Intestinal microbiota is a symbiotic ecosystem containing trillions of bacteria, which plays a key role in human health (7). The dysbiosis of intestinal microbiota is strongly associated with various diseases such as inflammatory bowel disease, diabetes, chronic kidney disease, and tumor (8). A study that enrolled 780 individuals showed that the relative abundance of *Bilophila*, *Desulfovibrio* and *Mogibacterium* was significantly higher in patients with adenomatous polyps (9), suggesting a crucial role of the intestinal microbiota in the development of colorectal polyps. Resection of colorectal polyps requires a bowel preparation in which flushing of large amounts of fluid and disruption of the anaerobic environment can lead to severe alterations in the intestinal microbiota, especially the reduction of Bacteroidetes and Firmicutes (10). Moreover, injury to the intestinal mucosa during resection of multiple polyps can cause varying degrees of mucosal inflammation (11), which may exacerbate intestinal microbiota dysbiosis and lead to abdominal symptoms. In addition, it has been shown that the intestinal microbiota composition didn't change significantly 3 months after colorectal polypectomy, and this preoperative-like intestinal ecology may be responsible for the recurrence of colorectal polyps (12). Therefore, the resection of colorectal polyps may lead to a severe imbalance of intestinal microbiota for a short period of time, which may result in the onset of symptoms such as abdominal pain and bloating, while the long-term effects are not significant.

Probiotics are live microorganisms considered to be beneficial to the host if consumed sufficiently (13). As an essential member of probiotics, *Bifidobacterium*, a Gram-positive anaerobic bacterium, has the ability to immunomodulate, inhibit pathogens, produce bacteriocins and maintain intestinal microbiota homeostasis (14). An *in vitro* experiment demonstrated that *Bifidobacterium* could inhibit the proliferation of several human colorectal cancer cell lines (15), suggesting that it has tumor suppressive effects. Our previous studies showed that oral *Bifidobacterium* reduced gastrointestinal symptoms, decreased inflammation and promoted restoration of intestinal microbiota diversity in patients after gastric cancer surgery (16). Another study showed that administration of *Bifidobacterium* after colorectal cancer surgery also reduced levels of inflammatory factors such as TNF-α, IL-6, IL-10, IL-12, IL-17A, IL-17C and IL-22 (17). Despite the significant role of *Bifidobacterium* in the prevention and adjuvant treatment of tumor, clinical trials on the effect of *Bifidobacterium* in patients receiving resection of multiple colorectal polyps have not been seen.

In this study, *Bifidobacterium animalis* subsp. *lactis* MH-02 was used to evaluate its effect on the symptoms and recovery of intestinal function in patients receiving resection of colorectal polyps, and high-throughput sequencing was performed to evaluate the effect of MH-02 on postoperative intestinal microbiota, in order to provide a scientific basis for the application of probiotics after resection of colorectal polyps.

MATERIALS AND METHODS

Study Design and Participants

This study was a single-center, double-blind, parallel group design, placebo-controlled trial. Patients were recruited from the gastroenterology inpatient unit of the First Hospital of Nanchang. All patients included in this trial underwent high-quality bowel preparation under professional guidance into the day before the colonoscopy procedure. Adult patients (≥ 18 years) diagnosed postoperatively with multiple colorectal polyps (at least 3) and resected endoscopically met inclusion criteria. Patients who had undergone abdominal surgery, had significant malignant lesions or inflammatory bowel disease under colonoscopy, had poor general condition, or had a history of allergy to drugs or probiotics were excluded. Patients who had been taking antibiotics, immunosuppressants or probiotics for the last three months were also excluded.

All patients participating in this study signed informed consent. This study was supervised by the Ethics Committee of the First Hospital of Nanchang (No. KY2021040) and registered in the Chinese Clinical Trial Registry with the registration number ChiCTR2100046687. All surgeons had rich experience in endoscopic operation. Patient demographic data, surgical information, past medical history, postoperative symptoms, defecation and laxative use were recorded, and the largest polyp diameter and highest pathological grade were recorded for multiple polyps statistics. All clinical data collection was done at the First Hospital of Nanchang.

Randomization and Masking

Participants were assigned 1:1 to either the probiotic group (P-Bb) or the placebo group (P-N) using random number table method by a non-participating staff member who provided the probiotics to the investigator after patient enrollment. There were no significant differences in packaging, color, or odor between probiotics and placebo, thus ensuring a double-blind status between investigator and patient. The staff member and the investigator remained masked until the end of the experiment.

Trial Protocol

Patients who met the eligibility criteria were randomly assigned to either the probiotic group (P-Bb) or the placebo group (P-N). Patients enrolled in the group started eating (light and easily digestible food such as thin rice and crumbled noodles) at 4 hours postoperatively and were asked to take the probiotic preparation we provided continuously for 7 days postoperatively, during which spicy and stimulating diet and alcohol consumption were prohibited. The experimental probiotics was a mixture of MH-02 and maltodextrin with 2×10^9 colony-forming units per packet of live bacteria. The placebo contained only the same grams of maltodextrin. MH-02 was provided by Harbin Meihua Biotechnology Co, Ltd, Harbin, Heilongjiang, PR China, and was stored in a refrigerator at 4°C. Probiotics and placebo are both taken one packet per day. Treatment compliance of patients was obtained by counting the number of pouches used, and good compliance was defined as using more than 80% after 7 days.

Patients were evaluated daily by a trained physician using a questionnaire in the postoperative period. The questionnaire included 3 common symptoms after colorectal multiple polypectomy: abdominal pain, bloating, and dyspareunia. Other symptoms such as dizziness, diarrhea, and hematochezia were not included in the analysis because of their short duration of occurrence or low incidence. The above symptoms were scored using a 4-point Likert scale (0-3, 0 = 'symptom absent', 1 = 'mild ', 2 = 'moderate' and 3 = 'severe'). The time when the patient started to experience self-initiated bowel movement, the use of laxatives and adverse reactions were also recorded. Stool samples were collected 5-7 days postoperatively in centrifuge tubes containing 30% sterilized glycerol and stored at -80°C in a refrigerator pending sequencing analysis.

Outcomes

The primary clinical endpoint was the improvement in patients' postoperative symptoms (including abdominal pain, bloating, and difficult defecation), as demonstrated by the reduction in the mean total postoperative symptoms scores between the P-Bb group and the P-N group within 7 days after the procedure. Patients were interviewed face-to-face or by telephone using a questionnaire, and were scored according to severity for any of these symptoms. Patients with multiple symptoms at the same time were scored in parallel.

Secondary clinical endpoints were single symptom scores, time to recovery of bowel function, the proportion of patients who had difficult defecation, and the use of laxatives. Time to recovery of bowel function was defined as the number of days since patients first experienced self-initiated bowel movement (excluding bowel movements that occurred after laxative use, which was recorded as 7 days if no self-initiated bowel movement occurred for more than 7 days) (18). The secondary biological endpoint were the changes in intestinal microbiota, as demonstrated by the difference in αdiversity, β-diversity and species composition between the two groups, as well as the analysis of the microbiota with differences in species composition compared to normal subjects.

DNA Extraction and High-Throughput Sequencing

Methods were provided by the technicians at Personal Biotechnology, Co., Ltd. (Shanghai, China). DNA kits were used to extract the bacterial DNA from the collected stool samples. The primer sets 338F (5′- ACTCCTACGGGAGGCAGCA -3′) and 806R (5′- GGACTACHVGGGTWTCTAAT -3′) were used to amplify the hypervariable V3V4 region of the 16S rRNA gene. The PCR-amplified products were double-ended sequenced using the Illumina MiSeq platform. ASV/OTU signature sequences were obtained using the DADA2 method, followed by processing using Quantitative Insights into Microbial Ecology (QIIME). The taxonomic classification was performed using the Greengenes database v13.8 (19). The samples we collected (group P-Bb, P-N) were analyzed for α-diversity, β-diversity, and species differences. We also collected high-throughput sequencing results of 20 healthy subjects (group C) in the NCBI public database (PRJNA706061) and performed species composition analysis with both P-Bb and P-N. Group C was described as preoperatively collected stools based on the literature, and was confirmed as a healthy population by colonoscopy (20).

Data Analysis

Since there is no authoritative published analysis of the efficacy of MH-02 applied after resection of multiple colorectal polyps, it is impossible to make a reasonable power analysis. Based on a previous study (21), we assumed that the mean total postoperative symptom score was 1.00 for the P-Bb group and 0.70 for the P-N group, with a standard deviation of 0.5 in both groups, we needed to enroll 44 people in each group for study (power of 80% and $\alpha = 0{\cdot}05$). Considering a 10% dropout rate, we ultimately planned to enroll 48 people in each group.

Patients were analyzed based on intentional analysis for the final analysis. Missing data were imputed with last observation carried forward. Data were analyzed or charted by GraphPad Prism (v8.0) and SPSS (v22.0). Quantitative data are shown as mean ± standard deviation or median (interquartile range), and qualitative data are expressed as rates. The mean score of all symptoms and the mean score of each symptom after the procedure were analyzed by using multiple regression analysis. Covariates or factors were selected from gender, age, polyps size, number, location, technique type, and preoperative symptoms. Other outcomes were analyzed by unpaired t-test for quantitative data and Fisher's exact test or chi-square test for qualitative data. Two-sided $P < 0.05$ was used as the basis for significant

differences. Since probiotics were defined as foods rather than drugs, no Data Monitoring Committee (DMC) for clinical trials was used.

RESULTS

From June 01, 2021 to October 31, 2021, a total of 153 individuals were assessed for eligibility, with 100 individuals randomly assigned and included in the final analysis, 48 in the group P-Bb and 52 in the group P-N. During the study, 1 participant in group P-Bb failed to complete the full study due to withdrawal of consent while 3 participants in group P-N (2 withdrawal of consent, 1 lost to follow-up) (**Figure 1**). Baseline information (**Table 1**) showed no significant differences between the two groups of patients in terms of age, gender, BMI, polyps data and technique types.

Among the 100 participants included in the analysis, 74 participants had preoperative symptoms such as abdominal pain, bloating, abnormal bowel habits or others (33 in group P-Bb and 35 in group P-N), and 26 participants had no discomfort (**Supplementary Table 1**). The result of multiple regression analysis of the primary endpoint showed that there was no significant difference between the two groups in the mean total postoperative symptoms score ($P = 0.246$). Meanwhile, there were statistical differences in the effects of polyps size ($b = 0.57$, $t = 2.71$, $P = 0.008$) and preoperative symptoms ($b = 0.30$, $t = 2.18$, $P = 0.032$) on the primary endpoint. Statistical analysis of individual symptom score using this analytical model showed a difference between the two groups only for the symptom of difficult defecation ($P = 0.045$), while no difference was seen in abdominal pain and bloating (**Table 2**). In addition, there was a statistically significant difference in the days to first self-initiated bowel movement (3.62 versus 2.90, $P = 0.032$). More people in the P-N group had difficult defecation than in the P-Bb group and required the use of laxatives more frequently during the consultation ($P = 0.032$) (**Table 3**).

Finally, 85 fecal samples (41 P-Bb, 44 P-N) were collected. In α-diversity, the two groups were significantly different in Chao1 ($P < 0.01$) (**Figure 2A**), Observed species ($P < 0.01$) (**Figure 2C**), Shannon ($P < 0.05$) (**Figure 2D**). Goods coverage (**Figure 2B**) were approximately 1 for both groups. In β-diversity, the principal coordinates analysis (PCoA) (**Figure 2E**) exhibited that the microbial diversity in P-Bb group and P-N group were different. For the two groups, a clustered heat map (**Figure 2F**) was plotted by correlation of the top 20 intestinal bacteria of average abundance at the genus level, showing a higher relative abundance of some beneficial bacteria in the P-Bb group, such as *Bifidobacterium*, *Faecalibacterium*, *Dorea*, *Roseburia*, *Gemmiger Blautia*, and *Ruminococcus*. Among them, *Dorea*, *Roseburia*, *Gemmiger*, *Blautia*, and *Ruminococcus* were at the same taxonomic level in the clustering tree. The relative abundance of *Megamonas*, and *Clostridium* was higher in the P-N group.

When the two groups were compared with the healthy group (C) (**Figure 3G**), it was found that the taxonomic composition of the P-Bb group and P-N group differed significantly from the C group at the genus level. The relative abundance of *Bifidobacterium* was significantly lower in both the P-N and P-Bb groups than in the C group, but higher in the P-Bb group than in the P-N group (**Figure 3A**). Compared to C group, the relative abundance of *Ruminococcus*, *Blautia*, and *Gemmiger* was significantly reduced in P-N group, while P-Bb group was similar to C group (**Figures 3B, D, E**). *Roseburia* was also significantly reduced in P-N group compared to C group, but its relative abundance was significantly higher in P-Bb group than C group (**Figure 3C**). And the

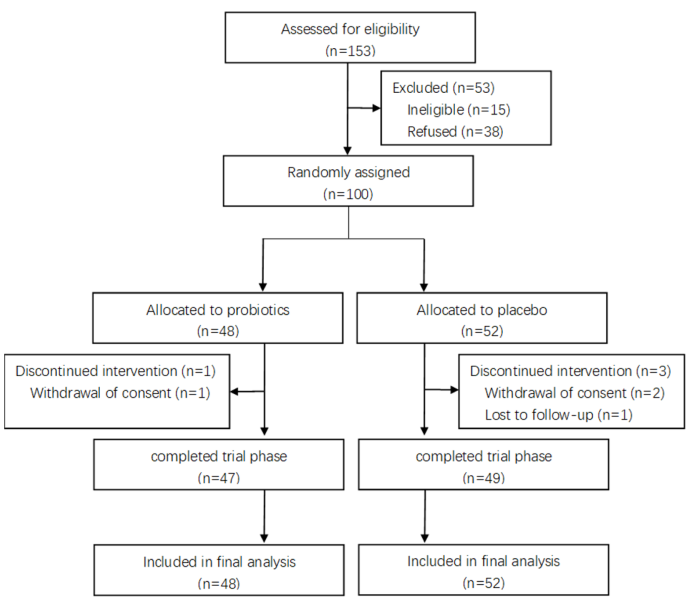

FIGURE 1 | Flowchart of the trial.

TABLE 1 | Baseline characteristics.

	P-Bb (n = 48)	P-N (n = 52)	*P*-value
Age (y) (mean ± SD)	58.67 ± 9.44	59.25 ± 11.33	0.781
Sex (female, n [%])	18 (37.50)	22 (42.31)	0.624
BMI (mean ± SD)	23.84 ± 3.33	24.03 ± 3.04	0.756
Size (mean ± SD)	0.61 ± 0.33	0.64 ± 0.34	0.614
Number (n [%])			0.858
3-9	43 (89.58)	46 (88.46)	
≥10	5 (10.42)	6 (11.54)	
Localization (n [%])			0.653
Single-site	10 (20.83)	9 (17.31)	
Multi-site	38 (79.17)	43 (82.69)	
Histology (n [%])			0.653
Inflammatory	1 (0.02)	3 (0.06)	
Hyperplastic	18 (0.38)	23 (0.44)	
Adenomatous	27 (0.56)	24 (0.46)	
Other	2 (0.04)	2 (0.04)	
Technique (n [%])			0.999
APC/EMR	46 (95.83)	49 (94.23)	
ESD	2 (4.17)	3 (5.77)	

Clostridium, which had low relative abundance in group C, was significantly higher in group P-N than in group P-Bb (**Figure 3F**).

There was no increase in adverse events with postoperative probiotics administration compared with placebo. In addition to the symptoms associated with the primary clinical endpoints described above, there was one case of hematochezia and one case of insomnia in the P-Bb group. The P-N group had one case of more severe diarrhea and two cases of hematochezia. The researchers concluded that the above symptoms may not be related to MH-02 intake and that oral MH-02 is considered to be very safe. No other serious adverse events or deaths occurred.

DISCUSSION

This prospective study showed efficacy of MH-02 in patients receiving resection of multiple colorectal polyps. Compared to placebo, MH-02 showed no significant improvement in postoperative symptoms, and among single symptoms, only difficult defecation was significantly improved. However, MH-02 allowed faster recovery of bowel function and reduced the frequency of laxative use. High-throughput analysis showed that MH-02 can help restore the diversity of intestinal microbiota, and increased the relative abundance of *Bifidobacterium*, *Roseburia*, *Gemmiger*, *Blautia* and *Ruminococcus*, while reduced the relative abundance of *Clostridium*, and the alteration of these bacteria was beneficial to health. Finally, administration of MH-02 is considered to have high acceptance and safety.

Colorectal polyps are a common and potentially dangerous intestinal disease that requires early endoscopic surgical resection (1, 4). The procedure is minimally invasive and the incidence of serious complications such as bleeding and perforation is less than 1% (22), however, minor complications such as abdominal pain and bloating occur in more than 30% of cases within 7 days after resection (5). In addition, difficult defecation is also a common symptom after surgery. Difficult defecation is the most common symptom in patients with constipation, with a prevalence of 68% in functional constipation (23). Patients are prone to constipation after gastrointestinal surgery (24), probably due to bowel preparation and surgical stress. A previous study showed that the administration of Lactobacillus acidophilus and Bifidobacterium lactis did not reduce abdominal pain and bloating in patients after colonoscopy, but the results were reversed in a subgroup analysis of preoperative symptomatic patients (21). Similarly, another study revealed that Bacillus subtilis and Streptococcus faecium, started 2 weeks before surgery, were effective in improving the onset of postcolonoscopy symptoms in patients with preoperative constipation, but the results were negative in the preoperative asymptomatic group (25). In this study, MH-02 provided no significant improvement in symptoms within 7 days after resection of multiple colorectal polyps, and performed a therapeutic effect only in difficult defecation when single symptom analysis was performed. Multiple regression analysis revealed that polyp size and preoperative symptoms were factors influencing the efficacy of MH-02 in improving postoperative symptoms. Bowel dysfunction can be commonly seen after colonoscopy (26), and it takes several days to return to

TABLE 2 | Postoperative symptoms.

Mean score - median (interquartile range)	P-Bb (n = 48)	P-N (n = 52)	*P*-value*
Total	0.29 (0.00-0.68)	0.43 (0.00-1.00)	0.246
Pain	0.00 (0.00-0.00)	0.00 (0.00-0.29)	0.968
Bloating	0.00 (0.00-0.29)	0.21 (0.00-0.57)	0.364
Difficult defecation	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.045

**All P- values are from a multiple regression analyses adjusted for the variables as the primary outcome.*

TABLE 3 | Postoperative bowel function.

	P-Bb (n = 48)	P-N (n = 52)	*P*-value
Time to recovery of bowel function (mean ± SD)	2.90 ± 1.39	3.62 ± 1.87	0.032
Difficult defecation (n [%])	4 (0.08)	12 (0.23)	0.057
Laxative use (n [%])	1 (0.02)	8 (0.15)	0.032

normal bowel habits. Postoperative constipation is the major reason affecting the recovery of bowel function. A previous study showed little effect of probiotics on intestinal function, with only subgroups showing such positive results (21). In this study, MH-02 significantly reduced the time to recovery of bowel function and reduced the incidence and severity of constipation in patients. Overall, MH-02 provided an adjuvant therapeutic effect after resection of colorectal polyps, especially in the recovery of bowel

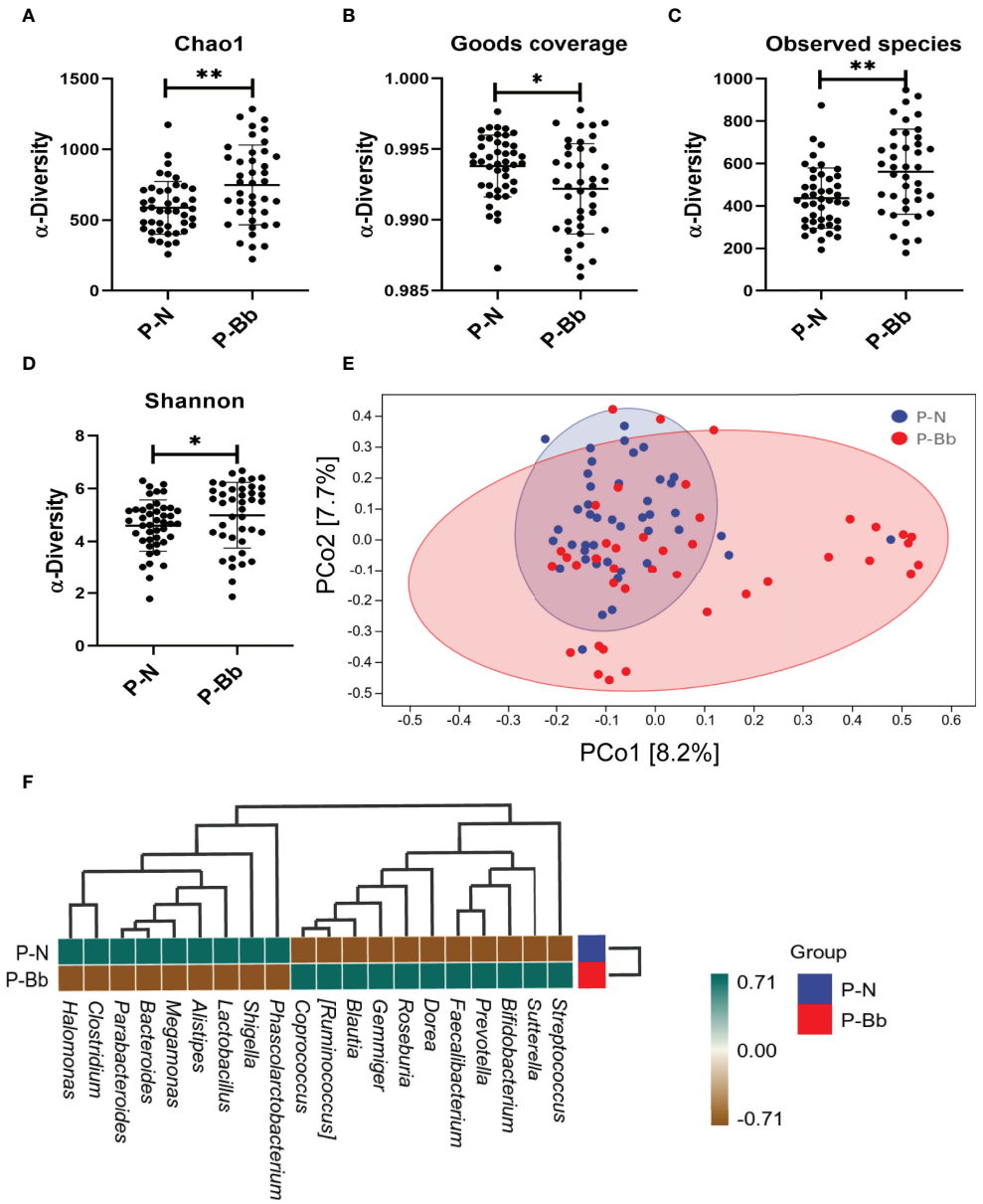

FIGURE 2 | MH-02 had an improvement effect on postoperative intestinal microbiota. Values are presented as means ± SD (41 P-Bb, 44 P-N). **(A)** The Chao1 index. **(B)** The Goods coverage index. **(C)** The Observed species index. **(D)** The Shannon index. **(E)** PCoA of β-diversity index. **(F)** The clustered heat map of P-Bb and P-N. *p < 0.05, **p < 0.01.

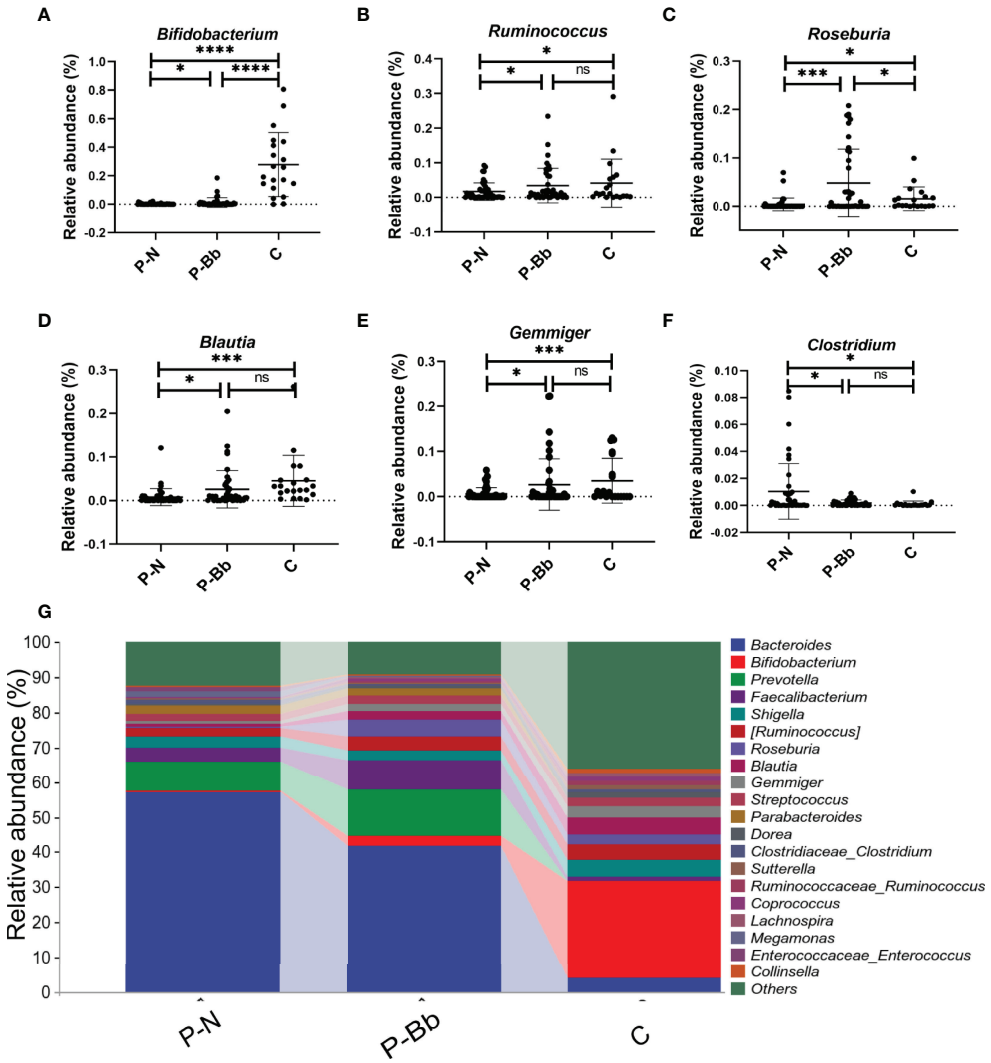

FIGURE 3 | MH-02 can help restore the majority of the postoperative intestinal microbiota towards healthy people. Values are presented as means ± SD (41 P-Bb, 44 P-N,20 C). **(A–F)** The relative abundance of *Bifidobacterium*, *Ruminococcus*, *Roseburia*, *Blautia*, *Gemmiger* and *Clostridium*. **(G)** The species composition analysis of P-Bb, P-N and C group. *p < 0.05, ***p < 0.001, ****p < 0.0001, ns, no significant.

function in patients, and there may be group differences in the therapeutic effect.

Resection of colorectal polyps may result in a severe dysbiosis of the intestinal microbiota. Bowel preparation is a key step in the gastrointestinal surgery, and our previous study showed that oral *Bifidobacterium* after bowel preparation significantly increased the diversity of the intestinal microbiota, and reduced the relative abundance of pathogenic *Acinetobacter*, while enriching the relative abundance of *Roseburia* and *Faecalibacterium* (10), suggested a critical role for *Bifidobacterium* in the recovery of intestinal microbiota. In this study, MH-02 significantly increased the αdiversity and β-diversity of the intestinal microbiota of postoperative patients. In addition, the clustering heat map showed that MH-02 could cause changes in the abundance of a variety of bacteria. *Bifidobacterium*, *Faecalibacterium*, *Dorea*, *Roseburia*, *Gemmiger*, *Blautia*, and *Ruminococcus* had higher abundance in patients taking MH-02, and these bacteria always play an active role in intestinal inflammation, immunity and tumor. *Bifidobacterium*, the main commensal flora of the intestine, has a high and stable relative abundance in the intestine of healthy adults (14), and can reduce inflammation after intestinal surgery and suppress tumors (15, 17). *Faecalibacterium* and *Roseburia* are the most important butyrate-producing bacteria in the human colon, and their presence may be associated with a reduced risk of chronic inflammation of the intestine (27, 28). Butyrate is a product of dietary fiber fermentation by bacteria and may exert tumor suppressive effects *via* pathways such as Gpr109a-butyrate signaling (29). *Dorea* is the main gas-producing bacterium in the

human intestine and may be associated with irritable bowel syndrome (30). *Ruminococcus*, one of the first stomach bacteria identified, has an important role in metabolism and has also been suggested to exert beneficial effects such as stabilizing the intestinal barrier and reducing the risk of colorectal cancer (31). The abundance of *Ruminococcus* and *Gemmiger* is negatively correlated with intestinal inflammation (32, 33). *Blautia* is widely present in the mammalian gut and considered to be a beneficial bacterium that plays a role in metabolic diseases, inflammatory diseases and biotransformation (34). Moreover, a study reported reduced abundance of *Blautia* in mucosal adherent microorganisms in patients with colorectal cancer (35). *Dorea*, *Roseburia*, *Gemmiger*, *Blautia*, and *Ruminococcus* were at the same taxonomic level in the clustering tree, indicating that these beneficial Bacteria had similar abundance in the samples and may have synergistic effects. In contrast, *Megamonas* and *Clostridium* were present in higher abundance in patients taking placebo after surgery. The abundance of *Megamonas* is significantly higher in Asian colorectal cancer population (36). *Clostridium* can produce exotoxins that become the cause of intestinal diseases, and it has been shown that specific species of *Clostridium* such as Clostridium difficile are closely associated with the development of colorectal cancer (37). However, age is also an important factor affecting the intestinal microbiota. In this experiment, the patients' ages were concentrated between 50 and 70 years, and our samples were collected within 1 week after the patients' surgery, when the intestinal microbiota disorder had not fully recovered and the probiotic intervention was the main influencing factor for this recovery process. To sum up, MH-02 can help restore intestinal microbiota balance and may provide evidence to further explain the mechanism of the effect of probiotics in patients with resection of colorectal polyps.

It is reported that 20-50% of patients with colorectal polyps are at risk of postoperative recurrence (6). Previous studies have suggested that the intestinal microbiota may be involved in the recurrence of colorectal polyps and even colorectal cancer after surgery, and that modulating the composition of the intestinal microbiota may be able to reduce recurrence outcomes (12, 38). In the present study, MH-02 altered the composition of the intestinal microbiota and changed the bacteria such as *Bifidobacterium*, *Ruminococcus*, *Roseburia*, *Blautia*, *Gemmiger* and *Clostridium* in a healthy direction. Longer time probiotics intervention is needed to determine whether it has the effect on stably altering the intestinal microbiota composition and influencing the outcome of colorectal polyp in the future.

The limitation of this study is that the homogeneity of preoperative symptoms of patients was not controlled. Preoperative symptoms are an important factor in the efficacy of probiotics, it is more appropriate to investigate people with homogeneous preoperative symptoms for relevant trials. Another limitation is that although we analyzed clinical symptoms, bowel function and changes in intestinal microbiota, direct evidence of the link among them was not explored. In addition, the sample size of this trial was estimated based on postoperative symptom scores, which are closely related to patients' preoperative symptoms, and the sample size required for the trial varies among different preoperative symptom populations, which may be the main reason for the small sample size estimate of this trial.

The strength of this trial is that we conducted a rigorous trial design, including strict inclusion and exclusion criteria, as well as detailed symptom scoring criteria. And the assessment was performed by the same highly trained person, which reduced other potential sources of variability. We also attempted to explain the possible mechanisms at the microbial level and succeeded in identifying some clue bacteria.

In conclusion, MH-02 showed efficacy in patients after resection of colorectal polyps, especially in the reduction of difficult defecation and restoration of bowel function. Meanwhile, MH-02 could help to restore the balance of intestinal microbiota, and the alteration of some bacteria may provide help to further explain its mechanism. Future studies should focus on the role of probiotics in different populations and need to further explore the mechanisms. Moreover, the effect of long-term probiotic intervention on the outcome of colorectal polyp recurrence could be investigated.

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 of the First Hospital of Nanchang. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NF: conceptualization, funding acquisition, supervision, writing review, and editing. TC: conceptualization, funding acquisition, writing review, editing, and assistance with formal analysis of data. HL: experimentation, references, original draft. KZ: experimental assistance, data formal analysis assistance. PL: experimental assistance. XX: editing assistance. YZ: experimental assistance. LG: editing assistance. LY: editing assistance. BL: experimental assistance. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.940500/full#supplementary-material

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Dietary supplementation with potassium-magnesium sulfate modulates the antioxidant capacity, immunity, and gut microbiota in weaned piglets

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The purpose of this study was to evaluate the effects of different levels of potassium magnesium sulfateon (PMS) on growth performance, diarrhea rate, intestinal morphology, antioxidant capacity, intestinal immunity, and gut microbiota in weaned piglets. A total of 216 weaned piglets were randomly divided into six dietary groups: the basal diet with 0% (CON), 0.15, 0.3, 0.45, 0.6, and 0.75% PMS. The results showed that the ADFI of 29–42 days and 1–42 days was linearly and quadratically increased by the PMS supplementation ($P < 0.05$), and significantly reduced the diarrhea rate in weaned piglets ($P < 0.05$). Moreover, dietary supplementation with PMS significantly reduced the serum adrenaline and noradrenaline levels in weaned piglets ($P < 0.05$). Furthermore, 0.3% PMS significantly increased the activity of glutathione peroxidase (GSH-Px) in the jejunum ($P < 0.05$) and tended to increase the activity of superoxide dismutase (SOD) in the jejunal mucosa of piglets ($P < 0.1$). Additionally, dietary supplementation with PMS significantly reduced the interleukin-1β (IL-1β) level in the jejunal mucosa ($P < 0.05$), and 0.3% PMS increased the serum IgM content in piglets ($P < 0.05$). Furthermore, the analysis of colonic microbiota by 16S RNA sequencing showed that the addition of PMS increased the Shannon index ($P < 0.05$) and Observed Species index ($P < 0.05$). Based on linear discriminant analysis effect size (LEfSe) and *T*-test analysis, the addition of PMS increased the relative abundance of *Ruminococcaceae* and *Peptostreptococcaceae* in the colonic digesta ($P < 0.05$). Spearman analysis showed that there was a positive correlation between intestinal GSH-Px activity and the relative abundance of *Peptostreptococcaceae*. These results showed that dietary supplementation

with PMS could improve growth performance, alleviate diarrhea incidence, and modulate the antioxidant capacity and intestinal immunity in weaned piglets, which was partially related to the significant changes in colonic microbiota composition.

KEYWORDS

potassium-magnesium sulfate, growth performance, antioxidant capacity, gut microbiota, weaned piglets

Introduction

Weaning stress caused dysfunctions in metabolism, digestion, and immune responses in piglets when suddenly facing nutritional, immunological, and psychological challenges (Upadhaya and Kim, 2021). Weaning is accompanied by intestinal injury, disorders of digestion, antioxidant system dysfunction, immune imbalance, diarrhea, and even death of piglets (Moeser et al., 2017; Cao et al., 2018; Cao et al., 2022). Hence, finding an effective nutritional strategy to prevent antioxidant and immune systems disruption in weaning piglets is important for animal husbandry.

Potassium and magnesium, as the essential major elements for animals, are used as cations to regulate the body's electrolyte balance in animals (Stone et al., 2016; Fiorentini et al., 2021). Potassium can regulate the appropriate osmotic pressure in cells and the acid-base balance of body fluids and participate in the metabolism of carbohydrates and proteins (Stone et al., 2016). The addition of potassium-containing compounds to drinking water could reduce the heat stress of animals (Ansari et al., 2020). Magnesium is involved in maintaining the stability of the nucleic acid structure, activating enzymes in the body, inhibiting nerve excitability, and participating in protein synthesis and muscle contraction (Fiorentini et al., 2021). Moreover, magnesium is an important activator of various enzyme systems in cell metabolism (Lötscher et al., 2022). Further, dietary magnesium supplementation improves lifespan and enhances antioxidant capacity in a mouse model of progeria (Villa-Bellosta, 2020). However, excessive addition of potassium and magnesium in animal diets led to renal function damage, electrolyte loss, and even hyperkalemia (Al Alawi et al., 2018; Gerhardt and Angermann, 2019). So, it remained unclear about the combination of potassium and magnesium on intestinal antioxidant capacity, immunity, and gut microbiota in weaned piglets.

Therefore, a natural compound mineral additive of potassium and magnesium (potassium-magnesium sulfate, PMS) was used to evaluate the effect of dietary supplementation with PMS on the growth performance, antioxidant capacity, intestinal immunity, and gut microbiota in weaned piglets.

Materials and methods

Animal ethics

All animal experimental protocols used in the current study were according to the Chinese guidelines for animal welfare and approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences (GAASIAS-2016-017).

Experimental animals, design, diets, and housing

A total of 216 piglets (Duroc × Landrace × Large White) weaned at 21 days with an initial average body weight of 7.53 kg, were randomly allotted into six dietary groups with six replicates of six piglets each replicate. Piglets were fed the basal diet supplemented with 0, 0.15, 0.3, 0.45, 0.6, and 0.75% PMS. The PMS products were obtained from Qinghai Lanhushancheng Bio-tech Co., Ltd. (Qinghai, China), which is composed of $K_2SO_4.MgSO_4.6H_2O$, Potassium: 21%, Magnesium: 6.5%. The potassium-magnesium sulfate ore was collected in the Qinghai Salt Lake, sorted by the screw machine, and removed the iron and plastic paper debris through a sieve. And then, the sorted potassium-magnesium sulfate ore was pulverized by a pulverizer and dried by the air-cooled dryer at a low temperature. Furthermore, the dried potassium-magnesium sulfate ore was further sorted by a 20-mesh sieve, and the particle size of potassium-magnesium sulfate ore smaller than 20 mesh was collected as the potassium-magnesium sulfate. The composition of the basal diets (**Table 1**) was formulated to meet or exceed the nutritional requirements for weaned piglets recommended by National Research Council (2012). All pigs were housed in an environmentally controlled room with slatted plastic flooring and an effective mechanical ventilation system. Each pen had two stainless feeders and four nipple drinkers. Pigs had *ad libitum* access to feed and water throughout the experimental period. The animal house temperature was controlled at 25–28°C and relative humidity was controlled at

TABLE 1 Ingredient and energy composition of weaned piglets' diets.

Item	1–28 days	29–42 days
Ingredients, %		
Corn	34.05	56.18
Expanded corn	12.00	13.00
Fermented soybean meal	10.00	10.00
Soybean meal	5.00	6.00
Expanded soybean	11.00	3.67
Fish meal	3.00	3.00
Whey powder	15.00	–
Whey protein concentrate	1.00	–
Soybean oil	1.50	1.00
White granulated sugar	2.00	2.00
Calcium citrate	1.40	–
Calcium carbonate	–	1.10
Calcium hydrogen phosphate	0.600	0.60
L-lysine hydrochloride	0.6	0.60
DL-Methionine	0.15	0.15
L-threonine	0.20	0.20
L-tryptophan	0.05	0.05
NaCl	0.30	0.30
50% Choline chloride	0.15	0.15
Premix[a]	2.00	2.00
Total	100	100
Energy and nutrient composition,[b] %		
Digestive energy	3600	3490
Crude protein	18.7	17.2
SID Lys	1.44	1.35
SID Met	0.44	0.45
SID Thr	0.85	0.83
SID Trp	0.26	0.24
Calcium	0.70	0.76
Phosphorus	0.55	0.53
Available phosphorus	0.35	0.31
Magnesium	0.94	0.64
Potassium	0.15	0.13

[a]The premix provided the following in diets: VA 4, 400 IU*kg^{-1}, VD_3 440 IU*kg^{-1}, VE 30 IU*kg^{-1}, VK 1 mg*kg^{-1}, VB_{12} 40 μg*kg^{-1}, VB_1 3 mg*kg^{-1}, VB_2 10 mg*kg^{-1}, Nicotinic acid 40 mg*kg^{-1}, D-pantothenic acid 15 mg*kg^{-1}, Folic acid 1 mg*kg^{-1}, VB_6 8 mg*kg^{-1}, Biotin 0.08 mg*kg^{-1}, ($FeSO_4 \bullet H_2O$) 120 mg*kg^{-1}, ($CuSO_4 \bullet 5H_2O$) 16 mg*kg^{-1}, ($MnSO_4 \bullet H_2O$) 70 mg*kg^{-1}, ($ZnSO_4 \bullet H_2O$) 120 mg*kg^{-1}, ($CaI_2 \bullet O_6$) 0.7 mg*kg^{-1}, (Na_2SeO_3) 0.48 mg*kg^{-1}.

[b]Nutrient levels were calculated values, except that the digestible energy, crude protein, and available phosphorus were determined values.

55–65%. There were no antimicrobial and anticoccidial drugs used during the trial.

Growth performance and diarrhea rate

Pigs were weighed individually on days 0, 24, and 42. The average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F: G) were recorded and calculated. The Feed:Gain ratio (F:G) = (Feed intake)/(Weight gain). The diarrhea rate was recorded every day during this experiment and quantified according to previous methods (Tang et al., 2021).

Sample treatment and collection

Piglets were weighed after fasting for 12 h at the end of the days 14, 28, and 42. After weighing at day 42, one piglet from each replicate was selected to slaughter. The blood was collected by the anterior vena cava. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature for 30 min, and collect the serum by centrifuging at 3,000 $\times g$ for 10 min in a refrigerated centrifuge. Subsequently, the duodenum, jejunum, ileum, cecum, and colon were quickly removed. About 2 cm segments of duodenum, jejunum, and ileum were immediately isolated and then fixed in 4% neutral paraformaldehyde solution. The mucosal samples from the jejunum were harvested by gently scraping with a glass slide and rapidly frozen in liquid nitrogen and stored at −80°C for further determinations. The colonic digesta samples were rapidly frozen in liquid nitrogen and stored at −80°C for the determination of gut microbiota composition and diversity.

Intestinal histological analysis

The specimens of the intestinal segments were embedded in paraffin and cut into 4-mm thickness sections for H&E staining according to the methods previously described (Yang et al., 2014). The tissue sections were measured under a microscope using an image processing and analysis system (Leica, Germany). The Program Image-pro Plus 6 (Media Cybernetics, Inc., GA, United States) was used to determine the villus height (VH), crypt depth (CD), and villus height-to-crypt depth (VH:CD) ratio. At least 25 villus samples with intact lamina propria were blindly selected and measured.

Analysis of antioxidant capacity in serum and intestine

Commercial kits were used to analyze the antioxidant capacity of serum and intestine mucosa, including activities of superoxide dismutase (SOD), total antioxidant capacity (T-AOC), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) content according to the manufacturer's protocols (Nanjing Jiancheng Biotechnology, Nanjing, China). Reactive oxygen species

(ROS) levels in serum and jejunum mucosa were determined according to the manufacturer's instructions (R&D Systems, MN, United States).

Analysis of immune factors in serum and intestine

The levels of immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), secretory immunoglobulin A (sIgA), interleukin-1β (IL-1β), tumor inflammatory factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-10 (IL-10) were determined according to the manufacturer's instructions (Enzyme-linked Biotechnology Co. Ltd., Shanghai, China), and measured with a multi-functional microplate reader (SynergyTM H1, BioTek, United States).

16S rRNA-based microbiota analysis

Total DNA was extracted from colonic digesta using a commercial kit according to the manufacturer's instructions (Omega Bio-Tek, Norcross, GA, United States). The DNA concentration was checked by NanoDrop 2000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, NC, United States), and the DNA quality was monitored by 1% agarose gel electrophoresis. The V3-V4 variable region of the 16S rRNA gene was amplified using the specific primers (341 F:5′-CCTAYGGGRBGCASCAG-3; 806 R:5′-GGACTACNNGGGTATCTAAT-3′). The PCR products were detected by agarose gel electrophoresis, and the 300 bp amplicon was cleaned and subjected to 16S rDNA sequencing on an Illumina HiSeq 2500 PE 250 platform (Novogene Bioinformatics Technology Co., Ltd., Tianjin, China). All sequence data processing was performed using the QIIME software package. Sequences were paired-end and high-quality sequences were aligned against the SILVA database (Ribocon GmbH, Bremen, Germany). The UCHIME software (Tiburon, CA, United States) was used to identify and remove chimeric sequences. Operational taxonomic units (OTUs) were assigned at a 97% identity using the SILVA database. The Venn diagram with shared and unique OTUs was used to identify the similarity and differences among treatments. The clustered OTUs were used to calculate the alpha-diversity within groups including Shannon index, Simpson index, Chao 1 richness, Good's coverage, and Observed species of the whole tree. Beta diversity index, principal coordinate analysis (PCoA plots, weighted UniFrac distance), non-metric multidimensional scaling (NMDS plots, weighted UniFrac distance), and unweighted pair-group method with arithmetic means (UPGMA) clustering were accessed to calculate the β-diversity between groups. The differences in the relative abundances of microbiota among treatments were compared using the linear discriminant analysis effect size (LEfSe) and *T*-test analyses.

Statistical analysis

Data were presented as mean ± SEM. All data were statistically analyzed by ANOVA using the LSD multiple comparisons by the SPSS 23 software after the Levene test and the Kolmogorov Smirnov test. $P < 0.05$ and $0.05 < P < 0.1$ was considered as statistically significant and tendency, respectively.

Results

Growth performance and diarrhea rate

There are no differences in the initial body weight, final body weight, ADG, ADFI, and F:G of piglets between different PMS levels groups ($P > 0.05$) (Table 2). Piglets fed with 0.6% PMS tended to have higher ADFI at 29–42 days compared with other treatments ($P < 0.1$). Moreover, the ADFI of 29–42 days and 1–42 days was linearly and quadratically increased by the enhanced PMS level ($P < 0.05$). There were no animals that died during the experiment.

Additionally, supplementation with 0.3, 0.45, 0.6, and 0.75% PMS could significantly reduce the diarrhea rate in weaned piglets at 29–42 days ($P < 0.05$). The piglets in the 0.3% PMS group had a lower diarrhea rate in comparison with other groups ($P < 0.05$). From 1 to 42 days, the diarrhea rate was quadratically decreased by dietary PMS treatments ($P < 0.05$).

Serum biochemical indices

As shown in Table 3, the PMS treatments tended to enhance the serum glucose content of weaned piglets ($0.05 < P < 0.1$). Moreover, the serum glucose content of weaned piglets showed a significant quadratic relationship with PMS levels ($P < 0.05$, Quadratic). However, the levels of total protein, creatinine, alanine aminotransferase, glutamic oxalacetic transaminase, alkaline phosphatase, albumin, blood urea nitrogen, urea, triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein were not significantly influenced by dietary PMS treatments ($P > 0.05$).

Serum hormone contents

The dietary supplementation with PMS significantly reduced the contents of serum norepinephrine and epinephrine (Table 4) ($P < 0.05$). However, dietary PMS treatments did not affect the serum levels of cortisol and glucocorticoid ($P > 0.05$).

TABLE 2 Effect of dietary supplementation with PMS on growth performance in weaned piglets.

Item	PMS, %						*P*-value		
	0	0.15	0.30	0.45	0.60	0.75	ANOVA	Linear	Quadratic
ADG, g/d									
1–14 days	314 ± 19	314 ± 41	316 ± 32	316 ± 19	323 ± 38	318 ± 19	0.99	0.66	0.91
15–28 days	524 ± 30	532 ± 11	531 ± 13	521 ± 16	541 ± 34	540 ± 22	0.98	0.51	0.85
29–42 days	554 ± 20	600 ± 27	616 ± 15	594 ± 12	619 ± 31	607 ± 15	0.32	0.09	0.11
1–42 days	464 ± 24	482 ± 35	488 ± 10	477 ± 17	494 ± 42	488 ± 21	0.48	0.11	0.22
ADFI, g/d									
1–14 days	412 ± 10	411 ± 8	412 ± 13	412 ± 7	423 ± 10	420 ± 5	0.90	0.33	0.57
15–28 days	745 ± 34	762 ± 20	757 ± 35	750 ± 9	779 ± 25	777 ± 21	0.91	0.32	0.60
29–42 days	862 ± 26	960 ± 35	966 ± 13	941 ± 15	975 ± 47	963 ± 22	0.09	0.04	0.03
1–42 days	673 ± 20	711 ± 17	712 ± 10	701 ± 9	726 ± 22	720 ± 11	0.23	0.04	0.09
F: G									
1–14 days	1.31 ± 0.05	1.32 ± 0.05	1.31 ± 0.03	1.30 ± 0.02	1.32 ± 0.06	1.32 ± 0.02	0.99	0.87	0.93
15–28 days	1.43 ± 0.11	1.43 ± 0.06	1.42 ± 0.09	1.44 ± 0.08	1.46 ± 0.15	1.44 ± 0.06	0.99	0.61	0.88
29–42 days	1.56 ± 0.14	1.60 ± 0.09	1.57 ± 0.09	1.59 ± 0.07	1.58 ± 0.13	1.59 ± 0.05	0.98	0.83	0.96
1–42 days	1.45 ± 0.05	1.48 ± 0.04	1.46 ± 0.03	1.47 ± 0.04	1.47 ± 0.05	1.48 ± 0.02	0.80	0.33	0.60
Diarrhea rate, %									
1–14 days	4.36 ± 0.79	5.36 ± 1.05	2.78 ± 0.59	5.16 ± 1.18	4.96 ± 1.28	4.36 ± 0.79	0.49	0.89	0.98
15–28 days	8.13 ± 1.21	8.73 ± 1.29	5.16 ± 1.86	5.95 ± 1.92	4.56 ± 1.04	8.73 ± 0.25	0.13	0.48	0.11
29–42 days	8.53 ± 1.28^{a}	7.93 ± 0.85^{a}	4.36 ± 1.33^{b}	4.76 ± 1.27^{b}	4.56 ± 1.04^{b}	3.97 ± 0.59^{b}	0.02	0.00	0.00
1–42 days	7.01 ± 0.89	7.34 ± 0.85	4.10 ± 0.90	5.29 ± 0.95	4.69 ± 0.95	5.69 ± 0.20	0.05	0.07	0.04

ADG, Average daily gain; ADFI, Average daily feed intake; F: G, Feed to gain ratio.
^{a,b}Within a row, means without a common superscript letter differ at $P < 0.05$. Data were presented as mean + SEM.

Intestinal morphology

As shown in Table 5, the ratio of villus height to crypt depth in the ileum showed a significant linear relationship with PMS levels ($P = 0.05$). Moreover, the depth of duodenal crypt depth showed a significant quadratic relationship with PMS levels in weaned piglets ($P < 0.05$). However, dietary PMS treatments did not affect the intestinal morphology of the duodenum, jejunum, and ileum ($P > 0.05$).

Serum and intestinal antioxidant index

In Figure 1, 0.3% PMS significantly increased the activity of GSH-Px (Figure 1J) in the jejunum ($P < 0.05$) and tended to enhance the activity of GSH-Px (Figure 1D) in serum and SOD activity (Figure 1H) in the jejunum ($P < 0.1$). Moreover, the levels of T-AOC (Figure 1A), GSH-Px (Figure 1D) in serum and GSH-Px (Figure 1J) activity in jejunum showed a significant quadratic relationship with PMS levels ($P < 0.05$, Quadratic). And we found that there was no significant difference in the ROS contents in serum and jejunum of weaned piglets treated with different PMS levels ($P > 0.05$) (Figures 1F,L).

Intestinal immune cytokines and immunoglobulin levels

In Figure 2, the addition of 0.6 and 0.75% PMS significantly reduced IL-1β (Figure 2D) in the jejunum of weaned piglets ($P < 0.05$). The content of IgM (Figure 2B) reached the peak at 0.3% ($P < 0.05$, Linear, Quadratic), and decreased linearly with the increase of the addition of PMS.

Microbial composition analysis by 16S RNA sequencing

There are 715 OTU clusters shared by the six groups, while 0.3% PMS has 561 unique OTU clusters (Figure 3A). The top 10 abundance of bacteria at the phylum (Figure 3B) included *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Spirochaetota*, *Euryarchaeota*, *Unified bacteria*, *Desulfobacterota*, *Cyanobacteriota*, *Actinobacterita*, and *Actinobacteria*. At the family level, the dominant top 10 bacteria are *Lactobacillaceae*, *Prevotellaceae*, *Eubacterium coprostanogenes group*, *Burkholderiaceae*, *Lachnospiraceae*, *Muribaculaceae*, *Peptostreptococcaceae*, *Clostridiaceae*, *Spirochaetaceae*, and *Oscillospiraceae* (Figure 3C). The addition

TABLE 3 Effect of dietary supplementation with PMS on serum biochemical indices in weaned piglets.

Item	PMS, %						*P*-value		
	0	0.15	0.30	0.45	0.60	0.75	ANOVA	Linear	Quadratic
TP, g/L	57.53 ± 2.89	60.9 ± 3.48	57.33 ± 1.42	60.4 ± 2.09	58.46 ± 1.33	57.38 ± 1.46	0.76	0.79	0.72
HDL, mmol/L	0.96 ± 0.06	0.99 ± 0.05	0.95 ± 0.06	0.94 ± 0.07	0.90 ± 0.06	0.91 ± 0.07	0.92	0.32	0.60
LDL, mmol/L	1.13 ± 0.19	1.05 ± 0.19	0.94 ± 0.13	1.11 ± 0.09	0.89 ± 0.09	1.02 ± 0.10	0.80	0.43	0.63
AST, U/L	73.68 ± 9.99	79.57 ± 16.35	53.88 ± 5.50	81.3 ± 15.37	80.2 ± 15.87	75.62 ± 10.59	0.67	0.72	0.87
ALP, U/L	215.08 ± 21.61	203.45 ± 35.42	166.45 ± 16.53	217.88 ± 46.95	214.53 ± 25.06	206.51 ± 11.84	0.81	0.86	0.83
ALB, g/L	29.59 ± 1.51	29.91 ± 2.21	29.74 ± 0.72	29.88 ± 1.41	27.74 ± 1.08	29.79 ± 1.78	0.91	0.66	0.91
BUN, mmol/L	1.93 ± 0.31	1.69 ± 0.09	1.73 ± 0.14	1.88 ± 0.29	1.80 ± 0.23	2.03 ± 0.21	0.89	0.58	0.54
UA, μmol/L	23.94 ± 3.16	37.5 ± 3.72	28.96 ± 4.90	32.04 ± 4.49	32.53 ± 6.80	32.34 ± 4.85	0.51	0.46	0.58
GLU, mmol/L	2.93 ± 0.22	2.00 ± 0.47	2.21 ± 0.39	2.38 ± 0.27	3.21 ± 0.42	3.19 ± 0.21	0.07	0.10	0.03
TG, mmol/L	0.62 ± 0.18	0.59 ± 0.05	0.45 ± 0.04	0.53 ± 0.09	0.50 ± 0.05	0.49 ± 0.03	0.73	0.21	0.37
TC, mmol/L	2.33 ± 0.15	2.33 ± 0.22	2.05 ± 0.15	2.24 ± 0.12	1.96 ± 0.11	2.12 ± 0.07	0.39	0.11	0.24
CRE, μmol/L	121.65 ± 12.07	113.40 ± 4.48	103.32 ± 3.86	103.19 ± 5.76	114.19 ± 4.78	113.27 ± 5.33	0.36	0.49	0.11
ALT, U/L	56.88 ± 4.07	47.42 ± 4.39	42.93 ± 1.97	52.48 ± 3.32	50.87 ± 4.21	51.20 ± 3.28	0.17	0.79	0.22

TP, Total protein; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; AST, Osteocalcin; ALP, Alkaline phosphatase; ALB, Albumin; BUN, Blood urea nitrogen; UA, Urea; GLU, Glucose; TG, Triglycerides; TC, Total cholesterol; CRE, Creatinine; ALT, Alanine aminotransferase.

TABLE 4 Effect of dietary supplementation with PMS on serum hormone in weaned piglets.

Item	PMS, %						*P*-value		
	0	0.15	0.30	0.45	0.60	0.75	ANOVA	Linear	Quadratic
COR, ng/ml	1.50 ± 0.01	1.50 ± 0.01	1.51 ± 0.00	1.51 ± 0.00	1.50 ± 0.00	1.50 ± 0.01	0.68	0.84	0.45
NE, ng/ml	7.69 ± 0.53^{a}	6.34 ± 0.37^{b}	5.74 ± 0.39^{b}	5.60 ± 0.46^{b}	5.94 ± 0.23^{b}	5.93 ± 0.32^{b}	0.02	0.01	0.00
EP, ng/ml	5.11 ± 0.35^{a}	4.39 ± 0.15^{ab}	4.10 ± 0.18^{b}	3.87 ± 0.29^{b}	4.17 ± 0.22^{b}	4.17 ± 0.30^{b}	0.05	0.03	0.00
GC, ng/ml	3.89 ± 0.56	4.96 ± 0.58	3.75 ± 0.47	2.59 ± 1.02	3.66 ± 1.37	4.50 ± 1.04	0.57	0.79	0.62

COR, Cortisol; NE, Norepinephrine; EP, epinephrine; GC, Glucocorticoid.
^{a,b}Within a row, means without a common superscript letter differ at $P < 0.05$. Data were presented as mean ± SEM.

TABLE 5 Effects of dietary supplementation with PMS on intestinal morphology in weaned piglets.

Item	PMS, %						*P*-value		
	0	0.15	0.30	0.45	0.60	0.75	ANOVA	Linear	Quadratic
Duodenum									
Villus height	537 ± 43	532 ± 27	500 ± 19	490 ± 19	494 ± 20	514 ± 42	0.68	0.18	0.20
Crypt depth	365 ± 30	368 ± 25	341 ± 10	334 ± 26	315 ± 26	411 ± 20	0.11	0.76	0.03
V:C ratio	1.47 ± 0.23	1.40 ± 0.12	1.49 ± 0.09	1.51 ± 0.16	1.51 ± 0.08	1.44 ± 0.06	0.77	0.45	0.58
Jejunum									
Villus height	450 ± 21	479 ± 38	473 ± 50	449 ± 26	435 ± 10	450 ± 33	0.93	0.57	0.72
Crypt depth	269 ± 24	274 ± 15	242 ± 11	265 ± 15	277 ± 25	291 ± 25	0.64	0.39	0.23
V:C ratio	1.73 ± 0.14	1.77 ± 0.14	1.98 ± 0.22	1.70 ± 0.06	1.63 ± 0.14	1.56 ± 0.08	0.40	0.20	0.20
Ileum									
Villus height	387 ± 8	344 ± 13	375 ± 22	355 ± 17	370 ± 9	409 ± 27	0.15	0.26	0.04
Crypt depth	311 ± 24	325 ± 23	267 ± 13	269 ± 17	248 ± 16	305 ± 31	0.11	0.16	0.08
V:C ratio	1.27 ± 0.08	1.17 ± 0.07	1.41 ± 0.09	1.33 ± 0.09	1.52 ± 0.09	1.37 ± 0.09	0.14	0.05	0.57

V:C ratio: The ratio of villus height to crypt depth. Data were presented as mean ± SEM.

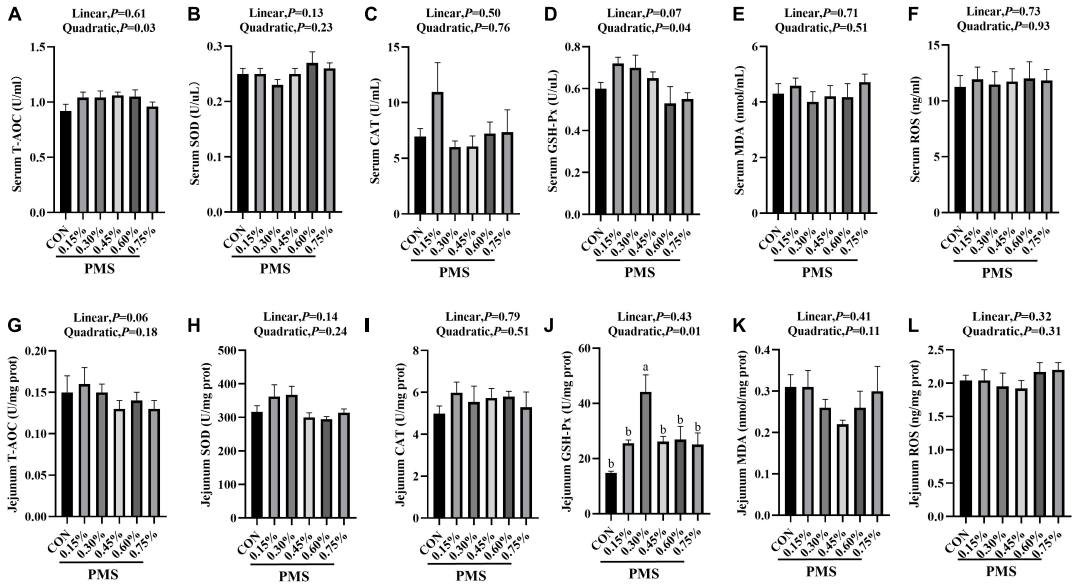

FIGURE 1
Effects of dietary supplementation with PMS on serum and intestinal antioxidant indexes in weaned piglets. **(A)** Serum T-AOC level; **(B)** Serum SOD activity; **(C)** Serum CAT activity; **(D)** Serum GSH-Px activity; **(E)** Serum MDA content; **(F)** Serum ROS content; **(G)** Jejunal T-AOC level; **(H)** Jejunal SOD activity; **(I)** Jejunal CAT activity; **(J)** Jejunal GSH-Px activity; **(K)** Jejunal MDA content; **(L)** Jejunal ROS content. T-AOC, Total antioxidant capacity; SOD, Total superoxide dismutase; CAT, Catalase; GSH-Px, Glutathione peroxidase; MDA, Malondialdehyde; ROS, Reactive oxygen species. CON: basal diet; 0.15%: the diet contained 0.15% PMS, 0.3%: the diet contained 0.3% PMS, 0.45%: the diet contained 0.45% PMS, 0.6%: the diet contained 0.6% PMS, 0.75%: the diet contained 0.75% PMS. PMS, Potassium-magnesium sulfate. ^{a,b}Within a row, means without a common superscript letter differ at $P < 0.05$. Data were presented as mean ± SEM.

of 0.15, 0.45, and 0.75% PMS significantly enhanced the abundance of *Firmicutes* and decreased the abundance of *Bacteroidota* (Figures 3D,E).

Microbial α-diversity of the colon contents

Compared with the control group, the addition of 0.15 and 0.6% PMS significantly increased the Shannon index of colonic digesta (Figure 3F) in weaned piglets ($P < 0.05$). Moreover, the Simpson index (Figure 3G) was not influenced by the addition of PMS ($P > 0.05$), while the Chao1 index (Figure 3H) was quadratically increased with the addition of PMS ($P < 0.05$). The addition of 0.30% and 0.60% PMS significantly increased the observed species index ($P < 0.05$) (Figure 3I).

Microbial composition analysis by 16S RNA sequencing

The effects of PMS on the β-diversity of colonic microbiota in weaned piglets are shown in Figure 3. The PCoA (Figure 4A) and NMDS plots (Figure 4B) based on weighted UniFrac distance were applied to evaluate the microbial β-diversity. As revealed by both PCoA and NMDS plots, the microbial community structure of different samples is not clearly separated among different groups (Figures 4A,B). The UPGMA clustering based on unweighted UniFrac showed significant differences in the microflora composition of colonic contents among the six groups (Figure 4C). In particular, *Firmicutes* in the PMS addition group had significantly prominent clusters compared with the control group (Figure 4C).

LEfse analysis and *T*-test analysis

The differential microbiota from different treatments is presented based on LEfSe analysis. The results in Figure 5A show that the control group was enriched with *Ralstonia* (genus), *Ralstonia pickettii* (species), *Burkholderiaceae* (family). On the other hand, 0.15% PMS addition group is enriched with *Negativicutes* (class), 0.45% PMS addition group is enriched with *Bacteroidaceae* (family), *Bacteroides* (genus), 0.75% PMS addition group is enriched with *[Eubacterium] coprostanogenes group*, *Firmicutes* (phylum), and *Clostridia* (class). In Figure 5B, *T*-test analysis results showed that the 0.3% PMS addition group increased the relative abundance of *Lachnospiraceae bacterium GAM79* at the boundary level ($P < 0.05$) and decreased the relative abundance of *Clostridium*, *Butyricum* compared with the control group ($P < 0.05$). Moreover, the relative abundance of *Clostridiaceae*, *Unidentified Chloroplast*,

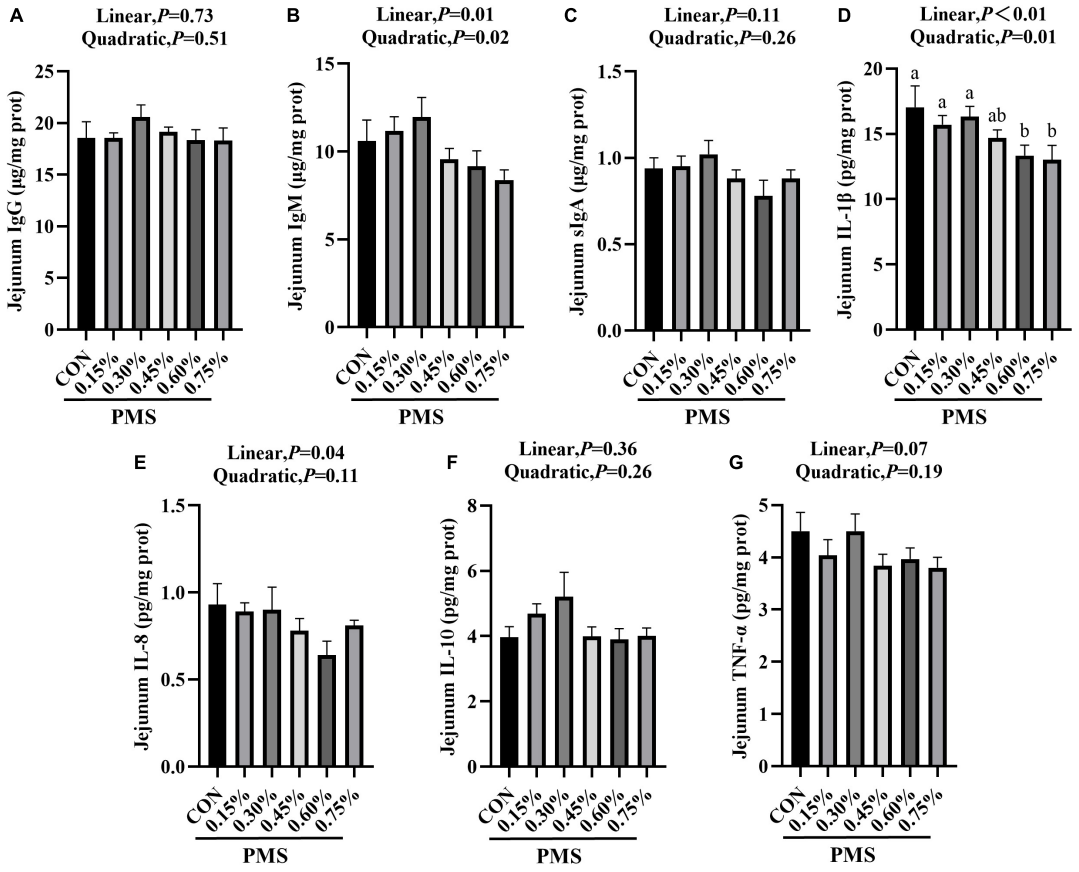

FIGURE 2
Effects of dietary supplementation with PMS on intestinal immune factors in weaned piglets. **(A)** IgG content; **(B)** IgM content; **(C)** sIgA content; **(D)** IL-1β content; **(E)** IL-8 content; **(F)** IL-10 content; **(G)** TNF-α content. IgG, Immunoglobulin G; IgM, Immunoglobulin M; sIgA, Secretory Immunoglobulin A; IL-1β, Interleukin-1β; IL-8, Interleukin-8; IL-10, Interleukin-10; TNF-α, Tumor inflammatory factor-α. CON: basal diet; 0.15%: the diet contained 0.15% PMS, 0.3%: the diet contained 0.3% PMS, 0.45%: the diet contained 0.45% PMS, 0.6%: the diet contained 0.6% PMS, 0.75%: the diet contained 0.75% PMS. PMS, Potassium-magnesium sulfate. [a,b]Within a row, means without a common superscript letter differ at $P < 0.05$. Data were presented as mean ± SEM.

Ruminococcaceae, and *Peptococcaceae* was increased by 0.3% PMS addition at the family level ($P < 0.05$) (Figure 5C). Compared with the control group, the 0.75% PMS addition group increased the relative abundance of Firmicutes at the phylum level ($P < 0.05$), as well as *Erysipelotrichaceae*, *Erysipelatoclostridiaceae*, *Ruminococcaceae*, *Peptococcaceae*, *Bifidobacteriaceae*, and *Oscillospiraceae* at the family level ($P < 0.05$) (Figures 5D,E).

Spearman correlation analysis

There was a significant correlation between colonic microbiota at the family level with growth performance and jejunal antioxidant capacity of weaned piglets (Figure 6). The relative abundance of *Butyriccoccaceae* was positively correlated with ADFI ($P < 0.01$) and ADG during 1–42 days ($P < 0.05$), and the relative abundance of *T34* was negatively correlated with diarrhea rate ($P < 0.05$). Furthermore, T-AOC activity in jejunal mucosa was positively correlated with *Lachnospiraceae* ($P < 0.05$). SOD activity in jejunal mucosa was positively correlated with the relative abundances of *Veillonellaceae*, *Succenivibrionaceae*, and *Streptcoccaceae* ($P < 0.05$). The GSH-Px activity was positively correlated with the relative abundances of *Muribaculaceae*, *Peptostreptococcaceae*, *Desulfovibrionaceae* ($P < 0.01$), and *Clostridiaceae*, *Erysipelotrichaceae* ($P < 0.05$). Interestingly, there was a negative correlation in CAT activity with the relative abundances of *Lactobacillaceae* and *Erysipelotrichaceae* ($P < 0.05$), but positively correlated with *UCG 010* ($P < 0.05$).

Discussion

Weaning often results in intestinal digestion and absorption dysfunction, diarrhea, and even death, which caused huge

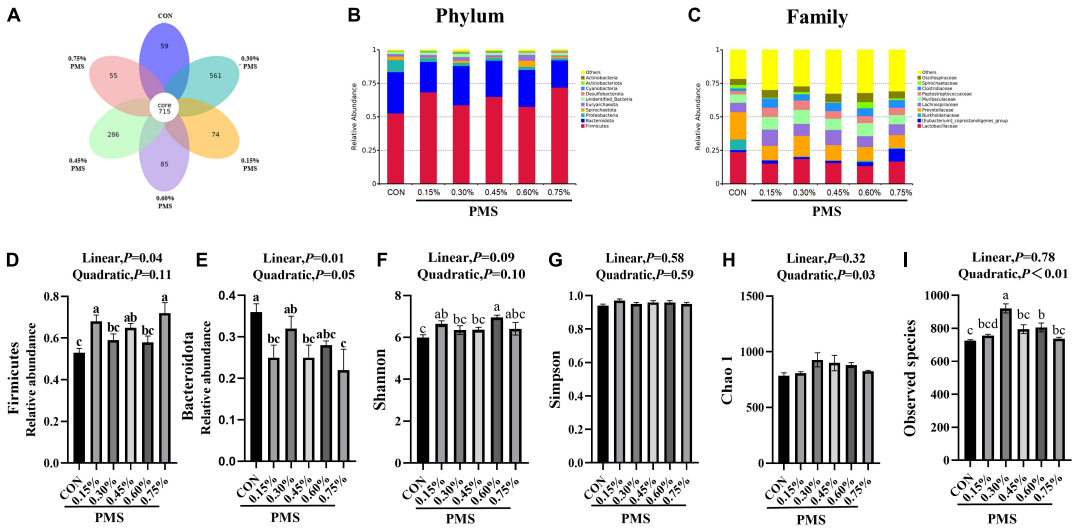

FIGURE 3
Effect of PMS supplementation on the composition and diversity index of colon microbiota in weaned piglets. **(A)** The common and special OTUs distribution among the different levels of PMS treatments is shown by the Petal diagram. **(B,C)** The relative abundance of top ten phyla **(B)** and families **(C)**. **(D,E)** The relative abundance of Firmicutes **(D)** and Bacteroidota **(E)**. **(F–I)** Alpha diversity index, including Shannon index **(F)**, Simpson index **(G)**, Chao1 index **(H)**, and observed species **(I)**. CON: basal diet; 0.15%: the diet contained 0.15% PMS, 0.3%: the diet contained 0.3% PMS, 0.45%: the diet contained 0.45% PMS, 0.6%: the diet contained 0.6% PMS, 0.75%: the diet contained 0.75% PMS. PMS, Potassium-magnesium sulfate. ^{a,b,c}Within a row, means without a common superscript letter differ at $P < 0.05$. Data were presented as mean ± SEM.

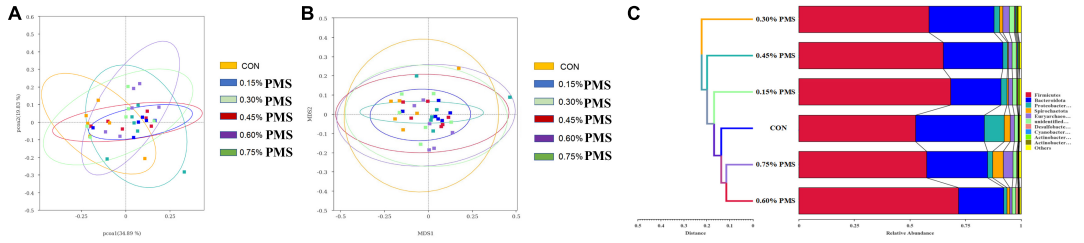

FIGURE 4
Effect of PMS supplementation on the β-diversity of colon microbiota in weaned piglets. **(A,B)** PCoA plot **(A)** and NMDS plot **(B)** based on ASV weighted unifrac distance. **(C)** UPGMA clustering was conducted based on unweighted unifrac distance. CON: basal diet; 0.15%: the diet contained 0.15% PMS, 0.3%: the diet contained 0.3% PMS, 0.45%: the diet contained 0.45% PMS, 0.6%: the diet contained 0.6% PMS, 0.75%: the diet contained 0.75% PMS. PMS, Potassium-magnesium sulfate.

economic losses in livestock husbandry (Upadhaya and Kim, 2021). We found that the addition of different doses of PMS linearly increased the ADFI and ADG, and significantly reduced the diarrhea rate of weaned piglets. Magnesium and potassium levels were found to affect the appetite, growth, and behaviors of the animals (Adam and Dawborn, 1972; McCaughey and Tordoff, 2002; Laires et al., 2004). A previous study reported that oral administration of magnesium by capsule to lambs resulted in the restoration of appetite, but intravenous injection of magnesium sulfate had a lesser effect (Martin et al., 1964). Livingston et al. (2019) Broilers fed supplemental potassium diets trended toward an improved feed conversion ratio from 29 to 35 days compared to broilers fed basal potassium deficiency diets (Livingston et al., 2019). So, we speculate that the addition of PMS in the feed enhanced the appetite of weaning piglets, and further led to the enhanced average daily feed intake of piglets. In this study, the addition of different doses of PMS linearly increased the ADFI and ADG, and significantly reduced the diarrhea rate of weaned piglets. Similarly, previous studies reported that supplemental magnesium significantly increased the piglet's weight at birth (Zang et al., 2014). Moreover, Plush et al. (2018) found that maternal magnesium sulfate supplementation in a pre-farrow diet improves factors important for piglet viability (Plush et al., 2018). Under stressful conditions, the hypothalamus pituitary adrenal (HPA) axis is activated (Sarkodie et al., 2019), leading to an enhancement in cortisol and catecholamine stress hormone secretion (Pulopulos et al., 2020). In this study, we found that dietary addition of PMS

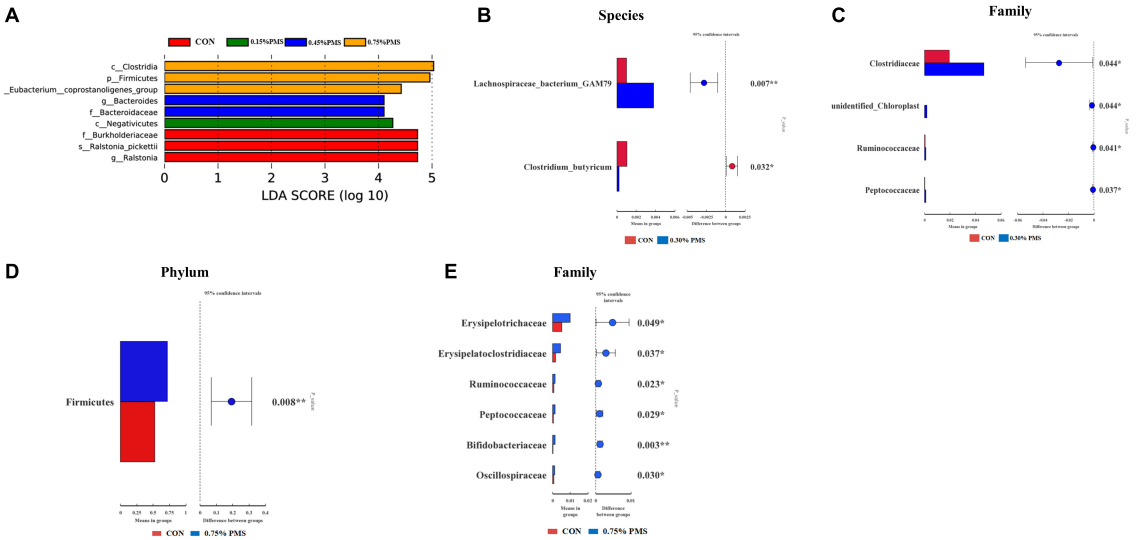

FIGURE 5
LEfSe analysis and *T*-test analysis for the significant changes of differential colon microbiota at different levels in weaned piglets. **(A)** The LEfSe analysis (LDA score ≥ 4) identified the biomarker bacterial species in the six groups. **(B,C)** Group A and C different microbiota *T*-test analyses based on Species **(B)** and Family **(C)** levels. **(D,E)** Group A and F different microbiota *T*-test analysis based on Phylum **(D)** and Family **(E)** levels. CON: basal diet; 0.15%: the diet contained 0.15% PMS, 0.3%: the diet contained 0.3% PMS, 0.45%: the diet contained 0.45% PMS, 0.6%: the diet contained 0.6% PMS, 0.75%: the diet contained 0.75% PMS. PMS, Potassium-magnesium sulfate. $^{*}P < 0.05$ and $^{**}P < 0.01$.

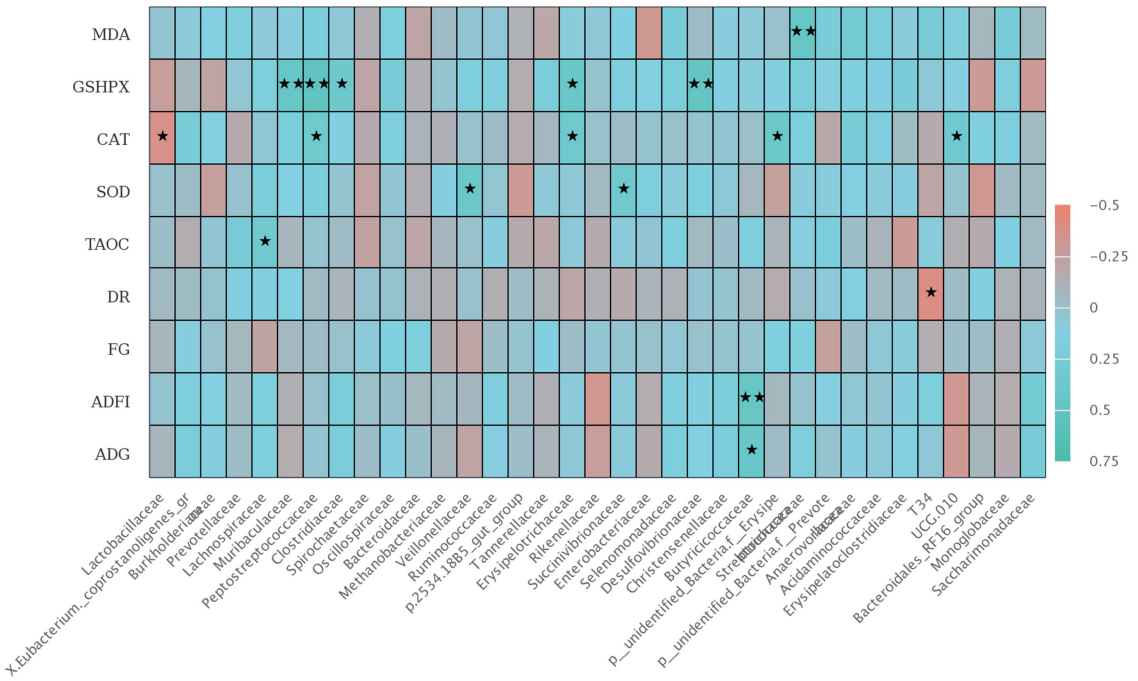

FIGURE 6
The Spearman correlation analysis of colon microbial species at the family level with ADG, ADFI, F: G, MDA, GSH-Px, CAT, SOD, and T-AOC differential metabolites in weaned piglets. The heatmap with red indicated a negative correlation, while blue represented a positive correlation. ADG, Average daily gain; ADFI, Average daily feed intake; F: G, Feed to gain ratio; TAOC, total antioxidant capacity of jejunum; SOD, total superoxide dismutase of jejunum; CAT, catalase of jejunum; GSH-Px, glutathione peroxidase of jejunum; MDA, malondialdehyde of jejunum. $^{★}P < 0.05$ and $^{★★}P < 0.01$.

significantly reduced the contents of serum norepinephrine and epinephrine, suggesting that PMS may alleviate the weaning stress of piglets.

Weaning stress leads to the excessive production of oxygen free radicals, resulting in lipid peroxidation and damage to the structure and function of biofilm (Cao et al., 2018; Degroote et al., 2020; Novais et al., 2021). A previous study reported that the exogenous addition of magnesium can alleviate the decrease of GSH-Px activity in mitochondria of premature aging mice caused by oxidative stress (Villa-Bellosta, 2020). Morais et al. (2017) showed that magnesium plays a key role in several metabolic reactions, particularly in oxidative stress in obese individuals (Morais et al., 2017). Similarly, we found that the addition of 0.3% PMS significantly increased the GSH-Px activity and tended to increase the SOD activity of the jejunum of weaned piglets, indicating that PMS could improve the intestinal antioxidant capacity and alleviate the intestinal oxidative stress in piglets during weaning.

Cytokines such as the interleukin family play a critical role in immune stress. Especially, circulating IL-1β, IL-6, and IL-8 are upregulated in systemic and chronic inflammatory conditions (Bester and Pretorius, 2016). Xie et al. (2019) reported that magnesium isoglycyrrhizinate suppresses LPS-induced inflammation and oxidative stress by inhibiting NF-κB and MAPK pathways in RAW264.7 cells (Xie et al., 2019). Lopez-Baltanas et al. (2021) found that magnesium supplementation reduces inflammation in rats with induced chronic kidney disease (Lopez-Baltanas et al., 2021). Moreover, magnesium sulfate differentially modulates fetal membrane inflammation in a time-dependent manner (Cross et al., 2018). In this experiment, we found that dietary addition of PMS linearly reduced the inflammatory factor IL-1β and IL-8 levels in the jejunum. Immunoglobulin is one of the components of the immune system of animals (Cao et al., 2022). Newborn piglets lack protective immunity and they mainly rely on the absorption of immunoglobulin from colostrum to obtain passive immunity (Levast et al., 2014; Cao et al., 2022). Some studies have found that the anionic salt in the sow diet is positively related to the content of immunoglobulin (Wang et al., 2020; Yang et al., 2022). So far, the information regarding the effects of PMS on immunoglobulin levels and cytokines levels in weaning pigs is limited. We found that the addition of PMS had a trend to enhance IgM in the intestine, which may be due to the increase of dietary cations for stimulating the expression of immunoglobulin by the addition of PMS in weaned piglet diet.

Intestinal barrier dysfunction and immune-mediated injury are usually related to the disruption of the host gut microbial composition. We found that the addition of PMS significantly increased the relative abundance of Firmicutes, and decreased the abundance of Bacteroidetes. Previous research found that the increased feed intake of weaning pigs may be related to the change in gut microbiota structure (Ban-Tokuda et al., 2017), with Firmicutes being the dominant bacteria affecting pig feed efficiency (Myer et al., 2015). Moreover, the data showed that the addition of PMS increased the Shannon index, Observed Species, Simpson index, and Chao1 index in the colonic microbiota of weaned piglets. Similarly, Crowley et al. (2018) found that the addition of magnesium-rich marine minerals significantly improved the intestinal microbial diversity of adult male rats (Crowley et al., 2018). Further Lefse and *T*-test analysis showed that PMS improved antioxidant and intestinal immunity. However, high-dose magnesium supplements can lead to the decline of microbial diversity and ecological imbalance in rats (García-Legorreta et al., 2020). Our results showed that the relative abundance of *Clostridiaceae*, *Unidentified chloroplast*, *Ruminococcaceae*, and *Peptostreptococcaceae* in the 0.3% PMS group was higher than that in the control group. Furthermore, the 0.75% PMS group increased the relative abundance of *Erysipelotrichaceae*, *Erysipelatoclostridiaceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Bifidobacteria CEAE*, and *Oscillospiraceae* in the family level compared with the control group. A previous study reported that the enhanced *Peptostreptococcaceae* abundance in rat intestines is related to the reduced oxidative stress induced by a high-fat diet (Yang et al., 2019). Consistently, we found that PMS increased the abundance of *Peptostreptococcaceae*, which may involve alleviating the weaning stress of piglets.

The gut immune and antioxidant homeostasis are associated with distinct alternations in the intestinal microbiome (Ghaisas et al., 2016; Gershon and Margolis, 2021). A previous study found that *Erysipelotrichaceae* may worsen intestinal inflammation by enhancing the response of Th17 cells and molecular simulation of myelin oligodendrocyte glycoprotein (MOG) (Maghzi and Weiner, 2020). In this study, the addition of high-dose PMS leads to the increase of *Erysipelotrichaceae*, which may affect intestinal health and growth performance. Spearman's analysis showed that there was a correlation between intestinal GSH-Px was positively correlated with *Muribaculaceae*, *Peptostreptococcaceae*, and *Desulfovibrionaceae*, suggesting that PMS may affect the activity of GSH-Px by increasing the abundance of *Peptostreptococcaceae*. Several studies reported that intestinal flora and antioxidant capacity are interrelated (Tomasello et al., 2016; Wang et al., 2017). According to the previous studies, Yi et al. (2021) found that the MDA (liver) was positively correlated with *Erysipelotrichaceae* in colon microorganisms and negatively correlated with *Ruminococcus* through the protective effect of antrodina on alcohol-induced intestinal microbiota and liver metabolomic disorders in mice (Yi et al., 2021). The results show that intestinal microorganisms can affect the antioxidant ability of the different organs. Miao et al. (2021) found that it enhanced the GSH-Px activity in the small intestine of mice and increased the relative abundance of *Lactobacillus* after

the 5 ml/kg walnut oil gavage test on mice (Miao et al., 2021). In our experiment, we analyzed the correlation between intestinal oxidative stress and colonic microbial changes and found that there was a correlation between intestinal GSH-Px was positively correlated with *Muribaculaceae*, *Peptostreptococcaceae*, and *Desulfovibrionaceae*, suggesting that PMS may affect the activity of GSH-Px by increasing the abundance of *Peptostreptococcaceae*. This finding can provide a potential target for improving the antioxidant capacity of weaned piglets.

Conclusion

In conclusion, dietary supplementation with PMS could improve the growth performance, antioxidant activity, and immune capacity in weaned piglets, which was partially related to the significant changes in colonic microbiota composition. Thus, PMS may be a potential feed additive to improve growth performance and intestinal health of weaning piglets.

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

This animal study was reviewed and approved by Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences.

Author contributions

SC, KH, and XW: investigation, methodology, data curation, validation, visualization, and writing—original draft. SH, BC, and JG: visualization, validation, and formal analysis. KY, XZ, QW, and HX: investigation, methodology, formal analysis, and resources. LW, ZJ, CZ, and SC: conceptualization, supervision, funding acquisition, and writing—review and editing. 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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Bifidobacterium animalis KV9 and *Lactobacillus vaginalis* FN3 alleviated β-lactoglobulin-induced allergy by modulating dendritic cells in mice

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Food allergy is a serious public health problem because of its high incidence and risk. Probiotics can induce immune regulation in patients with allergic diseases, but its mechanism is not fully clear. In this paper, β-lactoglobulin (β-LG)-sensitized mice were used as models to explore the mechanism of *Bifidobacterium animalis* KV9 (KV9) and *Lactobacillus vaginalis* FN3 (FN3) on reducing allergic reactions and regulating immune cell function. The results showed that oral administration of KV9 and FN3 significantly reduced the scores of allergic symptoms, hypothermia symptoms, and serum levels of β-LG-specific immunoglobulins E (β-LG-sIgE), histamine, and mast cell protease in allergic mice. Flow cytometry analysis of intestinal dendritic cells (DCs) showed that the proportion of CD11c+major histocompatibility complex (MHC)-II+DCs, CD11c+CD80+DCs, and CD11c+ CD86+DCs increased after KV9 and FN3 intervention, indicating that the strains induced immature DCs and decreased the antigen-presenting capacity of DCs. Meanwhile, the toll-like receptor 4 (TLR4)-NF-κB signaling pathway was activated in DCs. The secretion of interleukin-12 (IL-12) was significantly increased, while interleukin-4 (IL-4) was decreased by DCs after KV9 and FN3 intervention, indicating that DCs have the potential to promote T-cell differentiation into T helper type 1 (Th1) cells. Furthermore, the proportion of CD3+CD8–IFN-γ+ T cells in the spleen increased, while CD3+CD8–IL-4+T cells decreased after oral administration of KV9 and FN3, correcting the T helper type 2 (Th2)-skewed immune responses. These results indicate that KV9 and FN3 reduce β-LG-induced allergic symptoms in mice, and suggest that the two potential probiotics might be used as an alternative therapeutic agent for mitigating food allergy.

KEYWORDS

anti-food allergy, β-lactoglobulin, probiotics, toll-like receptor 4, immunity

Introduction

The incidence of food allergy has increased rapidly in recent years, affecting nearly 5% of adults and 8% of children worldwide (1). Milk, egg, peanut, tree nuts, soy, wheat, fish, and crustacean shellfish are considered the eight major allergenic foods (2). Milk allergy is an allergic reaction caused by allergenic proteins in milk and dairy products, β-lactoglobulin (β-LG) is one of the most important allergens of the whey fraction, and accounts for 10% of total milk proteins (3). In addition, approximately 62% of cows' milk allergy patients are sensitive to β-LG (4). Common food allergy is mediated by immunoglobulin E (IgE), which involves the uptake of antigens by antigen-presenting cells (APCs), such as dendritic cells (DCs), across the intestinal barrier. IgE intermediated by the binding of multivalent antigens at the surface of mast cells triggers the release of many allergy-related mediators such as histamine and leukotrienes. The production of IgE is affected by T helper type 1 (Th1)/T helper type 2 (Th2) cell balance (5). Treatment targeting the Th1/Th2 balance has become an effective approach. The most effective way to prevent food allergy is to strictly avoid food allergens; however, accidental ingestion is difficult to be absolutely avoided in our life. The first-line treatment is intramuscular epinephrine after the diagnosis of anaphylaxis. Epinephrine can maintain blood pressure, improve respirations, and decrease edema that may be causing airway collapse (6). H1 antihistamines are the next treatment that should be used for anaphylactic symptoms. As allergic reactions can cause histamine release, giving medications that block the H1 and H2 receptors can improve the vasculature integrity and maintenance of the blood pressure and heart rate (7). Medicine treatment only temporarily alleviates allergic symptoms and the suffering of anaphylaxis and cannot prevent the occurrence of food allergy in advance (8). Therefore, a new type of food allergy therapy is urgently needed.

Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts (9), and may be a safe alternative due to their capacity to affect the innate and adaptive immune system (10). There have been several studies showing that oral administration of probiotics could alleviate food allergy. Shandilya et al. reported that *Lactobacillus acidophilus* LaVK2 and *Bifidobacterium bifidum* BbVK3 reduced whey protein-induced intestinal anaphylaxis in mice by reversing the Th1/Th2 immune imbalance (11). In another study, *Lactobacillus rhamnosus* LA305, *Lactobacillus salivarius* LA307, and *Bifidobacterium longum* subsp. *Infantis* LA308 could contribute to alterations in immune responses in a mouse model of β-LG allergy, as well as anergy that could contribute to oral tolerance acquisition (12). DCs are commanders-in-chief of the immune system, in charge of the mechanisms of immune response/tolerance in the gut (13). In a previous study, bone marrow-derived mouse DCs were exposed to irradiated lethal lactic acid bacteria, and the results showed that all strains upregulated major histocompatibility complex (MHC)-II and B7-2 (CD86) on the surface of DCs, with *Lactobacillus casei* strongly inducing IL-12 secretion and *Lactobacillus reuteri* inducing IL-10 secretion, stimulating T cells to polarize into Th1 and Treg in the intestinal tract (14). Current studies have shown that probiotics can regulate immune function through DCs; nevertheless, the immunoregulation of DCs by probiotics is strain dependent and the knowledge of molecular mechanisms underlying probiotics–host interactions through DCs is still incomplete.

This study aimed to assess the effects of KV9 and FN3 on relieving β-LG-induced food allergy and to explore their potential mechanisms of action. We found that KV9 and FN3 alleviated the symptoms caused by food allergy and regulated the development and function of DCs and T cells. These results provide further insight into probiotics as a new type of food allergy therapy.

Materials and methods

The strains and preparation of bacterial suspension

Bifidobacterium animalis KV9 (KV9) and *Lactobacillus vaginalis* FN3 (FN3) were stored in the Functional Dairy and Probiotic Engineering Laboratory of Ocean University of China. The strains were cultured successively twice in de Man, Rogosa, and Sharpe (MRS) broth (Qingdao Hope Bio-Technology Co., Ltd., China) before use, then incubated anaerobically at 37°C for 24 h. The bacteria were washed twice by sterile phosphate-buffered saline (PBS) with pH 7.2 and collected by centrifugation and resuspended in PBS to give a concentration of 5×10^8 colony-forming units (CFU)/ml.

Animals and construction of food allergy model

Balb/c mice (female, 5 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. China. Mice were kept in plastic cages and allowed free access to diet (Beijing Keao Xieli Feed Co., Ltd., China) and water, animal rooms were maintained on a 12-h light/dark cycle, and the air was exchanged at 15 times/h with room temperature and humidity of 22 ± 1°C and 55 ± 10%, respectively. All experimental processes were approved by the Animal Ethics Committee of Ocean University of China (permission number: SPXY2021112401).

Mice were acclimatized to the animal facility for 1 week before experimentation. Briefly, the mice were divided randomly

into the following groups (n = 10 per groups): non-sensitized (control), β-LG-sensitized and challenged (allergy), hydrocortisone supplement and β-LG-sensitized and challenged (medicine), KV9 supplement and β-LG-sensitized and challenged (KV9), and FN3 supplement and β-LG-sensitized and challenged (FN3). Mice were sensitized according to previous research and modified by intraperitoneal injection of 50 μg of β-LG (Sigma Aldrich Co., LTD, USA) and 100 μg of Imject™ Alum Adjuvant (Alum, Thermo Fisher Scientific Co., LTD., USA) in 200 μl of sterile PBS on day 14, and boosted with 100 μg of β-LG and 100 μg of Alum on day 28 in 200 μl of sterile PBS (15). Bacterial suspension (0.2 ml, containing 10^8 live bacteria) and PBS (0.2 ml) were administered *via* oral gavage to β-LG-sensitized mice from day 7 to day 52 in the KV9 groups, FN3 groups, and control groups, respectively (16). Hydrocortisone (0.5 mg/kg bw, Aladdin Reagent Co., LTD., China) in 200 μl of PBS was administered *via* oral gavage to β-LG-sensitized mice from day 28 to day 52 in the medicine group. Mice were orally challenged six times with 5 mg of β-LG in sterile PBS every 2 days from day 42. Before challenge, mice were deprived of food for 2 h.

Evaluation of allergic reaction in animals

The core body temperatures were monitored every 15 min for 1 h after the β-LG challenge, and the temperature usually decreased when an allergy occurs. Allergic symptoms were monitored for 15 min and scored as follows (17): 0—no signs; 1—mice are scratching between 4 and 10 times for 15 min; 2—mice are scratching more than 10 times for 15 min, or display reduced activity or bristled fur; 3—mice have a strongly reduced activity, display liquid diarrhea, and have difficulty in walking normally, bristled fur, and sometimes labored respiration; 4—manifestations of degree 3 are stronger, and mice displayed cyanosis around the mouth and tail; 5—death.

Measurement of serum cytokine and β-LG-specific IgE

Blood was obtained 1 h after the final challenge, and serum was obtained by centrifugation. Serum levels of histamine, mMCPT-1, and β-LG-specific IgE (β-LG-sIgE) were determined using an ELISA kit (Nanjing Jiancheng Technology Co., Ltd., China) according to the instructions.

Culture of mouse lymphocytes and DCs

Spleen and intestinal lymph nodes were extracted from mice under aseptic conditions, cut into pieces, and ground on a 70-μm cell filter. Mouse lymphocytes and DCs were isolated by the Mouse Splenic Lymphocyte Isolation Kit (Beijing Solarbio Science & Technology Co., Ltd., China) and the Mouse Tissue Dendritic Cell Isolation Kit (Tianjin Haoyang Biological Manufacture Co., Ltd, China), respectively. The regulated cell concentration of 5×10^6 cells/ml was suspended in Roswell Park Memorial Institute (RPMI-1640, Corning Incorporated Co., Ltd., China) medium with 10% fetal bovine serum (FBS, Biological Industries Israel Beit Haemek Co., Ltd., Israel), 100 units/ml penicillin (Beijing Solarbio Science & Technology Co., Ltd., China), and 100 μg/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd., China), cultured for 3 days (18). IFN-γ and IL-4 from lymphocyte culture supernatants and IL-12 and IL-4 from DC culture supernatants were detected using an ELISA kit according to the manufacturer's instructions (Nanjing Jiancheng Technology Co., Ltd., China).

Flow cytometry analysis of DCs and T cells

Single cells isolated from spleen and intestinal lymph node were stained for FACS analysis as described previously (16). Lymphocytes were stained using the Zombie Violet™ Fixable Viability Kit (BioLegend Biotechnology Co., LTD, China) and were stained for surface markers including CD3-APC-Cy7 and CD8-APC (Thermo Fisher Scientific Co., LTD., USA); cells were then fixed and permeabilized by the Intracellular Fixation and Permeabilization Buffer Set (Thermo Fisher Scientific Co., LTD., USA) and stained for intracellular expression markers such as IFN-γ-PerCP and IL-4-PE (Thermo Fisher Scientific Co., LTD., USA). DCs were stained for surface markers including CD11c+-PE-Cy7, CD80-APC, CD86-FITC, and MHC-II-PE (Thermo Fisher Scientific Co., LTD., USA). Data were acquired with BD FACSVerse (Becton Dickinson Co., LTD., Canada) and analyzed with BD FACSuite (Becton Dickinson Co., LTD., Canada).

Gene expression determination by qPCR

DCs were employed to examine the toll-like receptor 4 (TLR4)-NF-κB relative expression of genes (19). The total RNA was extracted from spleens using Trizol Reagent (Tiangen Biotech CO., LTD., China), and total RNA was reverse transcribed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo CO., LTD., Japan). Quantitative PCR was performed using the SYBR Green Realtime PCR Master Mix (Toyobo CO., LTD., Japan) according to the instructions on a CFX96 Real-Time System (Bio-Rad Laboratories CO., LTD., USA). For qPCR, the reaction mixture contained 8 μl of ddH_2O, 12 μl of SYBR qPCR Mix, 2.5 μl of forward primer and reverse primer, and 2.5 μl of complementary DNA. The conditions for qPCR were as follows: 95°C for 60 s, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA levels, and β-actin was used as the

internal control. Primers for qPCR were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd (China). The primers are shown in Table 1.

Statistical analysis

All data were expressed as the mean ± SD. Statistical differences between the two groups were analyzed using Student's *t*-test; *p* values <0.05 were considered significant and are indicated by asterisks (*). Statistical differences between three or more groups were analyzed using a one-way analysis of variance and Duncan test for multiple comparisons by the Statistical Package for the Social Sciences 25 (SPSS 25), and the lack of significance was indicated by the same letters.

Results

Effect of oral probiotics on suppressing allergic sensitization of β-LG

In order to evaluate the effect of oral probiotics on allergic reactions in mice, we established a food allergy model in mice by intraperitoneal injection of β-LG; the protocol is shown in Figure 1. In allergic animals, continuous oral β-LG stimulation can cause a significantly higher allergic symptoms score, and the specific manifestation was scratching and bristling fur (Figure 2). At the same time, the core body temperatures of allergic animals decreased significantly (Figure 3). Oral administration of KV9 and FN3 led to a significant attenuation of allergic symptoms score 15 min after the final oral β-LG challenge with similar ability and no significant difference with the control group (Figure 2). Meanwhile, the core body temperature in allergic mice was reduced, and oral administration of KV9 and FN3 significantly alleviated hypothermia in allergic mice (Figure 3). The serum levels of β-LG-sIgE, histamine, and mMCPT-1 (Figure 4) in the allergic mice were higher than those of control mice after repetitive challenges, indicating severe allergic reactions in mice. The high levels of β-LG-sIgE were decreased by KV9 and FN3 supplementation and have no significant difference from the control group; the FN3 group was slightly better than the medicine group. The serum levels of mMCPT-1 and histamine were decreased by KV9 and FN3 supplementation in allergic mice. However, histamine levels in the KV9 and FN3 groups did not return to the same level as in the control group. These results confirm that the probiotics supplementation suppresses allergic sensitization to β-LG.

Effect of oral probiotics on regulating DCs' maturation and function

Allergic reactions to food begin with the uptake of food allergens by DCs, processed and presented to T cells. The ability of activated DCs to prime T-cell activity is attributable to DCs' expression of MHC-II and costimulatory molecules that are upregulated during maturation (20). The expression of maturation of DCs was analyzed to investigate the possible functional relevance of DCs to probiotics suppressing allergic sensitization. FACS analysis showed that oral administration of KV9 and FN3 significantly reduced the percentage of CD11c+ MHC-II+DCs, CD11c+CD80+DCs, and CD11c+CD86+DCs in CD11c+DCs (Figure 5). This indicated that the probiotics induced immature DCs. In order to gain insight into the effect of the administration of probiotics to allergic mice on expression patterns of cytokine production in DCs, cytokine production and gene expression were analyzed by DCs purified from the mice after the β-LG challenge. Oral β-LG significantly increased the *in vitro* IL-4 production and reduced the IL-12 by DCs (Figure 6). However, there was no significant difference in IL-4 and IL-12 production by DCs between the mice supplemented with probiotics and mice in the control group. The IL-4 and IL-12 gene expression confirmed the results (Figure 6).

Effect of oral probiotics on activating Toll-like receptor 4-NF-κB

TLR4 is a class of pattern recognition receptors that recognize the probiotics and activate adaptive immunity,

TABLE 1 RT-PCR amplified primers.

Gene name	Forward (5'-3')	Reverse (5'-3')
β-actin (M)	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC
TLR4	GGCATGGCTTACACCACCTC	TTGTCTCCACAGCCACCAGA
Myd88	AGCAGAACCAGGAGTCCGAGAAG	GGGCAGTAGCAGATAAAGGCATCG
TRAF6	GACTGCCCAACAGCTCCAATCC	AAGTGTCGTGCCAAGTGATTCCTC
IκB	TGGTGTGACTGTGGATCTCTGGAG	GGCTGGCTTCTCTGTGGTGATTC
NF-κB	GGATATGAGGAAGCGGCATGTAGAG	CCTGATACTGGCACTTCGGACAAC
IL-12	TCTTTGATGATGACCCTGTGCCTTG	GTGATTCTGAAGTGCTGCGTTGATG
IL-4	TACCAGGAGCCATATCCACGGATG	TGTGGTGTTCTTCGTTGCTGTGAG

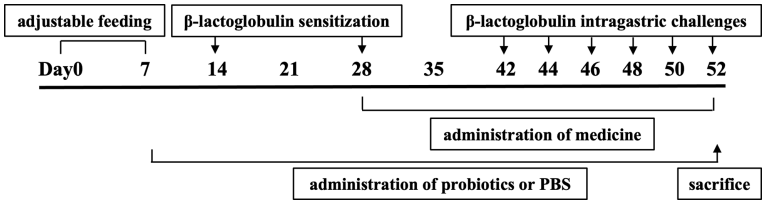

FIGURE 1
Protocol of mice sensitization and challenge.

which activates downstream signals when they bind to specific ligands. To explore the effect of the probiotics on TLR4 and downstream signal gene expression of DCs, gene expression of TLR4, Myd88, TRAF6, IκB, and NF-κB was detected. Compared with control mice, the expression of TLR4, Myd88, TRAF6, IκB, and NF-κB genes (Figure 7) was significantly increased after KV9 and FN3 supplementation; thus, the probiotics indicate activation of the TLR4-NF-κB signaling pathway.

Effect of oral probiotics on regulating T-cell differentiation and function

T cells play an important role in the food allergy process, especially in the Th1/Th2 immune balance (21). To investigate whether T-cell function changed after ingestion of the probiotics, spleen cells were isolated from mice after the last oral challenge, and their ability to produce Th1-related cytokine IFN-γ and Th2-related cytokine IL-4 *in vitro* was tested. Compared with control mice, the proportion of IFN-γ/IL-4 (Figure 8) in a splenic cell culture supernatant of allergic mice was significantly reduced. Oral administration of KV9 and FN3 increased the proportion of IFN-γ/IL-4 significantly compared with the allergic mice, but differed from mice in the control group. To directly evaluate the T-cell differentiation regulated by the probiotics, the percentage of Th1 cells and Th2 cells was measured by FACS (Figure 9). IFN-γ and IL-4 need to be stained after the Intracellular Fixation and Permeabilization Buffer Set treatment, which affects the expression of CD4+; thus, this expressed CD4+ gate by CD3+CD8−. The results showed that CD3+CD8−IL-4+T cells were predominant in the spleen of allergic mice and the proportion of CD3+CD8−IL-4+T cells in the spleen of KV9 and FN3 supplement mice decreased while CD3+CD8−IFN-γ+T cells increased, confirming that the

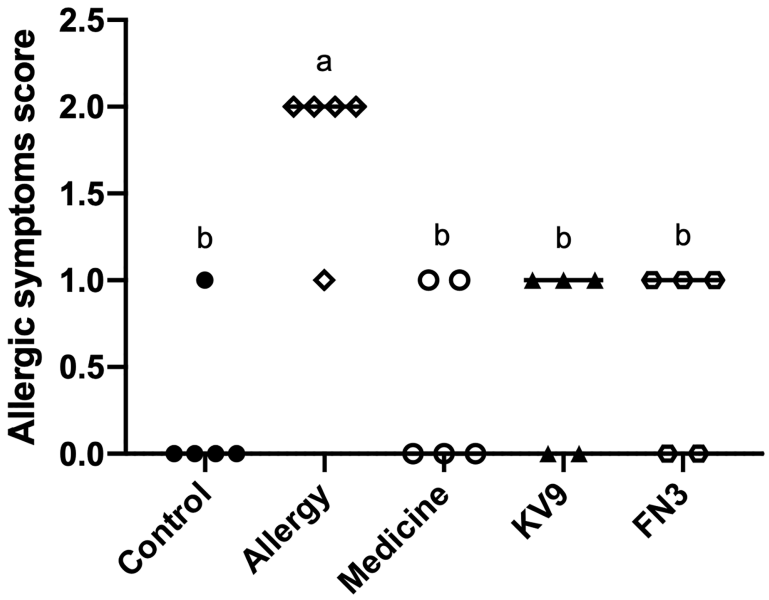

FIGURE 2
Allergic symptom scores in mice. Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

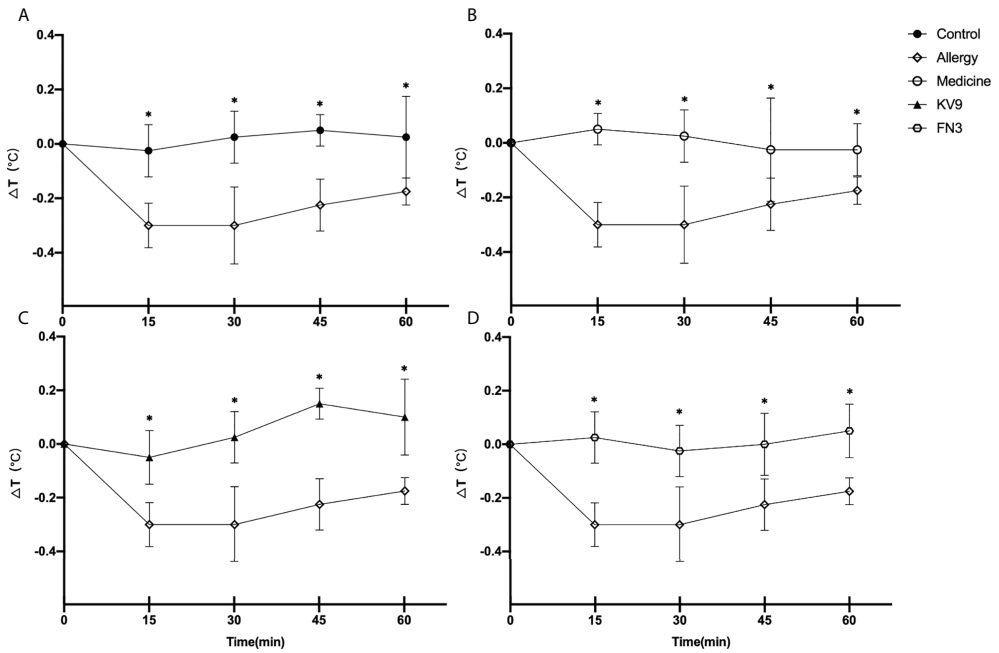

FIGURE 3
Changes in core body temperature at indicated time points following first challenge with β-LG in control **(A)**, medicine **(B)**, KV9 **(C)**, and FN3 **(D)** group mice compared with mice in the allergy group (*$p < 0.05$).

probiotics skewed the Th1/Th2 immune balance toward Th1 and thus suppressed downstream allergic reactions. This is similar to the result of exocrine cytokines from the cell body.

Discussion

The number of patients diagnosed with food allergies has been increasing in many countries. Evidence has shown that probiotics can exert a variety of probiotic effects by adhering to intestinal endothelial cells, producing antibacterial metabolites, competing with pathogenic microorganisms for nutrition, strengthening the epithelial barrier, changing the immune response pathway, and regulating the immune system (22, 23). In recent studies, probiotics such as *B. longum* and *Lactobacillus plantarum* have shown antiallergic potential in both animal models (12, 24). In the previous study of our laboratory, six probiotics capable of regulating the secretion of IFN-γ/IL-4 by peripheral blood mononuclear cells were screened out from 48 strains. Through the food allergy mouse model, two probiotics with good ability to alleviate food allergy were selected, namely, KV9 and FN3. In this study, oral administration of KV9 and

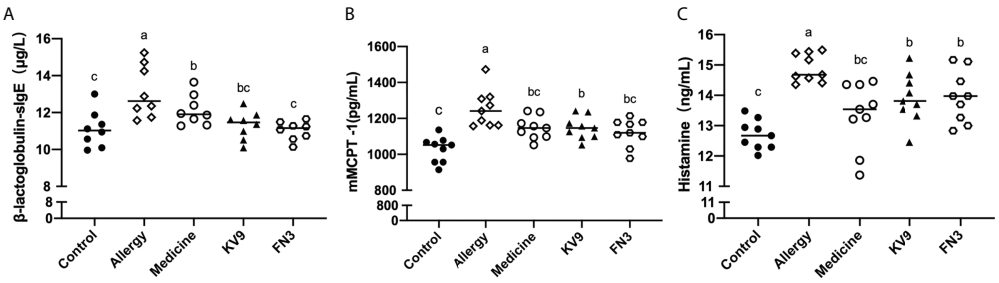

FIGURE 4
β-LG-sIgE **(A)** in the serum of mice. mMCPT-1 **(B)** in the serum of mice. Histamine **(C)** in the serum of mice. Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

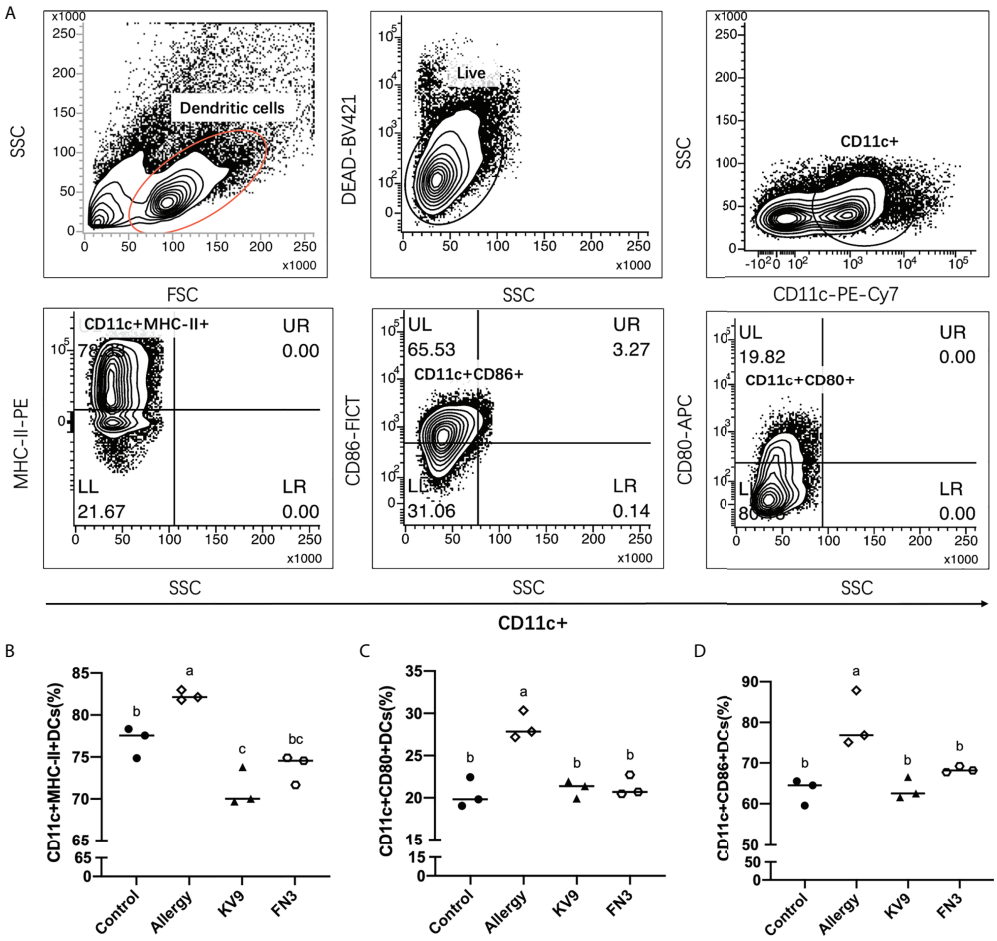

FIGURE 5
The effect of probiotics on DCs. The gating strategy **(A)** and the effect of oral administration of KV9 and FN3 on populations of CD11c+MHC-II +DCs **(B)**, CD11c+CD80+DCs **(C)**, and CD11c+CD86+DCs **(D)** in the intestinal lymph node from different groups of mice. Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

FN3 reduced the score of allergic symptoms, the production of β-LG-sIgE in serum, the activation of mast cells, and the concentration of histamine in serum, and relieved the decrease of core body temperature, confirming that KV9 and FN3 were functional probiotics that can suppress allergy in mice. The exploration of the mechanism found that the probiotics induced the immature state of DCs, and significantly increased the secretion of IL-12 in mouse DCs through activating the TLR4-NF-κB signaling pathway; IL-12 is a central cytokine for the development of Th1 cells (25), resulting in the immune balance of T cells being skewed towards Th1-dominated direction.

Food allergy is often associated with Th2-skewed immune responses; IL-4 as a marker of Th2 cells promotes IgE production by B cells, while Th1 cells' cytokine IFN-γ can inhibit Th2 cells' function (5). Oral administration of KV9 and FN3 can reduce the level of β-LG-sIgE in serum of allergic mice; IgE is the main immunoglobulin in mast cell degranulation, indicating that the two probiotics play an immune regulatory role. The DCs are the most powerful APCs, the initiator and modulator of the immune response. DCs support and fine-tune antigen presentation through costimulatory molecules and cytokines. Depending on the conditions, DCs can stimulate the growth and activation of a variety of T cells, thereby having varying effects on the immune response (14). It is well known that the function of naïve T cells needs two specific signals from APC to be activated (26). The first specific signal is provided by the interaction between T-cell receptors and the major histocompatibility complex (MHC)-II on APC. The second signal is provided by the interaction between APC-provided costimulatory molecule ligands and CD28 and CTLA-4 receptors on T cells. Inhibition of costimulatory molecules on cell surface or intracellular signal transduction can induce T cells to have no response (27). The interaction between B7-1(CD80)/B7-2(CD86) on APC and CD28 determines whether T-cell reaction occurs (28). DCs have

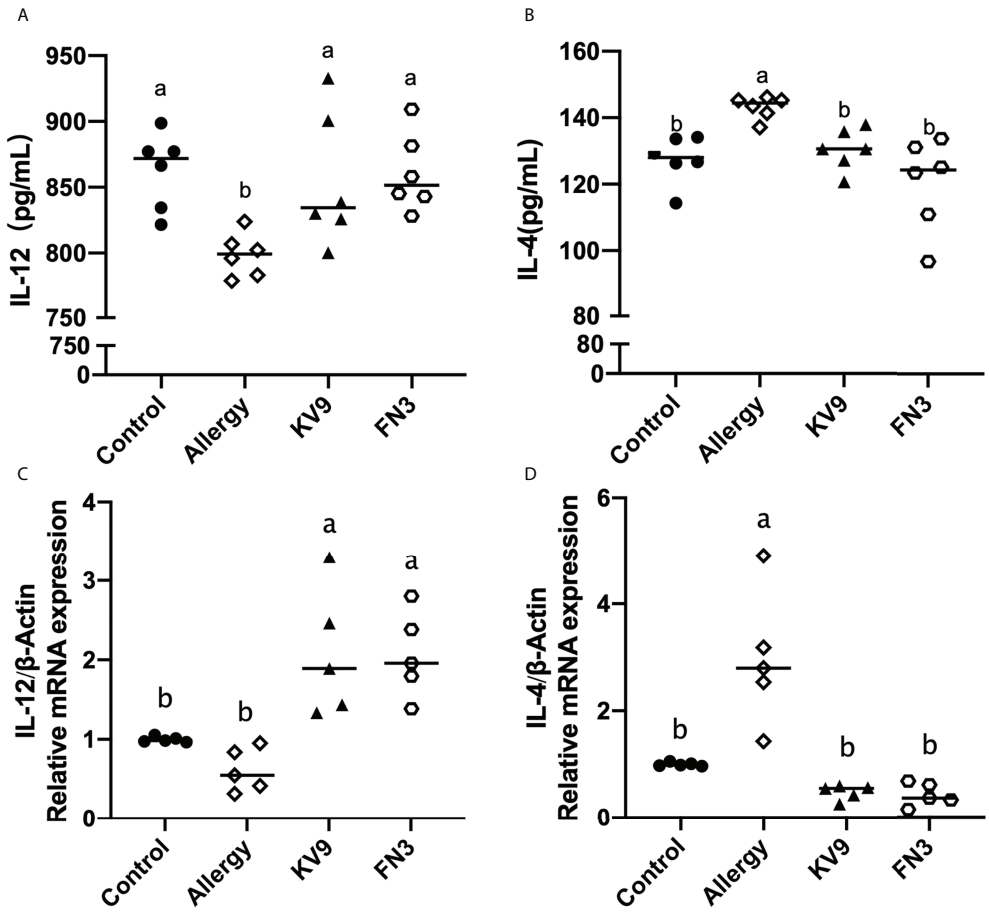

FIGURE 6
The IL-12 **(A)** and IL-4 **(B)** cytokine levels secreted from DCs and gene expression of IL-12 **(C)** and IL-4 **(D)** in DCs of each group of mice. Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

strong migration ability when they are immature, and DCs have strong antigen presentation ability when they are mature, which leads to a greater risk of food allergy (13). Furthermore, the expressions of DCs' surface MHC-II and CD80/CD86 can be used as a marker of maturation (29). In the presence of mature DCs and the IL-12, they produce T cells that tend to become Th1 cells, which secreted IFN-γ, while the DCs induced T cells to differentiate into Th2 cells that secreted IL-4 and IL-5 under the action of IL-4 (13, 25). The expression of costimulatory molecules and cytokines in DCs depends on signals from their environment, including pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns, and cytokines (30). The key mechanism of probiotics-mediated immune regulation may be the regulation of DCs' function. In a previous study, exposure of bone marrow-derived mouse DC to irradiated *L. casei* resulted in strong induction of IL-12 secretion, a potential strain that stimulates T cells to polarize into Th1 in the intestinal tract (14). The aforementioned study also revealed that *L. reuteri* can induce high expression of B7-2 (CD86) costimulatory in DC to polarize T cells into Th2 cells (14). Huang et al. confirm that oral *Lactobacillus murinus* suppressed the expression of OX40L in duodenal, a DC-derived costimulatory molecule known to promote Th2 responses, and indicates that DCs' functions are modulated (31). Furthermore, Ma's research on oral probiotics supplementation on DCs induced CD103+DCs' expression, which promoted differentiation of FoxP3+Tregs (32). In this study, the expression levels of MHC-II, CD80, and CD86 decreased in the CD11c+DCs in the intestinal lymph nodes of each group of mice after oral KV9 and FN3. This implied that immature DCs increased and the antigen presentation ability of DC was positively correlated with its maturity (33). At the same time, the secretion of IL-12 in DCs significantly increased after oral administration of KV9 and FN3 compared with allergic mice.

IL-12 is a central cytokine in the activation of immunity in the generation of Th1-type responses; defective IL-12 production was associated with lack of NF-κB activation (34). A study revealed that mice with TLR4 deficiency after allergen

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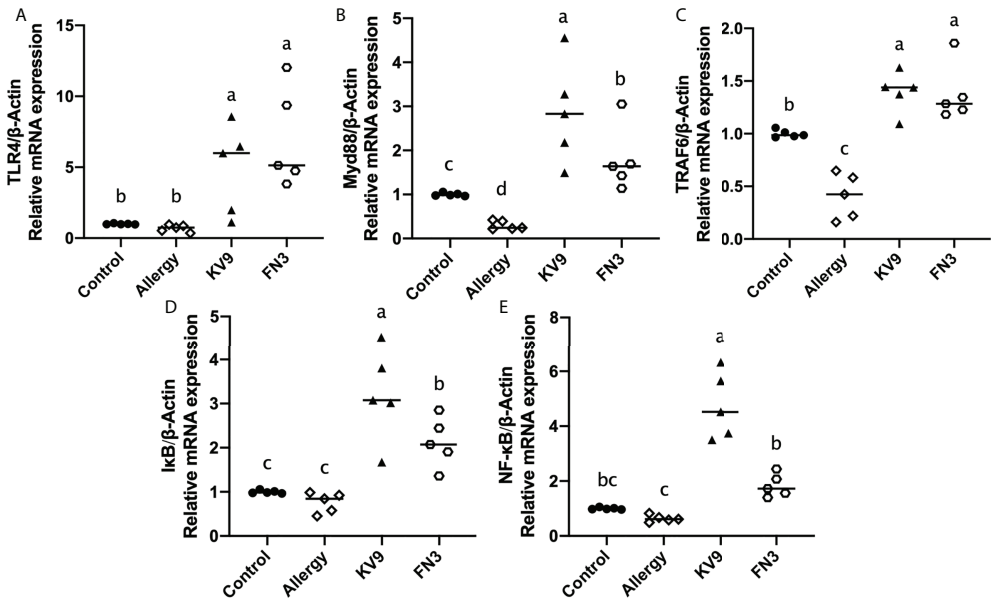

FIGURE 7
Gene expression of the TLR4-NF-κB signal pathway in DCs. The results were quantified as the density ratio between the gene of interest and the reference standard (β-actin). Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

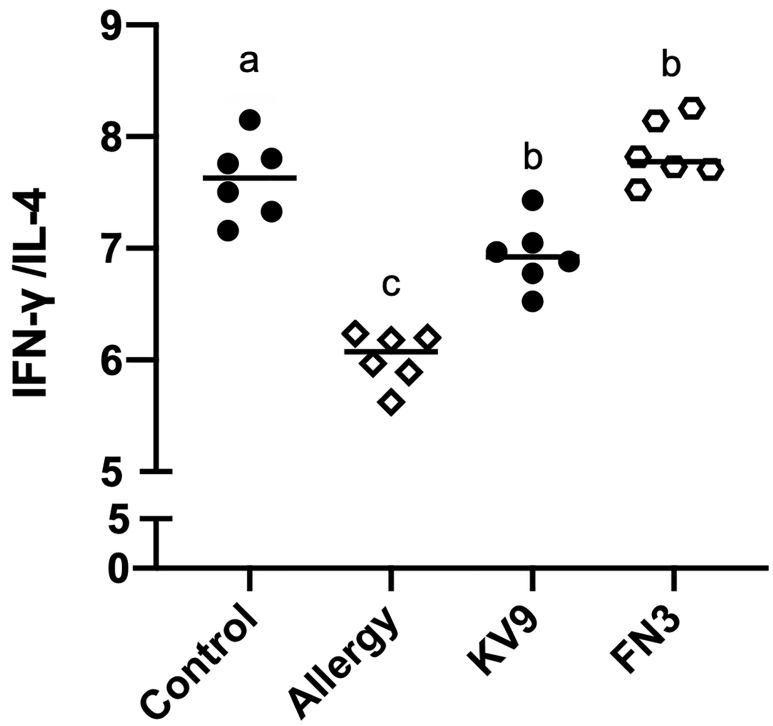

FIGURE 8
The ratio of IFN-γ (pg/ml)/IL-4 (pg/ml) cytokine levels secreted from spleen lymphocytes. Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

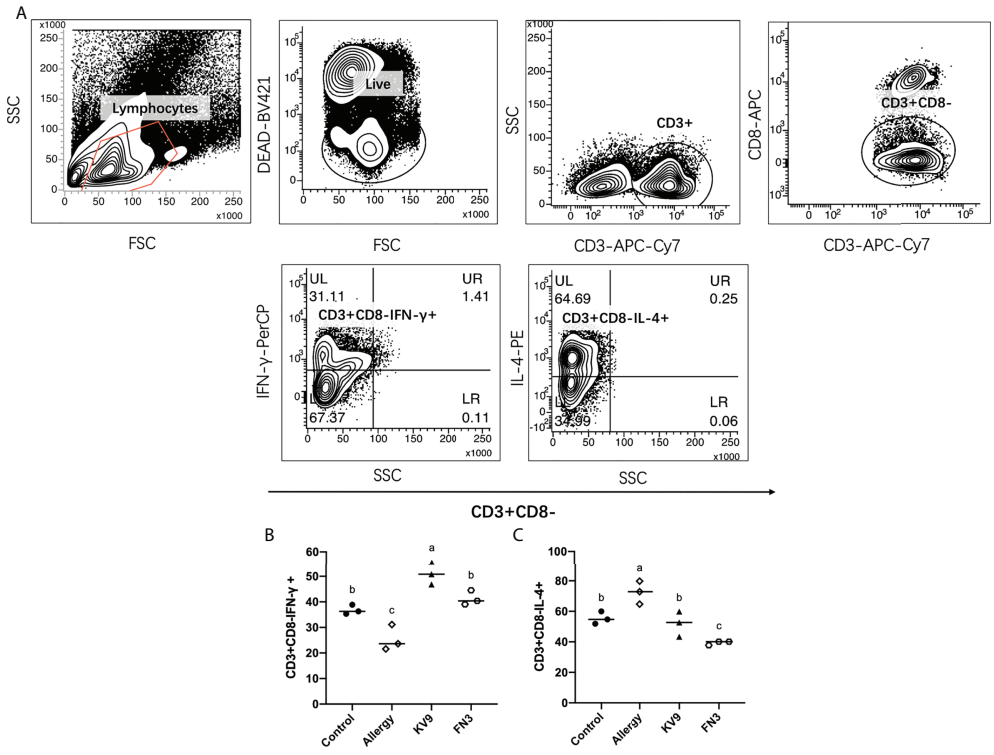

FIGURE 9
The effect of probiotics on T cells. The gating strategy **(A)** and the effect of oral administration of KV9 and FN3 on populations of CD3+CD8−IFN-γ+ T cells **(B)** and CD3+CD8−IL-4+ T cells **(C)** in the spleen from different groups of mice. Identical lowercase in the figure indicates the lack of significance ($p < 0.05$).

stimulation showed food allergy and immune tendency to Th2 (35). The author observed that after killing the intestinal flora of both healthy mice and TLR4-deficient mice with antibiotics, the mice showed food allergy. In addition, the healthy mice showed oral tolerance to food antigens, while allergy reactions persisted in the TLR4-deficient mice, indicating that intestinal microbes play an important role in food tolerance through the TLR4 signaling pathway (35). However, C3H/HeJ mice with corrected TLR4 alleles were sensitized and challenged with peanuts in a study (36), revealing that mice were resistant to anaphylaxis after the oral peanut challenge; restoring TLR4 function in mice did not protect them from anaphylaxis. A similar conclusion was obtained in Berin's study, which revealed that TLR4-deficient mice did not show Th2 immune advantage and food allergic reaction (37). Recent research suggested that TLR4, as an upstream target of the NF-κB pathway, plays an important role in oral tolerance to food antigens. In a study on *L.* casei, Zhang provided an intervention to achieve tolerant cell CD4+CD25+Treg differentiation by regulating NF-κB signal in which TLR2 and TLR4 were activated (16). Notably, in our research, the activation of TLR4-NF-κB signaling pathway by KV9 and FN3 in DCs resulted in the significant increase in the secretion of IL-12. This suggests that the supplementation of KV9 and FN3 enabled DCs to induce T cells to differentiate into Th1 cells.

In line with this notion, each group of mouse spleen lymphocytes in FACS analysis was extracted to determine whether oral KV9 and FN3 had an impact on allergic mice T-cell function and differentiation. The results showed a significantly lower proportion of Th1 cells in allergic mice spleen and a higher proportion of Th2 cells compared with mice in the control group. KV9 and FN3 supplementation increased the Th1 proportion, decreased the Th2 proportion significantly, and regulated T-cell cytokines of the IFN-γ/IL-4 secretion ratio in the supernatant of mouse spleen cells cultured *in vitro*. These results indicated that KV9 and FN3 alleviated the Th2 immune skew induced by food allergy.

In conclusion, our research demonstrated that *B. animalis* KV9 and *L. vaginalis* FN3 are potential probiotics against food allergy. The strains of KV9 and FN3 regulate DCs' development and IL-12 production to promote Th1 immune and reduce allergic reactions in mice. These findings suggest that strains KV9 and FN3 may be used as candidate probiotics to prevent Th2-mediated allergic diseases. The functional substances of KV9 and FN3 to relieve food allergy will be studied in the future.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of Ocean University of China.

Author contributions

Conceptualization: LZ and PG. Methodology: XT, LZ, and PG. Formal analysis: XT, RF, and HH. Supervision: LZ and PG. Data curation: XT and QC. Writing—original draft preparation: XT, XL, HY, and QL. Writing—review and editing: TL, KL, and ZZ. Project administration: LZ. Funding acquisition: LZ. 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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Bifidobacterium breve intervention combined with environmental enrichment alleviates cognitive impairment by regulating the gut microbiota and microbial metabolites in Alzheimer's disease mice

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Alzheimer's disease (AD) is characterized by behavioral and cognitive impairments and its increasing prevalence imposes a healthcare burden on society. To date, most intervention studies have only focused on a single AD-related factor and have yielded modest cognitive improvements. Here, we show that environmental enrichment (EE) training combined with *Bifidobacterium breve* CCFM1025 intervention significantly alleviated amyloid-β (Aβ)-induced cognitive impairment and inhibited neuroinflammation in mice. Moreover, we found that EE combined with *B. breve* CCFM1025 treatment restored AD-associated gut microbiota dysbiosis and reversed microbial metabolites changes. By integrating behavioral and neurological data with metabolomic profiles, we corroborated the microbiota–metabolite–brain interactions, with acetate and tryptophan metabolism as potential drivers. Taken together, our results provide a promising multidomain intervention strategy to prevent cognitive decline and delay the progression of AD through a combination of dietary microbiome-based approaches and lifestyle interventions.

KEYWORDS

Alzheimer's disease, *Bifidobacterium breve*, environmental enrichment, gut microbiota, acetate, tryptophan metabolism, gut–brain axis

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the deposition of amyloid plaques, the formation of neurofibrillary tangles, cognitive impairment, and neuroinflammation (1). Accumulating evidence from both animal models and human cohorts indicate that AD is related with gut microbiota dysbiosis, including quantitative and qualitative changes in bacterial diversity and microbiome composition (2). Among the differentially abundant bacteria, salutogenic taxa, such as *Bifidobacterium* and *Turicibacter*, are decreased in abundance, whereas pathogenic taxa, such as *Bilophila* and *Gemella*, are significantly increased in patients with AD compared with healthy individuals (3). Further research has demonstrated that the clinical indicators of AD are negatively correlated with the abundance of lactate- and propionate-producing bacteria, such as *Akkermansia* and *Bifidobacterium* (4). Indeed, as the gut microbiota has been shown to bidirectionally communicate with the brain, the gut–brain axis has been proposed (5). Moreover, mounting mechanistic evidence suggests that the gut microbiome impacts brain function through microbiota–gut–brain connections, which may be regulated by neuronal and immune-mediated signaling (6). At the intersection of neuroscience and microbiology, the gut microbiome is a dynamic entity that changes in composition and structure throughout the host's lifespan. Thus, the modulation of the intestinal microbiota through dietary intervention offers a novel strategy to delay the progression of AD.

As determined by metabolomic screening, the gut microbiome generates various small molecule metabolites to communicate with the host (7). Broadly, these microbial metabolites may be categorized into three types: 1) dietary metabolites unique to bacteria, including short-chain fatty acids (SCFAs) and amino acids; 2) bacteria-modified bile acids; and 3) host–bacteria co-metabolites, such as neurotransmitters (7). A large proportion of microbiota-derived metabolites are able to cross the blood–brain barrier (BBB) and thus enter the brain and affect brain function and host health (8). Recent technological advancements in metabolomic analyses have made it possible to detect microbiota-derived metabolites. Moreover, the development of multi-omics techniques has pushed the limits of the delineation of their regulatory mechanisms. Specific metabolites closely linked with cognition have been observed in the hippocampus, indicating that these metabolites may serve as active drivers of the microbiota–gut–brain interaction (9). Despite increased awareness of the potential function of the intestinal microbiota and microbial metabolites in AD, mechanistic links underlying the gut–brain interaction remain to be elucidated.

Lifestyle interventions to promote brain health, including diet, exercise, and social and intellectual stimulation strategies, can improve cognition in older individuals with AD (10). However, most previous intervention studies have only targeted a single factor and have yielded modest cognition improvements. Given the multifactorial aetiology of AD, multidomain interventions that target several risk factors and mechanisms simultaneously may be critical for an optimal preventive effect (10). Psychobiotics, which produce and deliver neuroactive substances, are a class of probiotics that benefit patients with psychiatric illnesses (11). As a major modulator of gut microbiota dysbiosis and brain function, the central role of selective psychobiotics in neurodegenerative diseases, through interactions with the gut–brain axis, is gaining broader acceptance (12). Environmental enrichment (EE) refers to the use of housing conditions that facilitate enhanced sensory, cognitive, motor, and social stimulation (13). Typically, EE for animals involves housing them in relatively spacious cages containing objects of different types and shapes (14). As a combination of cognitive training and physical exercise, EE has numerous protective effects on the brain, such as promoting adult hippocampal neurogenesis, improving memory and learning, and enhancing synaptic plasticity (13). Moreover, exposing mice to EE changed the gut microbiota and microbial metabolites; further, the transfer of fecal microbiota from EE-mice enhanced brain plasticity in standard environment mice (15). Thus, EE is considered to be a promising strategy to improve brain function and lower the risk of AD (16). However, few studies have assessed whether EE training combined with probiotic intervention ameliorates AD-associated pathology and behavioral symptoms in mice, and the underlying mechanisms remain poorly understood.

Bifidobacterium breve CCFM1025 is a promising psychobiotic strain that has been isolated from fecal samples of healthy humans (17, 18). It has been shown to have several neuroprotective effects against AD and depression, and the underlying mechanisms involve the suppression of neuroinflammation, modulation of the microbiota, and the ability to colonize the gastrointestinal tract (19, 20). The aim of this study was to investigate the effects of combined *B. breve* CCFM1025 with EE treatment on cognition and the gut microbiome and its metabolites in mice with AD. To this end, we established a mouse model of AD and evaluated the changes in behavioral deficits, neuroinflammation, and the diversity and composition of the gut microbiota after the exposure of AD mice to EE in conjunction with *B. breve* CCFM1025 intervention. We also identified significantly altered metabolome signatures and further assessed the role of microbial metabolites in regulating AD. Here, we report that combined EE and *B. breve* CCFM1025 significantly improves cognitive function and inhibits neuroinflammation by restructuring the gut microbiome and regulating acetate and tryptophan metabolism.

Materials and methods

Bacterial treatment

B. breve CCFM1025 was cultured under anaerobic conditions at 37°C in modified De Man, Rogosa, and Sharpe broth supplemented with 0.05% (w/v) L-cysteine. Freshly cultured bacterial cells were collected by centrifugation (8,000 × g for 10 minutes at 4°C), and then washed and resuspended with 30% (v/v) glycerol. Aliquot resuspended bacteria cells in sterilized tubes and store at -80°C until use. The gradient dilution method was used to determine the number of bacteria cells. For oral administration, each aliquoted bacteria sample was washed twice and resuspended in phosphate-buffered saline (PBS) to reach a concentration of surviving bacteria at 1×10^9 colony-forming units/mL.

Animal experiments

Male adult C57BL/6J mice (8 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained at the Animal Center of Jiangnan University as previously described (17). The experimental procedures were approved by the Animal Experimentation Ethics Committee of Jiangnan University (JN. No20200630c0960909[123]).

After acclimatization, the mice were divided into five groups. The AD model was established by intrahippocampal injection of an amyloid beta ($A\beta_{1\text{-}42}$) oligomer as previously described (17). The volume of intrahippocampal $A\beta_{1\text{-}42}$ injection was 1 μL at a concentration of 2 μg/μL, whereas mice in Control group received equal volume of PBS. The mice were either housed in standard laboratory cages without any stimulation or in an EE cage (4–5 mice per cage). The mice were administered 200 μL of bacterial suspension or sterile PBS as a vehicle control by gavage once a day for 6 weeks. The details of the treatment protocol for each group and the experimental timeline are shown in Figure 1.

Environmental enrichment

For EE, the mice were kept in large cages (2.893×104 cm^3) and provided with running wheels, a red transparent mouse house, colored plastic tubes, wooden blocks, pressed cotton squares, and objects of different shapes (14). All enrichment items were disinfected or sterilized. Some of the items in the EE cages were replaced once a week, but the running wheel was kept for the entire experimental period. The shape and arrangement of some of the items were changed weekly to increase novelty.

Behavioral tests

Behavioral tests were performed in a blinded manner to measure different types of learning and memory, and mice were allowed to rest between tests. As previously described (9, 17), we performed the following four behavioral tests: 1) a Y-maze to evaluate spontaneous and short-term working memory, 2) a Morris water maze (MWM) to assess spatial learning and memory, 3) a novel object recognition task to measure short-term recognition memory, and 4) a passive avoidance test to assess retention memory. The tests were conducted under dimmed lighting. The detailed protocol for behavioral tests is described in the Supplementary methods. All behavioral tests were recorded and analyzed using Ethovision 11.5 (Noldus, Wageningen, the Netherlands).

Fecal and tissue sample collection

After the completion of behavioral tests, the mice were placed in an empty cage until defecation. Fecal samples were immediately collected, snap-frozen, and stored at -80°C for subsequent microbiome and metabolome sequencing. The mice were then deeply anaesthetized and decapitated. Brains were collected and hippocampal tissues were dissected. Aliquoted hippocampal tissues were stored at -80°C for subsequent analyses.

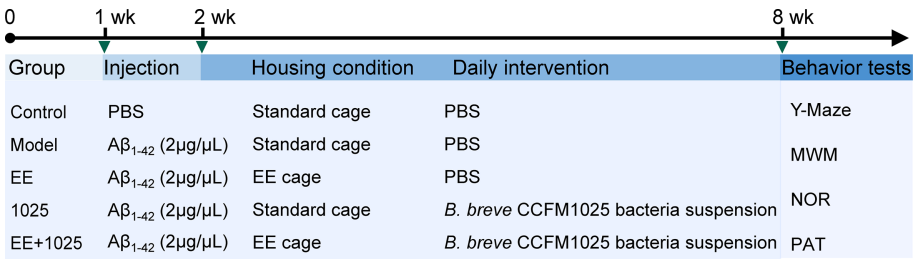

FIGURE 1
Schematic overview of the experimental design and study timeline. Vertical bars represent 1 week. wk, week; EE, Environmental enrichment; Aβ, Amyloid-β; PBS, phosphate-buffered saline; MWM, Morris water maze; NOR, novel object recognition; PAT, passive avoidance test.

Enzyme-linked immunosorbent assays

Hippocampal tissues were homogenized in pre-cooled PBS supplemented with protease inhibitor. The samples were centrifuged at 5,000 × g for 10 minutes at 4°C to collect supernatants. The supernatants were then used to measure $A\beta_{1\text{-}42}$, brain-derived neurotrophic factor (BDNF), and interleukin 6 (IL-6) levels. For the analyses of synaptophysin (SYP), fibronectin type III domain-containing protein 5 (FNDC5), and transforming growth factor-β1 (TGF-β1), the supernatants were further diluted in cold PBS. All of these protein levels were measured using enzyme-linked immunosorbent assay kits from Elabscience (Wuhan, Hubei, China) according to the manufacturer's protocol.

Fecal 16S rRNA sequencing and bioinformatic analysis

Microbial genomic DNA was extracted from fecal samples using a FastDNA Spin Kit (MP Biomedical, Irvine, CA, USA). The V3-V4 region of bacterial 16S rRNA was then amplified using a universal primer pair (341F and 806R). The differential detection of *Bifidobacterium* species was performed using primers designed to target the *groEL* gene. PCR products were purified and quantified using a Qubit dsDNA Assay Kit (Life Technologies, Invitrogen, Carlsbad, CA, USA). Purified amplicons were pooled and paired-end sequencing was performed on a MiSeq PE300 platform (Illumina, San Diego, CA, USA).

Raw data were processed using the QIIME tools version 2.0 and bioinformatic analyses of the microbiota were performed. α-Diversity was calculated based on species richness and evenness using Chao 1, Shannon, and Simpson indices. β-Diversity was assessed based on the Bray–Curtis distance and was visualized *via* principal coordinate analysis (PCoA). The differential abundance of microbial taxa was calculated using linear discriminant analysis effect size (LEfSe) and visualized using a taxonomic cladogram tree. Metagenomes of the gut microbiome were computed from 16S rRNA sequences based on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and functional pathways were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology.

Metabolomics

For fecal metabolomic analyses, metabolites were extracted as detailed in Mars et al. (21). The metabolite samples were analyzed with an UItiMate 3000 high performance liquid–chromatography (HPLC) system (Thermo Fisher Scientific, MA, USA) coupled to a high-resolution Q Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA). The detailed protocol for fecal metabolomics analysis, including metabolite sample preparation and the HPLC-MS analysis parameters are described in the Supplementary methods. The metabolomic data analysis were detailed in a previous report (19).

Fecal SCFAs, including acetate, propionate and butyrate, were extracted and quantified as previously described (22). For metabolites extraction, 30 mg fecal samples were weighed and soaked in saturated sodium chloride for 30 min. After homogenizing, 20 μL of H_2SO_4 (10% (v/v)) were added to each sample for acidification. Then, 800 μL of diethyl ether were added and mixed thoroughly. The supernatants were analyzed on gas chromatography–mass spectrometry (Thermo Fisher Scientific, MA, USA) with a Rtx-Wax column (column length: 30 m, inner diameter: 25 μm).

Statistical analysis

Statistical analysis was performed using Prism version 8.0.2 (GraphPad, San Diego, CA, USA) and SPSS version 22 (IBM, Armonk, NY, USA). Data are presented as mean values ± standard error of the mean or the median ± interquartile range. Before further statistical analyses, data were tested for normal distribution using the Shapiro–Wilk test and Q-Q plots. A one-way analysis of variance (ANOVA) with Holm–Sidak test or Student's t-test was used for the parametric analysis of differences between groups and a Kruskal–Wallis test followed by Dunn's test or Welch's t-test was performed for nonparametric data variance analysis. Values of $P < 0.05$ were considered to be statistically significant. Details of the statistical tests, the number of samples, and significance cut-offs are described in each figure legend. The network correlation between variables was determined using Spearman's correlation coefficients and visualized with Cytoscape version 3.8.2 (Institute for Systems Biology, Seattle, WA, USA).

Results

EE + *B. breve* CCFM 1025 treatment alleviates AD-induced cognitive and behavioral deficits

To explore the ameliorative effects of *B. breve* CCFM1025 combined with EE on AD-associated cognitive impairment, we performed behavioral tests in AD mice treated with or without either *B. breve* CCFM1025 or EE for 6 weeks after intersection hippocampal injection of $A\beta_{1\text{-}42}$ (Figure 1). In Y-maze tests, the total numbers of arm entries and spontaneous alternation behaviors were significantly lower in the $A\beta_{1\text{-}42}$-treated group than the sham treatment group (Figures 2A, B). Mice treated with EE or CCFM1025 alone showed a slight, but not statistically

significant, increase in the total number of arm entries (Figure 2A). Mice treated with EE alone failed to show improvement in alternation behavior, whereas those treated with *B. breve* CCFM1025 alone did show an improvement (Figure 2B). In contrast, mice treated with EE + *B. breve* CCFM1025 showed improved working memory in the Y-maze (Figures 2A, B). Similarly, in a passive avoidance test, EE or *B. breve* CCFM1025 treatment alone resulted in no observed improvements in latency time; however, mice treated with EE + *B. breve* CCFM1025 showed a markedly prolonged latency time compared with model mice (Figure 2C).

In the retention memory task of the MWM probe test, on day 6, all three groups receiving EE and/or *B. breve* CCFM1025 showed significantly shortened escape latency compared with model mice, whereas EE + *B. breve* CCFM1025 mice performed better (Figure 2D). Additionally, of all the groups tested, untreated AD mice spent the shortest amount of time in the targeted quadrant, whereas the time spent in the targeted quadrant was significantly extended for mice treated with EE + *B. breve* CCFM1025 (Figure 2E). In the novel object recognition task, AD mice exposed to EE, with or without *B. breve* CCFM1025 treatment, showed similar increases in discrimination ratio (Figure 2F).

EE + *B. breve* CCFM1025 treatment inhibits AD-related neuroinflammation and synaptic impairments in the brain

To compare the extent of brain damage in AD mice, we examined amyloid deposition in brain samples from each group. Significant accumulation of hippocampal $A\beta_{1\text{-}42}$ was seen in Aβ-injected model mice compared with sham-treated control mice (Figure 3A). Notably, oral *B. breve* CCFM1025 treatment decreased hippocampal $A\beta_{1\text{-}42}$ levels. Moreover, the accumulation of hippocampal $A\beta_{1\text{-}42}$ was significantly decreased in the EE-treated groups, with the EE + *B. breve* CCFM1025 group showing the greatest effect.

Next, to confirm whether EE and *B. breve* CCFM1025 treatments enhance synaptic plasticity, we measured the levels of BDNF, SYP, and FNDC5 in the hippocampal homogenates of Aβ-injected mice. We observed a highly

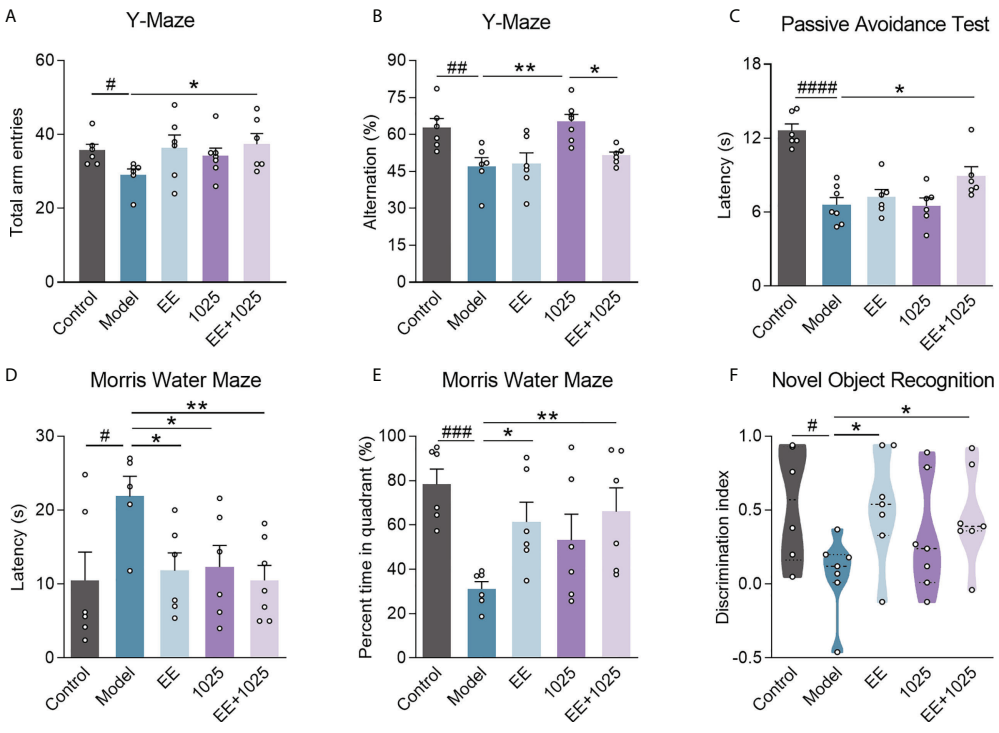

FIGURE 2
EE combined with *B. breve* CCFM1025 treatment alleviates AD-induced cognitive and behavioral deficits. Total arm entries **(A)** and spontaneous alternation behavior **(B)** in Y-maze test (n = 6-8). **(C)** Escape latency of passive avoidance test (n = 6-8). The escape latency **(D)** and percent time in target quadrant **(E)** during the probe phase (day 6) of the Morris water maze. **(F)** Discrimination index in the novel object recognition task (n = 6-8). Black horizontal dashed lines in violin plots depict medians, the bottom and top lines represent, respectively, the 25th and 75th percentile. Data are presented as mean ± standard error of the mean (mean ± SEM). Each data point represents one independent mouse. Control vs. Model: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ by unpaired student's t-test; $^{*}P < 0.05$, $^{**}P < 0.01$ as determined by one-way ANOVA.

significant increase in all three synaptic proteins in mice treated with EE compared with those housed in a standard cage (Figures 3B–D). Notably, mice treated with EE + *B. breve* CCFM1025 showed greater increases in the levels of hippocampal BDNF, FNDC5, and SYP than mice treated with EE only. Moreover, we found that treatment with *B. breve* CCFM1025 alone increased the levels of hippocampal SYP and FNDC5, but not the levels of BDNF. These findings indicated that the exposure of mice to EE was critical for enhancing synaptic plasticity.

To further explore the neuroprotective effect of EE combined with *B. breve* CCFM1025 on neuroinflammation, we measured cytokine concentrations in the brains of AD mice. The TGF-β1 levels increased in the hippocampal samples of mice in all three groups treated with EE and/or CCFM1025 compared with untreated AD mice housed in a standard cage (Figure 3E). Specifically, only treatment with EE + *B. breve* CCFM1025 significantly increased IL-6 levels in hippocampal tissues (Figure 3F). Given that TGF-β1 and IL-6 have been reported to regulate neurogenesis and are selectively elevated following exercise, EE-induced exercise combined with the psychobiotic potential of *B. breve* CCFM1025 may improve brain function under the pathological conditions of AD.

EE + *B. breve* CCFM1025 treatment improves gut microbial dysbiosis in Aβ-injected mice

Compared with control mice, gut microbiota dysbiosis in Aβ-injected model mice was observed using fecal 16S rRNA gene sequencing analysis. We analyzed α-diversity using Chao 1, Shannon and Simpson indices. While model mice showed no difference compared with control mice, oral treatment with *B. breve* CCFM1025 resulted in lower diversity and richness than EE group (Figures 4A–C). However, these decreased α-diversity indices in CCFM1025 group were significantly restored by EE+1025 treatment.

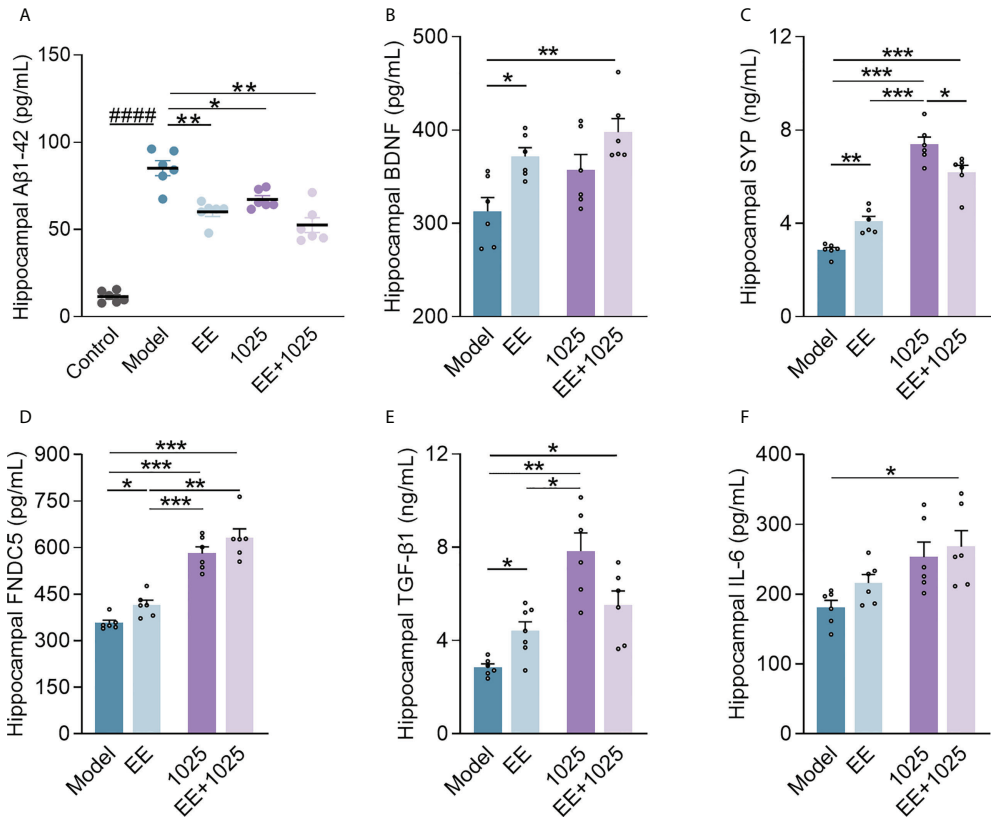

FIGURE 3
EE combined with *B. breve* CCFM1025 treatment inhibits AD-related neuroinflammation and synaptic impairments in the brain. **(A)** Hippocampal $A\beta_{1\text{-}42}$ levels (n = 6–8). **(B)** Hippocampal BDNF levels (n = 6–8). **(C)** Hippocampal SYP levels (n = 6–8). **(D)** Hippocampal FNDC5 levels (n = 6–8). **(E)** Hippocampal TGF-β1 levels (n = 6–8). **(F)** Hippocampal IL-6 levels (n = 6–8). Data are presented as mean ± standard error of the mean (mean ± SEM). Each data point represents one independent mouse. Control vs. Model: $^{\#\#\#\#}P < 0.0001$ by unpaired student's t-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as determined by one-way ANOVA.

To analyze the β-diversity of the gut microbiota, we performed PCoA based on the Bray–Curtis distance. Significant differences in gut microbiome structures and signatures were observed between the model and control groups (Figure 4D). Specifically, *B. breve* CCFM1025-treated mice showed a greater change in β-diversity than EE-treated mice, suggesting that *B. breve* CCFM1025 administration led to a dramatic change in the microbiome signature (Figure 4D).

At the phylum level, the most abundant taxa in each group of mice were Firmicutes and Bacteroidetes. Notably, there was a greater abundance of members of the Actinobacteria phylum in *B. breve* CCFM1025-treated mice than in Aβ-injected model mice. However, in EE-treated mice, the phylum Proteobacteria was enriched (Figure 4E). At the genus level, probiotic bacteria, such as *Bifidobacterium*, *Mucispirillum*, and *Ruminococcus*, were markedly enriched in the *B. breve* CCFM1025 group, whereas *Enterorhabdus* and *Gordonibacter* (classified in the order Coriobacteriales) were more prevalent in the EE + *B. breve* CCFM1025 group (Figure 4F). We also focused specifically on abundance of species from *Bifidobacterium* in each group of mice. Notably, the relative abundance of *Bifidobacterium* spp. was significantly different in each group (Figure 5A), and the abundance of *B. breve* was greater in in the *B. breve* CCFM1025-treated groups (Figure 5B), which may be due to the colonization of *B. breve* induced by its administration by oral gavage for 6 weeks. However, the abundance of *B. longum* was markedly higher in EE treated group as compared with other groups (Figure 5C). Importantly, EE alone or combined with *B. breve* CCFM1025-treated mice showed a decreased abundance of *B. pseudolongum*, which was a prevalent species in model mice (Figure 5D).

To gain insights into the potential pathways that may influence the host metabolic output, we performed KEGG pathway analysis of gut microbiome genes. Eleven pathways were identified differential between model and *B. breve* CCFM1025 groups, among which valine, leucine and isoleucine biosynthesis, and phenylalanine, tyrosine and tryptophan biosynthesis were statistically activated by *B. breve* CCFM1025 intervention ($P < 0.05$, Figure 5E). In contrast, of the seven differential pathways between model and EE + *B. breve*

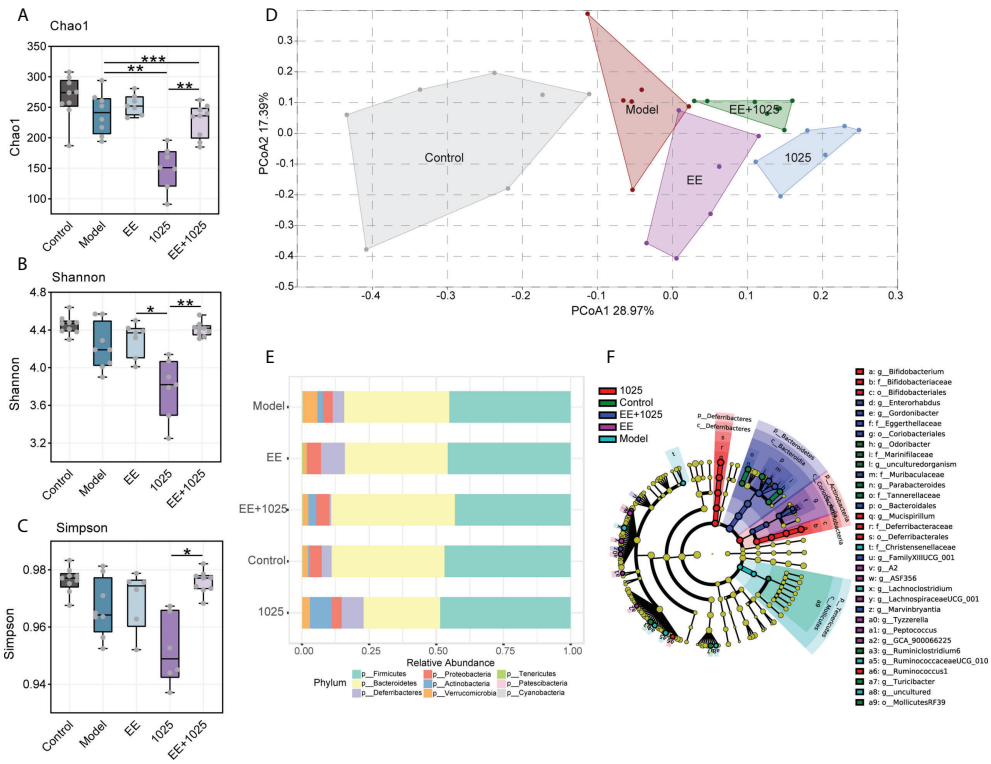

FIGURE 4
Changes in the diversity and structure of gut microbiome in different groups. **(A–C)** Microbial α-diversity, indicted by Chao 1, Shannon and Simpson indices (n = 6–8). In the box plot, the bottom and top are, respectively, the 25th and 75th percentile, a line within the box marks the median. Whiskers above and below the box indicate 1.5 interquartile range of the lower and upper quartile, respectively. Each data point represents one independent mouse. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as determined by one-way ANOVA. **(D)** Principal coordinates analysis (PCoA) based on Bray-Curtis distance (PERMANOVA, $P = 0.0001$; n = 6–8). **(E)** The relative abundance of bacteria at the phylum level (n = 6–8). **(F)** Linear discriminant analysis (LDA) effect size (LEfSe). Differential taxa are labeled with tags and annotated in the right panel. Data were computed with an LDA score above 2.00 and *P* value below 0.05 for the factorial Kruskal–Wallis test.

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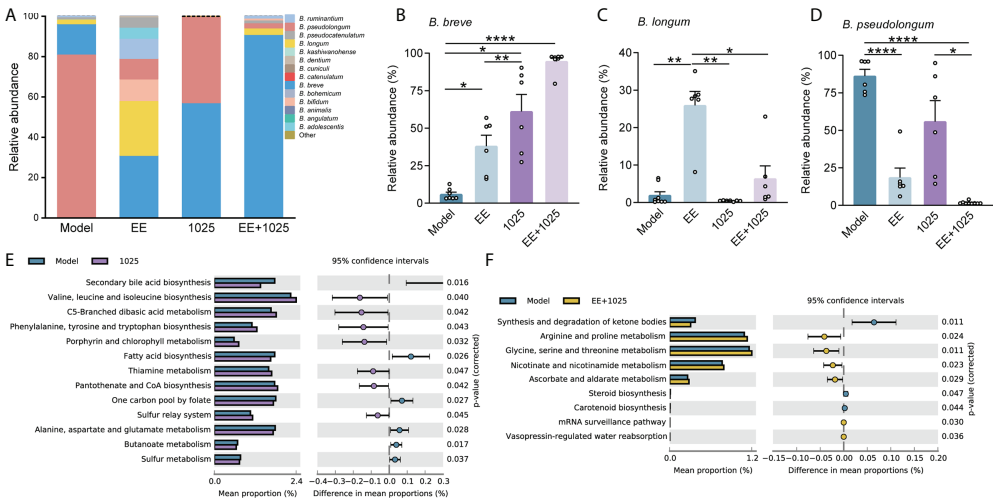

FIGURE 5
EE combined with *B. breve* CCFM1025 treatment alters the *Bifidobacterium* spp. and affects the gut microbiome on functional level. **(A)** Distribution of *Bifidobacterium* species in the *Bifidobacterium* genus (n = 6). **(B–D)** The relative abundance of *B. breve*, *B. longum* and *pseudolongum* (n = 6). Data are presented as mean ± standard error of the mean (mean ± SEM). Each data point represents one independent mouse. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ as determined by one-way ANOVA. **(E)** Differential microbial functions between model and CCFM1025 groups. **(F)** Differential microbial functions between model and EE+1025 treated groups. Statistical difference of the gene contents between the two groups is screened by Welch's-t test with $P < 0.05$.

CCFM1025 groups, tryptophan metabolism, arginine and proline metabolism, and glycine, serine and threonine metabolism were particularly upregulated in the EE + *B. breve* CCFM1025 group ($P < 0.05$, Figure 5F). Together, these metabolic outputs may be regulated by *B. breve* CCFM1025 alone or in combination with EE, predominantly related to amino acid metabolism.

EE + *B. breve* CCFM1025 treatment regulates microbial metabolites in Aβ-injected mice

Based on the role of the gut microbiome in regulating brain deficits induced by AD, we hypothesized that microbial metabolites may be potential factors that affect cognition. Thus, we first performed untargeted metabolomic analyses to detect candidate microbiota-derived metabolites in fecal samples of control, model, and EE + *B. breve* CCFM1025-treated mice.

A total of 5406 metabolites (3240 with defined names) were identified in fecal samples. Aβ-injection induced significant differences in fecal metabolites, with model and control group clustering separately (Supplementary Figure 1A). Among all of these metabolites matched to the database, we found the abundance of 87 metabolites were significantly altered between control, model, and EE + *B. breve* CCFM1025 groups (tested by one-way ANOVA, $P < 0.05$; Supplementary Figure 1B and Supplementary Table 1). Further, we performed multivariate analysis to identify discriminatory features between model and EE + *B. breve* CCFM1025 groups. Results from both orthogonal partial least squares-discriminant analysis (OPLS-DA) and hierarchical cluster analysis indicated distinct clusters of metabolites in fecal samples (Figures 6A, B, and Supplementary Table 2). As compared to model group, 44 differential metabolites (38 up-regulated and 6 down-regulated) were identified in the EE + *B. breve* CCFM1025 group by volcano plot (Figure 6C, Supplementary Figure 2 and Supplementary Table 3), thus contributing greatly to metabolome discrimination. Functionally, these differentially altered metabolites were enriched in three metabolic pathways in feces, predominantly relevant to tryptophan metabolism (Figure 6D). Notably, three tryptophan metabolites, including tryptophan, xanthurenic acid, and indole-3-acetic acid altered in model mice were restored towards control levels by EE+1025 treatment (Figures 6E–H). Moreover, widespread decreases in 5-hydroxyindole acetic acid in fecal samples were observed in EE + *B. breve* CCFM1025 group compared to model group.

Given the role of SCFAs, which are also essential microbiota-derived metabolites, in modulating gastrointestinal physiology and neurological function, we further detected the concentrations of SCFAs in fecal samples using a GC–MS technique. We found that the levels of propionate, butyrate, and acetate were significantly decreased in the feces of model mice compared with control mice (Figure 7A). Notably, acetate, propionate, and butyrate levels were increased in the EE + *B. breve* CCFM1025 group, with acetate levels showing a greater

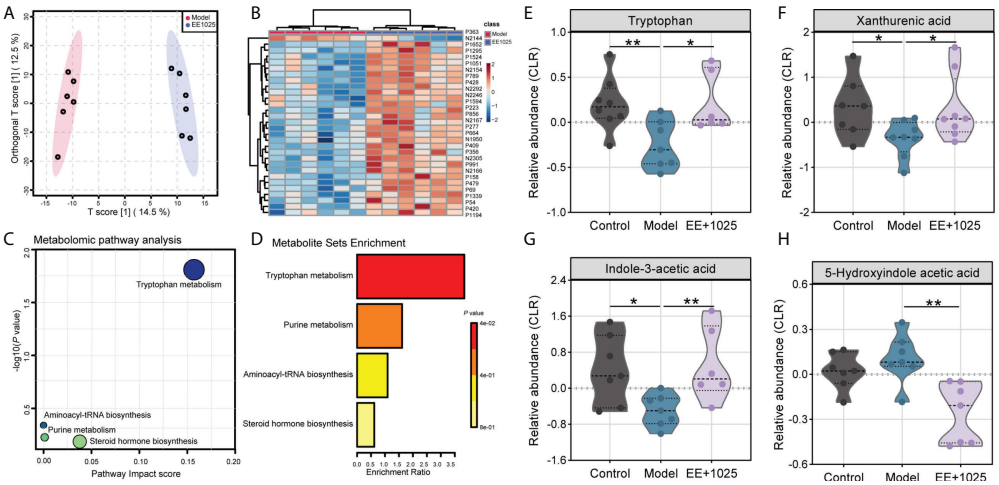

FIGURE 6
EE combined with *B. breve* CCFM1025 treatment regulates microbial metabolites in Aβ-injected model mice. **(A)** OPLS-DA score plot between model and EE + *B. breve* CCFM1025 groups. **(B)** Hierarchical cluster analysis of differential metabolites between model and EE + ***(B)*** *breve* CCFM1025 groups in fecal samples (top 30). The name of differential metabolites was listed in Supplementary Table 2. **(C)** Scatterplot showing results of MetaboAnalyst Pathway analysis using the Mus musculus (mouse) pathway library. **(D)** Overview of metabolite set enrichment in feces according to the KEGG database. **(E–H)** The relative abundance of tryptophan metabolites tryptophan, xanthurenic acid, indole-3-acetic acid, and 5-hydroxyindole acetic acid. The y axis shows CLR-transformed metabolite concentrations. Black horizontal dashed lines in violin plots depict medians, the bottom and top lines represent, respectively, the 25th and 75th percentile. *$P < 0.05$, **$P < 0.01$ as determined by Mann–Whitney test.

increase than propionate and butyrate levels (Figure 7A). These results indicated that treatment with EE + *B. breve* CCFM1025 modulated the levels of microbiota-derived metabolites, some of which may contribute to the altered levels observed in the brain, and thus, confer neuroprotective effects against AD-associated brain deficits.

Integrated analysis reveals that brain function is associated with changes in microbial metabolites

To identify putative microbiota–metabolite–brain interactions, we integrated behavioral and neurological data with metabolite levels using a correlation network. In this network, we found that specific metabolites differentially contributed to brain function (Figure 7B). Notably, the levels of $A\beta_{1\text{-}42}$ were negatively correlated with BDNF, acetate, and xanthurenic acid levels. Moreover, we found that the MWM latency was positively correlated with FNDC5 levels (Figure 7B). This may be partially due to that the EE + *B. breve* CCFM1025 treatment induced higher BDNF and FNDC5 levels are beneficial to brain function. In addition, strong positive correlations were also observed between indole-3-acetic acid level and behavioral improvements (Figure 7B). These results were consistent with tryptophan metabolite being key regulator of neurotransmitters and crucial modulator of cognition.

Discussion

In this study, we evaluated the effects of combined *B. breve* CCFM1025 and EE treatment on cognition, the composition of the gut microbiome, and microbial metabolite levels in AD mice. We found that cognitive impairment, neuroinflammation, and gut microbiota dysbiosis were key symptoms associated with the progression of AD. Compared with EE or *B. breve* CCFM1025 treatment alone, EE combined with *B. breve* CCFM1025 treatment showed a greater capacity to improve cognition and memory, reduce Aβ deposition, and inhibit neuroinflammation. Moreover, the findings from microbiome and metabolome analyses showed that EE combined with *B. breve* CCFM1025 treatment restored the structure of the gut microbiome, which was dysregulated in model mice, and altered microbial metabolite levels. Furthermore, metabolomic data integrated with behavioral and neurological data corroborated the microbiota–metabolite–brain interactions, with acetate and tryptophan metabolism as potential drivers. Given the numerous effects of EE and psychobiotics on cognition, behavior, synaptic plasticity, and microbial community structure, we propose that an intervention consisting of EE and *B. breve* CCFM1025 protects against cognitive deficits and slows the progression of AD, possibly by restructuring the gut microbiome and regulating acetate and tryptophan metabolism.

Amyloid accumulation is a core pathological hallmark of AD (1). In animal models of AD, intrahippocampal injection of $A\beta_{1\text{-}}$

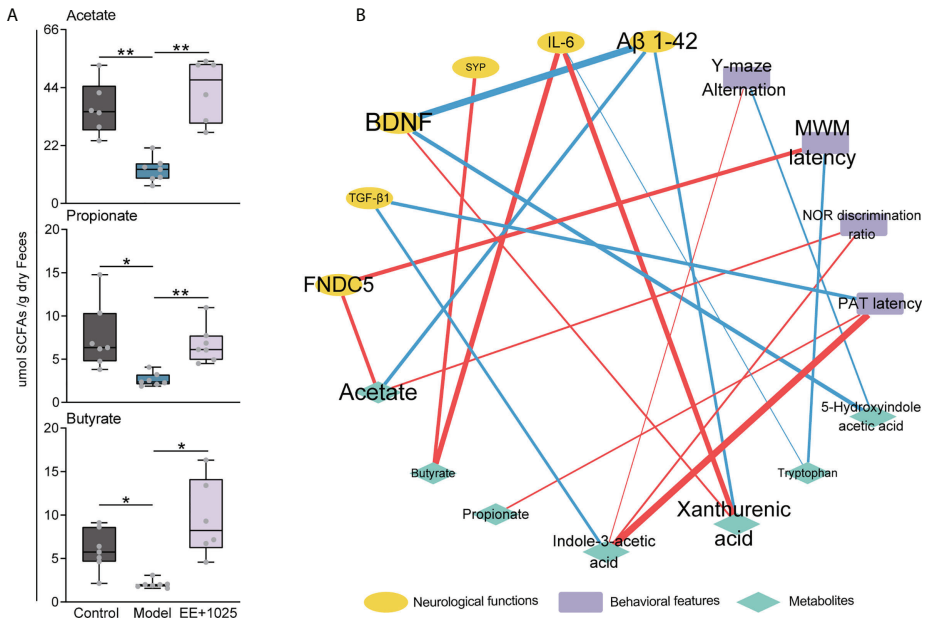

FIGURE 7
Integrated analysis reveals that brain function is associated with changes in microbial metabolites. **(A)** SCFA concentrations in feces. Data are presented as the median ± interquartile range. In the box plot, the bottom and top are, respectively, the 25th and 75th percentile, a line within the box marks the median. Whiskers above and below the box indicate 1.5 interquartile range of the lower and upper quartile, respectively. Each data point represents one independent mouse. *$P < 0.05$, **$P < 0.01$ as determined by one-way ANOVA. **(B)** Spearman correlation network containing behavioral features, neurological data and metabolites. Red edges indicate a positive correlation and blue edges indicate a negative correlation.

$_{42}$ mimics the Aβ-related neuropathological features of the AD brain, as its infusion can lead to behavioral alterations and cognitive deficits. Consistently, significant behavioral deficits, cognitive impairment, and synaptic damage were observed in Aβ-injected AD mice in this study. Recent studies have reported that psychobiotic interventions improve cognition and ameliorate depression in both animal and human studies (23). Additionally, previous studies have described the effect of exercise and probiotic treatment on cognition in different mouse models of AD (24–26). However, whether EE training alleviates AD-related pathology or probiotic administration has a neuroprotective effect have remained elusive. In this study, we confirmed that *B. breve* CCFM1025 treatment was sufficient to improve spatial reference memory, but its effect was not more significant than the effect of *B. breve* CCFM1025 + EE. However, *B. breve* CCFM1025 treatment was not sufficient to improve other forms of cognition. In contrast, EE combined with *B. breve* CCFM1025 treatment led to improved cognitive function in all four types of memory tests. The comprehensive improvements in cognition and memory may be due to the effect of EE, which provides the mice with more opportunities to perform a species-specific behavioral repertoire (14).

Previous studies have also confirmed the various positive effects of EE on the brain, including the promotion of adult hippocampal neurogenesis, improvements in learning and memory, and the enhancement of synaptic plasticity (13). Here, we found that EE-treated mice showed significantly decreased Aβ accumulation and highly elevated levels of BDNF, SYP, and FNDC5 compared with model mice housed in a standard cage, suggesting that exposure of mice to EE was critical for inhibiting neuroinflammation and increasing synaptic protein levels. Considering that these synaptic proteins are key modulators of synaptic plasticity, their increased levels may contribute to improved behavioral outcomes (27). Specifically, mice treated with EE + *B. breve* CCFM1025 showed increased levels of hippocampal BDNF, which is an essential neurotrophin that promotes many aspects of brain development and synaptic plasticity, whereas mice treated with *B. breve* CCFM1025 alone did not. This may be partially due to the fact that EE-induced exercise induces FNDC5, which stimulates the hippocampal expression of BDNF (28, 29). Similarly, only treatment of EE + *B. breve* CCFM1025 significantly increased the levels of hippocampal IL-6, another cytokine with selectively increased levels following exercise and which benefits cognition (27). As the components of EE include motor, sensory, cognitive, and social stimulation, no single factor explains the complexity underlying the multivariate effects of EE on the brain (13). Despite findings pointing to the effect of EE on

cognition and synaptic plasticity, a more integrative view from different mechanistic perspectives is required.

It has become increasingly apparent that the gut microbiota is closely associated with neurological diseases through gut–brain connections, which may be regulated by neuronal and immune-mediated signaling (6). Moreover, accumulating evidence from both animal and clinical studies has revealed that gut microbiota dysbiosis plays a critical role in the progression of AD (2). Here, we found that Aβ injection markedly altered the structure of the gut microbiota. *Bifidobacterium* spp. is commonly considered as probiotic species. EE + *B. breve* CCFM1025 administration significantly increased the relative abundance of *B. longum*, and decreased the relative abundance of *B. pseudocatenulatum*. These alterations are similar to those associated with healthy brain function in normal individuals (30). Notably, *Bifidobacterium* was shown to be positively linked to behavior and cognition in mice. Consistently, previous studies have reported that the relative abundance of *B. adolescentis* is significantly decreased in mice with AD (17). In addition, one recently published study concluded that *Akkermansia muciniphila* and *Bilophila wadsworthia* abundance are strongly linked with cognitive impairment in patients with PD (31). Moreover, mice in the *B. breve* CCFM1025 group showed a marked increase in the abundance of *B. breve*, which may be due to the colonization of *B. breve* induced by its administration by oral gavage (32). Taken together, these results suggested that AD-related gut microbiota dysbiosis was recovered upon *B. breve* CCFM1025 treatment, which was critically associated with gut colonization by *B. breve* and in turn, stability of the gut microbiome.

One potential mechanism by which the gut microbiome may affect brain function and host health is *via* the microbes' production of various metabolites, which convey signals from the gut to whole organs, including the brain (6). The most extensively studied microbiota-derived metabolites involved in the regulation of neurodegenerative diseases are amino acids and SCFAs (6). Amino acids are precursors for the biosynthesis of important neurochemicals and neurotransmitters, which can further affect the function of the central nervous system. A previous cohort study reported that people with AD have impaired amino acid metabolism (33) Similarly, functional prediction of gut microbiome genes revealed that metabolic outputs may be regulated by *B. breve* CCFM1025 alone or in combination with EE, predominantly related to amino acid metabolism. Furthermore, untargeted metabolomic profiles from fecal samples identified an important role of tryptophan metabolism. As one of the extensively studied amino acids, tryptophan can be metabolized by the gut microbiota. One recent study reported that hippocampal 5-hydroxyindole acetic acid levels increase with age (9). Here, we found that increased 5-hydroxyindole acetic acid levels were markedly restored by EE + *B. breve* CCFM1025 treatment, and were closely linked with improved cognition. In addition to tryptophan metabolites, SCFAs generated by the microbial fermentation of indigestible foods have also been reported to mediate gut–brain interactions to affect brain function. One recent study demonstrated that microbiota-derived acetate modulates the microglial phagocytosis of Aβ and disease progression in a mouse model of AD (34). Moreover, SCFA supplementation rescues cognitive and synaptic impairments, and alleviates microglial maturation defects. Here, the EE + *B. breve* CCFM1025-treated group showed a significantly higher concentration of acetate, which was suppressed in model mice. Numerous microbiota-derived metabolites are able to cross the BBB and thus, enter the brain and affect brain function (8). As such, EE + *B. breve* CCFM1025 treatment conferred neuroprotective effects against AD-associated brain deficits by modulating microbiota-derived metabolites, potentially driven by effects on acetate and amino acid metabolism.

There are some limitations of this study that should be addressed. First, we only demonstrated that EE training combined with *B. breve* CCFM1025 intervention provided a cognitive benefit to mice with AD. Whether these lifestyle intervention findings translate to patients with AD needs to be determined. Second, we primarily focused on the metabolic outcomes of EE combined with *B. breve* CCFM1025, but we are aware that, as an environmental factor, EE alone is critical for the progression of AD. Third, although we identified potential mechanisms from integrated data and correlation analyses, these experimental findings are not proof of causation. Finally, despite our increased awareness of the contribution of the gut microbiome and microbial metabolites to brain function, the mechanistic links and signaling molecules remain to be elucidated.

In conclusion, we demonstrated that EE training combined with *B. breve* CCFM1025 intervention attenuated AD-associated cognitive impairments and neuroinflammation. Furthermore, we found that the potential mechanism underlying these effects may involve the modulation of the gut microbiome and the regulation of acetate and tryptophan metabolism through gut–brain interactions. Although it is challenging to translate these findings to daily guidelines or clinical therapies, we provide an attractive multidomain intervention approach, with a combination of lifestyle-targeted and dietary microbiome-based interventions, to promote brain function and delay the progression of AD.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information repository, accession number PRJNA847039 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA847039).

Ethics statement

The animal study was reviewed and approved by the Animal Experimentation Ethics Committee of Jiangnan University.

Author contributions

Conceptualization, GZ and GW. data curation, JZ and WC. formal analysis: GZ. funding acquisition, GW and WC. investigation, GZ. methodology, HZ and MG. project administration, GW. resources, JZ and WC. software, MG. supervision, JZ and GW. validation, GW. visualization, GZ. writing – original draft, GZ. writing – review and editing, GW and WC. 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/fimmu.2022.1013664/full#supplementary-material

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Mucin-degrading gut commensals isolated from healthy faecal donor suppress intestinal epithelial inflammation and regulate tight junction barrier function

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The intestinal epithelium surface is covered by a layer of mucus that harbors a complex and dynamic population of bacteria termed gut microbiota. In particular, some gut bacteria have the ability to degrade the mucin glycan for nutritional sources. However, the bacterial diversity of mucin-degrading bacteria in human gut microbiota and their role in the gut remains unclear. In this study, we characterized the diversity of mucin-degrading bacteria in the human gut microbiota by an established cultivation-based molecular profiling method. The results showed the gut commensals having the mucin degrading ability were widely distributed in the gut microbiota and were more abundant than previously thought. In addition, many previously uncharacterized mucin degraders were isolated from faecals samples, suggesting the mucin-degrading gut commensals were underappreciated. To gain a better understanding of the interaction between these mucin-degrading gut commensals and the host, the effect of the commensals on intestinal epithelial cells were examined, and the results revealed that the commensals (8 *Bacteroides* spp., 2 *Parabacteroides* spp, *Akkermanisa muciniphila* and *Bifidobacterial dentium*) incited low level of inflammatory response (IL-8 and TNF-α) but suppressed the inflammatory response induced by *E. coli* through downregulating the NF-κB pathway. The presence of gut commensals also showed potential in enhancing the epithelial tight junction (TJ) barrier function through regulating the mRNA expression of TJ protein genes such as Zo-1, Occludin, Claudin-1 and E-cadherin. Furthermore, the presence of commensal bacteria *P. distasonis*, *B. thetaiotaomicron* and *A. muciniphila* completely or partly restored the pro-inflammatory cytokine IL-1β induced TJ barrier disruption. In conclusion, these findings indicate that mucin-degrading gut

commensals were widely distributed in the gut microbiota and showed anti-inflammatory effect against pathogen infection and potential in modulating the epithelial barrier function.

KEYWORDS

mucin-degrading bacteria, 16S rRNA gene sequencing, tight junction barrier, gut commensal, Transepithelial Electrical Resistance (TEER), intestinal inflammation

Introduction

The intestinal mucus layer acts as a line of defense that separates the luminal environment, including gut microbiota and other xenobiotics, from the host epithelial and immune cells (1). Intestinal mucin is mainly composed of a single highly O-glycosylated protein called mucin glycoproteins, with fucose or sialic acid terminating the glycan chains (2). Certain bacteria have developed various enzymatic machinery that can cleave and catabolize the sugar moieties for colonization. The fucosidase and sialidase activity of certain symbiotic bacteria, such as *Bacteroides thetaiotaomicron*, can liberate mucosal glycans without compromising the integrity of the mucus layer to support colonization (3). This ability helps the establishment of early colonization and offers ecological advantages to the gut commensals by providing an alternative endogenous source of nutrients when nutrition is depleted (4).

Once established in the intestine, the microbiota plays a vital role in human health and disease, influencing host immunity and metabolism. The impact is expected to be more direct for mucus-associated bacteria because of their proximity to the gut epithelial cells and immune system (3, 5). A typical example is *Akkermanisa muciniphila*, a recently isolated mucin degrader. Its abundance has been inversely correlated with the many diseases such as IBDs, type 1 diabetes mellitus, atopic dermatitis, autism, type 2 diabetes mellitus, and obesity (6). *A. muciniphila* has been proposed as a candidate next-generation probiotic because it has been demonstrated to possess many beneficial properties, including modulation of goblet cell number, mucus production, mucus barrier protection, and host immune modulation (7).

However, excess mucin degradation may disrupt the integrity of the mucosal layer and facilitate the access of luminal bacteria/antigens to the intestinal epithelial cells and immune system and then incite or exacerbate inflammation disease (8). Indeed, an increase in total mucosa-associated bacteria was observed in patients with inflammatory bowel disease (9). Furthermore, it has been reported that IBD patients have a disproportionate representation of mucin-degrading bacteria (10). A ~100-fold and >4-fold increase in *Ruminococcus torques* and *R. gnavus*, respectively, was observed in macroscopically- and histologically-normal intestinal epithelia in cases of both Crohn's disease (CD) and ulcerative colitis (UC). Together, these observations suggest the relevance of mucin degradation by the gut bacteria in human health and progress of the conditions such as gut inflammation disease. However, the systematic contribution of mucin-degraders in gut homeostasis and dysbiosis has not yet been thoroughly investigated, as only a limited number of mucin degraders and related enzymes have been studied (11).

In this study, we used a strategy combining both cultivation-dependent and -independent methods to characterize the diversity of human gut microbiota that can degrade mucin for growth. Concurrently, we isolated many potential, yet undescribed, mucin degraders from faecal samples. These mucin-degrading gut bacteria significantly suppressed the inflammatory response stimulated by *E. coli* and showed potential in modulating the epithelial tight junction (TJ) barrier function.

Materials and methods

Sample collection and process

Multiple faecal samples were collected from three healthy Hong Kong Chinese adults eligible for faeces donation for transplantation. A stringent set of criteria and screening as previously described was used to define its eligibility (12). Fresh faecal samples were kept on ice once being passed and were processed within two hours to preserve the viability of anaerobic bacteria as far as possible. Culture media and reagents for anaerobic cultures were placed in the anaerobic chamber (Bugbox Plus UM-017, Baker) overnight before use for pre-reduction of oxygen. The anaerobic chamber was held at 37 °C with 10% carbon dioxide, 10% hydrogen, 80% nitrogen. Weighted faecal sample (~ 1 g) was homogenized in reduced PBS by thorough vortex and then serially diluted tenfold down to 10^{-2}. Plate culture was performed in the anaerobic chamber by spread plating 100 µl of the respective faecal dilutions on each

petri dish. Mucin medium (MM) was prepared according to Wlodarska et al. (13) with some modifications and containing 0.5% porcine mucin (M1778, Sigma), 100 mM KH_2PO_4 (pH 7.2), 15 mM NaCl, 8.5 mM $(NH4)_2SO_4$, 4 mM L-cysteine, 11.1 mM vitamin K1, 15.3 mM hemin solution, 100 mM $MgCl_2$, 1.4 mM $FeSO_4$■$7H_2O$, 50 mM $CaCl_2$, 1% trace mineral supplement (ATCC), and 1% vitamin supplement (ATCC). Plates were incubated anaerobically at 37 °C for 7 days. On day 7, plates containing confluent growth of discrete colonies were manually scraped off the media surface and rinsed with 5 ml PBS solution in 10 ml tubes. The collected tubes were centrifuged at 5, 000 g for 30 min at 4°C. The pelleted samples were stored at -80 °C for DNA extraction.

Isolation and identification of potential mucin degraders

Fresh stool samples from the same subjects were used to make serial dilution and spread plate culture as described above. On day 7, colonies on the plates were picked and sub-cultured for identification by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonics). For the isolates that identification scores below 1.7 were further identified by full-length 16S rRNA gene sequencing using one degenerate primer: 7F (5'-AGAGTTTGATYMTGGCTCAG-3') forward primer and 1510R (5'-ACGGYTACCTTGTTACGACTT-3') (14).

The mucin degrading ability of the isolates was tested by measuring the amount of mucin degraded in the supernatant using the anthrone reagent test as described before (10). In brief, after 72 h anaerobic culture in MM broth culture, an aliquot of 50 µL of the supernatants were loaded in 96-well microplate followed by 150 µL of anthrone reagent (0.1%). Subsequently, plates were incubated 20 min at 100°C in water bath followed by a cooling step treatment at room temperature before reading absorbance at 620 nm. Measurements were taken in triplicates. Colorimetric absorbance was compared to a standard curve based on glucose, and total carbohydrate content was expressed as µg/mL of glucose. Anthrone reagent tests were repeated three times.

Faecal DNA extraction, 16S rRNA gene sequencing and analysis

Faecal DNA was extracted using the DNeasy Power soil kit (Qiagen). Following extraction, the V3-V4 variable regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) using forward (341F: CCTACGGGNGGCWGCAG) and reverse (806R: GGACTACNVGGGTWTCTAAT) primers (15). A second PCR was applied to add the sequencing adapters and multiplex indices. PCR products were purified using the MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology). 2 × 300 bp sequencing was performed on an Illumina MiSeq platform (outsourced to the Genomics Resource Core Facility at Cornell University, New York). Approximately 40,000 reads were sequenced per sample. QIIME 2 pipeline (v2019.1) was employed to process the raw sequencing files (https://qiime2.org/). Briefly, paired-end demultiplexed fastq sequence data were imported into QIIME and de-noised by DADA2 workflow (16). OTUs with a total frequency of less than 10 was filtered from the OTU table produced by DADA2 since the number of reads between replicates was less reproducible below this depth (17). For taxonomy classification, a pre-trained classifier based on Silva database (https://www.arb-silva.de/download/archive/qiime/) was used to assign taxonomy to the representative sequences. Phylogenetic trees and dendrograms were visualized using iTOL.

Intestinal epithelial cell (IEC) culture

The colorectal adenocarcinoma cell line HT-29 cells and Caco-2 cells were maintained at 37°C in 5% CO_2 and 95% air in Dulbecco's Modified Eagle Medium (DMEM) and McCoy 5a media, respectively. 10% inactivated fetal calf serum (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich) were supplemented as necessary. The medium was changed every second day. At ~80% confluence, HT-29 cells were harvested by adding trypsin-EDTA solution (Invitrogen) (0.25% [wt/vol] trypsin–1 mM EDTA) and counted in a hemocytometer before seeding in 24-well tissue culture plates (Costar) at a concentration of 5×10^4 cells/well. HT-29 cells were cultured until the complete confluence had been reached. Complete growth medium without the antibiotics was added, and the assay was performed the following day. Caco-2 cells were seeded on 12-well transwell inserts (12 mm diameter, 0.4 µm pore size, Polyester Membrane, Costar) at a concentration of 1×10^5 cells/mL. The cells were cultured until the cell monolayer had been formed and differentiated, i.e., around 14-17 days post seeding according to previous publications (18, 19). At this stage, the transepithelial resistance between the apical and basolateral surfaces of the cell monolayers reaches a relatively stable status and is in the appropriate growth phase to evaluate how it is affected by external factors.

Bacterial treatment

Prior to bacterial treatment, the inoculums of the commensals were centrifuged at 10,000 g for 10 min. The bacterial pellet was suspended in PBS and adjusted to McFarland 1.0 which equals a bacterial concentration of around 3×10^8 CFU/mL. The bacterial suspension was centrifuged and then re-suspended in cell culture media for

bacteria-cell co-culture with a multiplicity of infection (MOI) of 100.

Prevention and competition assay

Prevention and competition assay procedures were employed to evaluate the protective effect of the gut commensals on the IEC against pathogenic *E. coli* NCTC9001 infection. For the competition assays, gut commensals and *E. coli* NCTC9001 were added to the HT-29 cells and incubated at 37 °C for 2 h. For the prevention assays, cells were preincubated with gut commensals at 37 °C for two hours. Following incubation, the unattached bacteria were removed by washing the monolayers with sterile PBS three times and then *E. coli* NCTC9001 was added. Cell monolayer co-culture with gut commensals or *E. coli* NCTC9001 was used as a control. Cells were then detached from the wells by incubating with 100 uL trypsin-EDTA per well for 5 min at 37°C. The bacteria attached to the cells were determined by the plating counting method after serial 10-fold dilutions. Adhesion results are presented as percentage of adhesion, as obtained by dividing the final count of attached bacteria by the initial bacterial number applied to co-culture.

Cell RNA extraction and qRT-PCR

Total RNA from the cells was extracted using TRIzol reagent (Life Technologies, CA, USA) according to the manufacturer's manual instructions. TURBO DNA-free™ Kit (Ambion, TX, USA) was used to remove genomic DNA in RNA before cDNA synthesis. The cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen, CA, USA) following the manufacturer's instructions. TJ- and immune response-related genes were quantified using SYBR Green PCR Master Mix (Invitrogen, CA, USA). The primer sequences are listed in Tables S1, S2. The amplification program consisted of a pre-cycling hold at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 60°C for 30 s. Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, MA, USA) was used to analyze the mRNA expression levels. The gene's mRNA expression was normalized to the levels of 18s rRNA and expressed as the fold change relative to untreated cells using the $2^{-\Delta\Delta Ct}$ method. Reactions were performed in at least three replicates.

Measurement of Transepithelial Electrical Resistance

Transepithelial Electrical Resistance (TEER) is an indicator of epithelial paracellular permeability to ionic solutes and was used to assess intestinal barrier function. The TEER values were measured using a Millicell Electrical Resistance System (ERS-2) meter (Millipore Corporation, Bedford, MA, USA). The TEER value of the cell monolayer is calculated by the equation: TEER = (Rm – Ri) × A. Rm is the transmembrane resistance of the treated group, Ri is the intrinsic resistance of a cell-free transwell, and A is the surface area (cm^2) of the membrane of the insert. The protective effect of gut isolates on *E. coli*-induced intestinal permeability dysfunction was measured as follows: differentiated Caco-2 cell monolayers were treated with pathogenic *E. coli* together with gut isolates, the mixture was incubated for 2 h. The TEER value was measured before the addition of the bacteria (time zero) and then at various time intervals and expressed as the ratio of TEER at time '*t*' in relation to the initial value (at time zero) for each series. In parallel, cell monolayers mono-cultured with the respective strains represented the control for each experiment.

Immunofluorescence staining

After bacteria-cell co-culture, Caco-2 cell monolayers were washed with PBS before being fixed with 100% ice-cold methanal at -20 °C for 5 min and permeabilized with 0.5% Triton X-100 in PBS room temperature for 10 min. The monolayers were then washed with PBS and blocked for 1 hour with 4% (w/v) bovine serum albumin (BSA) at room temperature. The cells were then incubated with either antibody of Zo-1(Cell Signaling Technology,13663S), Occludin (Cell Signaling Technology, 91131S), or E-cadherin (Cell Signaling Technology, 3195S). Cells were fixed with 4% paraformaldehyde when staining the Claudin-2 with Claudin-2 (Invitrogen, 51–6100) primary antibodies. The cells were rinsed again with washing PBS three times, followed by incubation with the Alexa Fluor 594 goat anti-rabbit secondary antibody (Cell Signaling Technology, 8889S) for 1 h at room temperature. The cells were rinsed again with PBS before the membranes were separated from the Transwell insert using a scalpel. The membrane was finally mounted with cell side up between a slide and coverslip with UltraCruz® Aqueous Mounting Medium (Santa Cruz Biotechnology). The mounting media contains DAPI to stain all the nuclei. The microscopy of the mounted membranes was performed on a Nikon Confocal Laser Scanning Microscope.

Data availability

All 16S rRNA sequence data generated from this study were deposited to the GeneBank Sequence Read Archive with accession number PRJNA635743.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc.). Statistical differences between experimental groups were evaluated by Student's t tests and one-way analysis of variance (ANOVA) with a Dunnett's test for multiple comparisons. All data were expressed as means ± standard deviations (SD). p values of < 0.05 were considered statistically significant.

Results

Targeted culture-dependent molecular profiling identifies mucin-degrading bacteria in the healthy intestinal microbiome

A targeted culture-dependent molecular profiling method was developed to gain a whole picture of the bacterial diversity of mucin-degrading bacteria in the human gut microbiota. A minimal culture media with mucin as the sole carbon source (MM media) was used to enrich mucin-degrading bacteria from faecal samples selectively. Then the colonies grow on the plates was applied to 16S rRNA gene sequencing for bacterial diversity analysis. Faecal samples without culture (culture-independent) were used as control. On average, 110 ± 18 OTUs per subject were recovered by MM media. Meanwhile, 290 ± 58 OTUs per subject were recovered by cultured-independent sequencing method (Figure 1A). In comparison, 21.1% of the OTUs detected from faecal samples by culture-independent sequencing were cultured by MM media, representing 73.3% of family-level taxonomic groups from the faecal samples (Figure 1B). Taxonomic analysis showed the OTUs recovered from MM media (Figure 1C, outer ring in blue) were widely distributed in the seven phyla, namely Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Synergistetes (Figure 1C, inner tree). A large proportion of the OTUs enriched by MM belong to the phylum of Proteobacteria (40.4%) and Bacteroidetes (49.4%), dominated by the bacterial species including *Escherichia* (37.8%), *Parabacteroides distasonis* (16.1%), *Bacteroides fragilis* (14.0%), *Bacteroides vulgatus* (8.6%), *Bacteroides dorei* (2.8%), *Bacteroides thetaiotaomicron* (1.4%) and other *Bacteroides species* (Figure 1C, the outer blue bar chart). Notably, 8.6% of OTUs falls under the phylum of Verrucomicrobia (*Akkermanisa*). MM media also enriched the broad bacterial diversity of OTUs belonging to Firmicutes. However, the relative abundance was relatively low (1.2%), indicating that most MM-enriched OTUs from the Firmicutes were rare bacterial species/strains. In addition, 0.32% and 0.01% of OTUs belonging to the phyla of Fusobacteria (*Fusobacterium*) and Synergistetes (*Cloacibacillus*), respectively were detected in MM as well (Figure 1C, the outer blue bar chart).

To further test the distribution of mucin degradation in the human gut microbiota, we screen 38 bacterial species for growth in minimal media broth containing commercially available pig-derived gastric mucin as a sole carbon source. These 38 bacterial species belong to 24 genera and represent 70.4% of the bacterial abundance at the genus level in faecal samples as determined by 16S rRNA gene sequence (Figure S1). 50% (19 out of the 38) test bacterial species are able to grow in MM media (change of OD_{595} >0.1) (Figure 1D). Consistent with the bacterial growth, most of them showed > 30% reduction of mucin in the broth as determined by the anthrone reagent test (Figure 1D), suggesting that the mucus-utilizing capacity of the intestinal microbiome has been underappreciated. Apart from the known mucin degraders like *A. muciniphila*, *B. thetaiotaomicron*, *B. fragilis* and *B. vulgatus*, importantly, we found many other bacterial species showed potential mucin-degrading ability as well. This potential, yet undescribed, mucin degraders include five *Bacteroides* (*B. intestinihominis, B. uniformis, B. salyersiae, B. ovatus* and *B. eggerthii*) and two *Parabacteroides* strains (*P. johnsonii* and *P. distasonis*), two *Bifidobacterium* strains (*B. dentium* and *B. adolescentis*), *Parasutterella excrementihominis*, *Gordonibacter faecihominis* and *Klebsiella oxytoca.*

Intestinal epithelial cells immune tolerance to the mucin-degrading gut commensals

Gut commensals with mucin-degrading ability are expected to have more chance to interact with mucosal immunity. We then evaluated the immunomodulating effect of 12 selected gut commensals with relatively high mucin-degrading ability by co-culture with the intestinal epithelial cells line HT-29. Real-time PCR was conducted to measure the transcription of pro-inflammatory cytokines IL-8 and TNF-α, and anti-inflammatory cytokines IL-10 and TGF-β. As a positive control, *E. coli* NCTC 9001 stimulated high-level mRNA expression of pro-inflammatory cytokines IL-8 (13.6 ± 3.9 fold) (Figure 2A) and TNF-α (30.5 ± 9.4 fold) (Figure 2B) but not anti-inflammatory cytokines IL-10 (0.64 ± 0.78 fold) (Figure 2C) and TGF-β (0.83 ± 0.24 fold) (Figure 2D). In contrast, most isolated mucin-degrading gut commensals induce small or little mRNA expression of pro-inflammatory cytokines (Figure 2). Four *Bacteroides* and *B. dentium* induced ~2-fold increase of IL-8 (Figure 2A), while two *Bacteroides*

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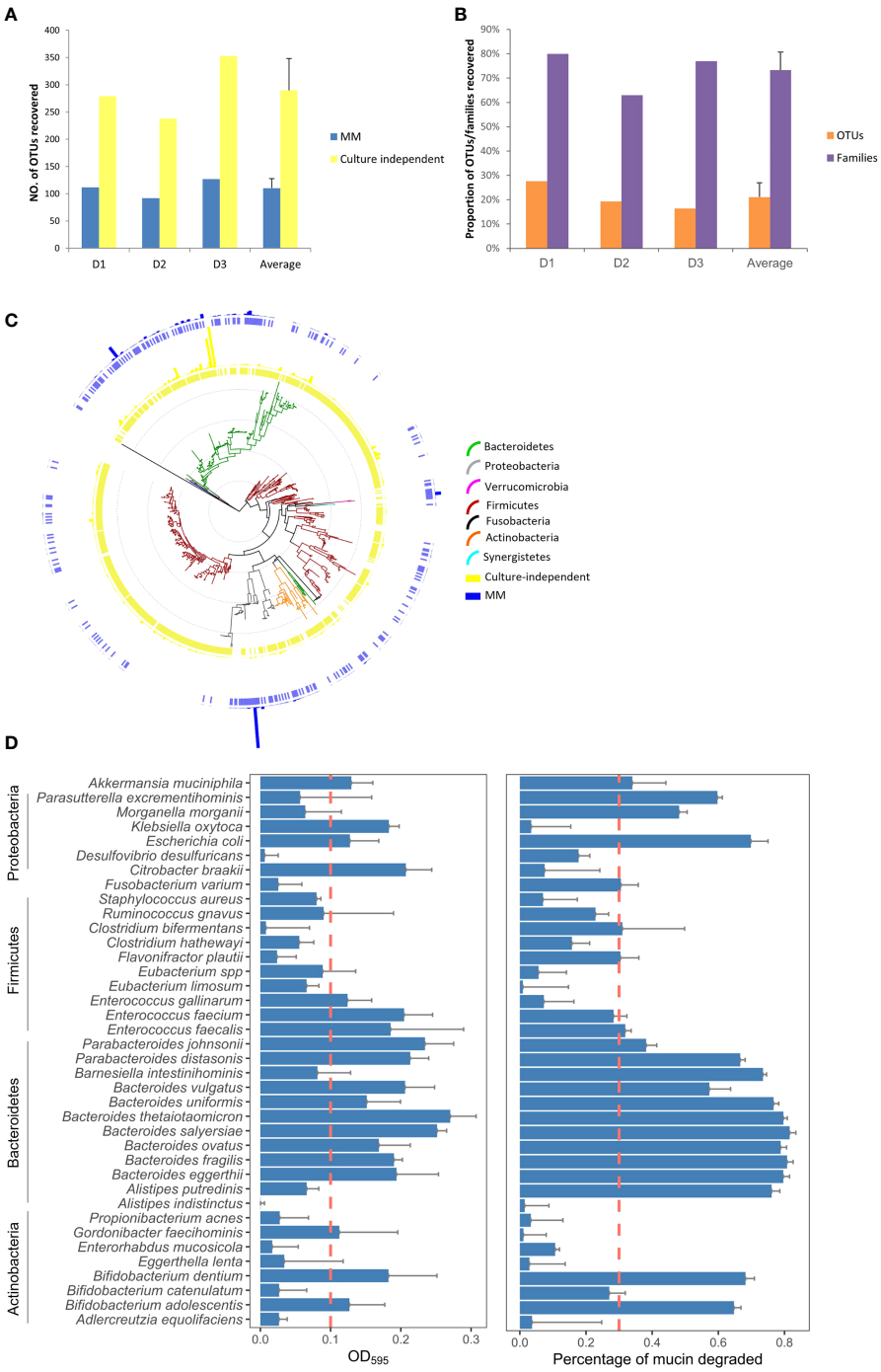

FIGURE 1
Mucin-degrading bacteria captured by cultivation-based molecular profiling. **(A)** Number of OTUs recovered from 16S rRNA gene sequencing of faecal samples from the three health Donor 1 (D1), Donor 2 (D2) and Donor 3 (D3) by MM media and culture-independent methods. **(B)** Proportion of OTUs and the corresponding bacterial families from culture MM media as compared to the culture-independent sequencing method. **(C)** The phylogenetic tree and relative abundance of the OTUs recovered from 16S rRNA gene sequencing of the faecal samples by MM culture and culture-independent methods. The inner ring is colored based on the phylum the OTUs are assigned. The outer ring shows the distribution of OTUs. The exterior bar chart shows the relative abundance of the OTUs. **(D)** Percentage of mucin degraded over 72 h by the 38 gut isolates in minimal media containing porcine gastric mucin as the sole carbon source. The amount of mucin degraded was measured by the loss of hexose in the supernatant using the anthrone reagent test. Data are means and standard deviations for three replicates.

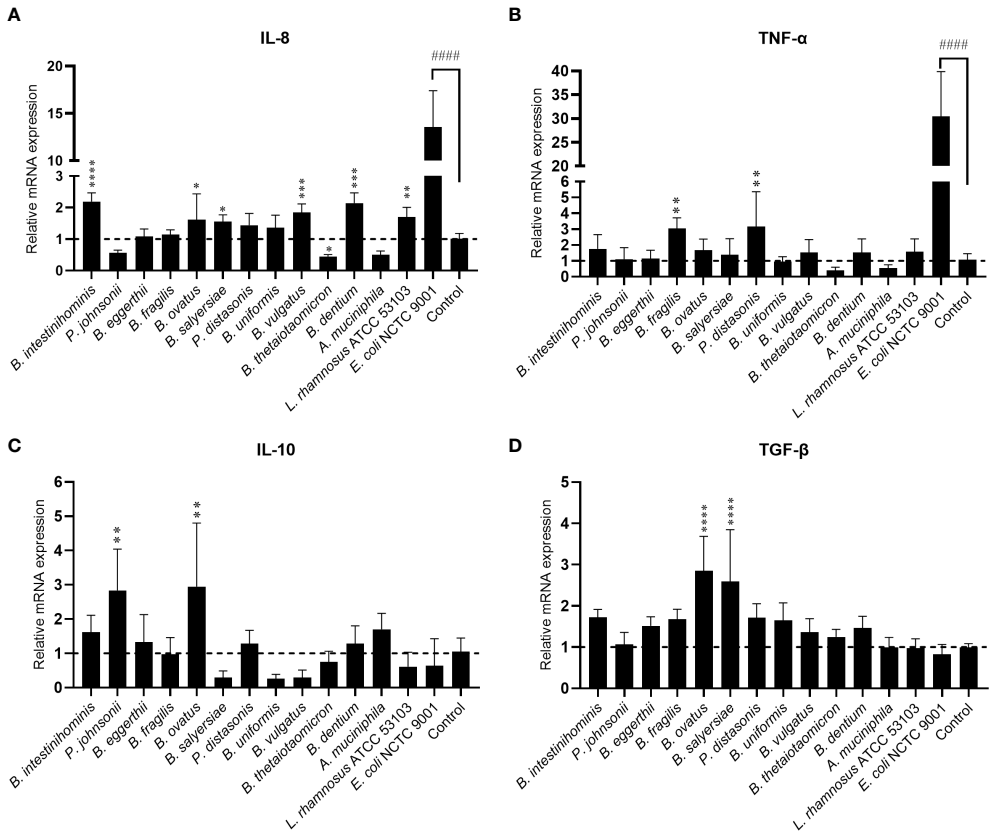

FIGURE 2
Effects of mucin-degrading gut commensals on the mRNA expression of IL-8 **(A)**, TNF-α **(B)**, IL-10 **(C)** and TGF-β **(D)** in HT-29 after 2-hour co-culture. *L. rhamnosus* ATCC 53103 and pathogenic *E. coli* NCTC9001 were included in the experiments as control strains. ####$p < 0.0001$ compared to the control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared to the *E. coli* NCTC9001 group as determined by one-way ANOVA followed by Dunnett's test for multiple comparisons.

stimulated around 3-fold increase of TNF-α (Figure 2B). Interestingly, *P. johnsonii*, *B. thetaiotaomicron* and *A. muciniphila* were able to down-regulate the cell basal level of IL-8 (~0.5 fold) or TNF-α (~0.5 fold) (Figures 2A, B). In addition, *P. johnsonii*, *B. ovatus* and *B. salyersiae* were able to induce anti-inflammatory cytokines IL-10 or TGF-β (Figures 2C, D). These observations suggested the immune tolerance of these mucin-degrading gut commensals to intestinal epithelial cells.

Mucin-utilizing gut commensal alleviate inflammation induced by pathogenic *E. coli*

To test the hypothesis that these mucin-degrading commensals have an anti-inflammation function in the intestine, we treated the HT-29 cell with selected mucin-degrading gut commensals followed by stimulation with *E. coli* NCTC9001 and examined the mRNA expression of pro-inflammatory cytokines IL-8 and TNF-α. As *L. rhamnosus* is known to have anti-inflammatory potential, we used this strain as a positive control. As shown in Figures 3A, B, the presence of all the isolated gut commensals, as well as the *L. rhamnosus* ATCC 53103, significantly decreased the *E. coli* induced IL-8 and TNF-α, except for *B. fragilis* and *P. distasonis* which showed no impact on the downregulation of *E. coli* induced mRNA expression of TNF-α. This suggests that anti-inflammation seems a common but bacterial species-specific property of the mucin-degrading gut commensals. Compared to the competition co-culture model, however, pretreatment of the cells with the gut commensals showed compromised effects on down-regulating the IL-8 and TNF-α (Figures 3C, D). For example, *B. intestinihominis* and *P. johnsonii*, were the best in decreasing the *E. coli*-induced TNF-α in the competition assay but showed no significant effect on the TNF-α level in the pretreatment assay. This result suggests that efficient anti-inflammation requires the presence of gut commensals.

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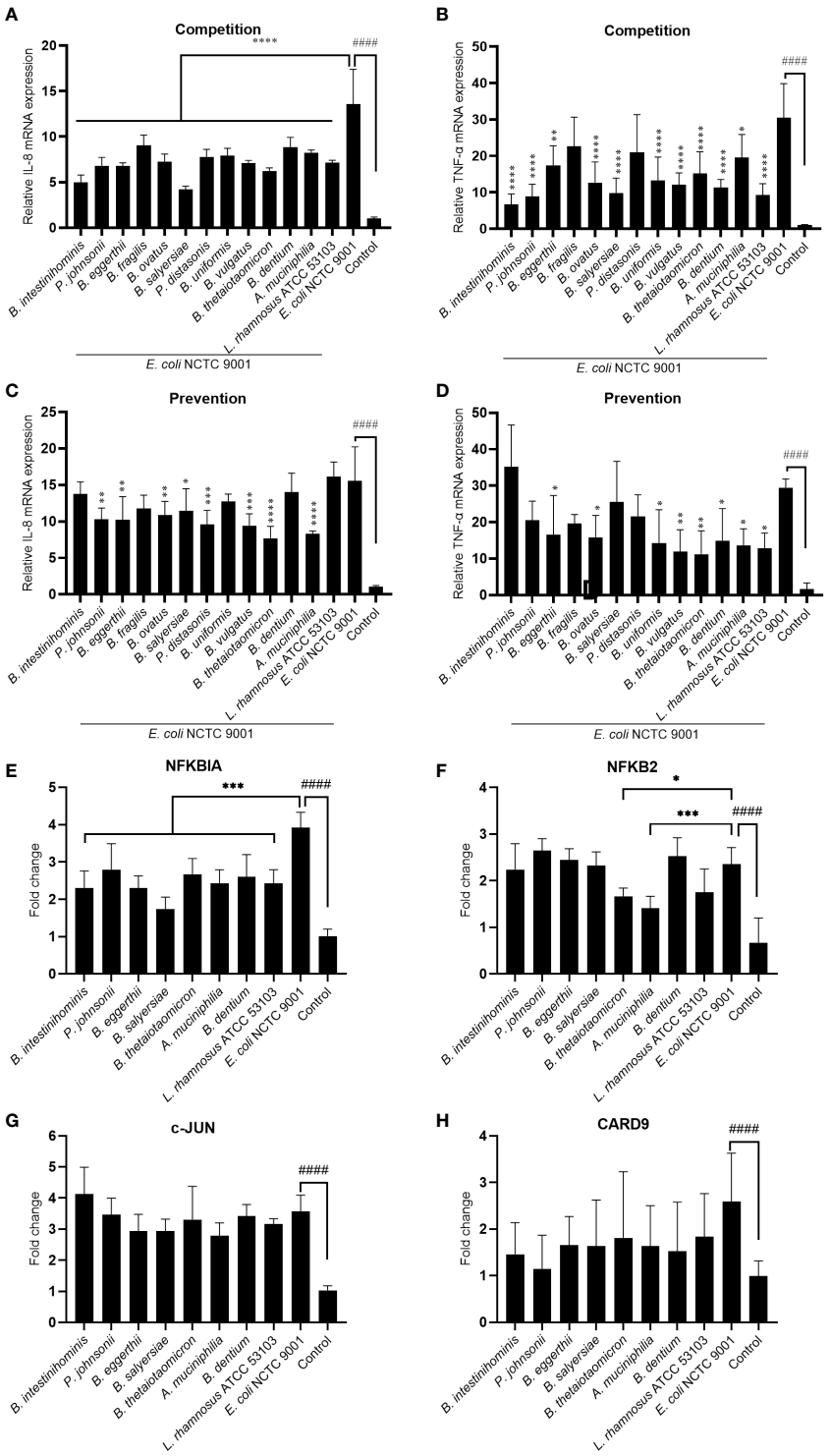

FIGURE 3
Mucin-degrading gut commensals alleviate the pathogenic *E. coli* induced inflammation by downregulating the NF-kB signaling pathway. HT-29 cells co-incubated (competition) or pre-incubated (prevention) with gut commensals were challenged by *E. coli* 2 h. The IL-8 **(A, C)** and TNF-α **(B, D)** mRNA expression in HT-29 cells was detected by qPCR. HT-29 cells incubated with gut commensals were challenged by *E. coli* NCTC9001 for 2 h, mRNA expression of NF-kB and MAPK pathway involved gene NFKBIA **(E)**, NFKB2 **(F)**, c-Jun **(G)** and CARD 9 **(H)** were measured by qPCR. All data are presented as the mean ± SD of three biological replicates with two technical replicates. ####p < 0.0001 compared to the control group; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to the *E. coli* NCTC9001 group as determined by one-way ANOVA followed by Dunnett's test for multiple comparisons.

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Antagonization of the adhesion of pathogen to the epithelial cell is one of the mechanism that mediated the anti-inflammatory effect of some probiotics. However, no significant antagonization of the *E. coli* NCTC9001 adhesion to HT-29 cells was observed in either prevention (Figure S2A) or competition model (Figure S2B). This data suggests the anti-inflammation property of these gut commensals observed above is not attributed to the antagonism of pathogen adhesion to HT-29 cells but may be due to direct bacteria-cell interaction. We further explored two intracellular signaling pathways involved in the regulation of immune response: NF-kB and MAPK pathways. *E. coli* induced an activation of both NF-kB and MAPK pathways (Figure 3 and Figure S3) as shown by a significant increase in NFKBIA (Figure 3E), NFKB2 (Figure 3F), c-Jun (Figure 3G) and CARD9 (H). However, all the selected gut commensals, together with the control *L. rhamnosus* ATCC 53103, significantly downregulated the *E. coli*-induced NFKBIA (Figure 3E). In addition, *B. thetaiotaomicron* and *A. muciniphila* significantly suppressed the *E. coli* induced NFKB2 (Figure 3F). However, these gut commensals have no effect on the *E. coli*-induced c-Jun (Figure 3G) and CARD9 (H), suggesting the anti-inflammation exert by gut commensals is due to the inhibition of NF-kB signaling pathway but not the MAPK pathway.

Mucin-degrading gut commensals affect Caco-2 intestinal epithelial TJ barrier function and mRNA expression of TJ protein genes

To examine the effect of mucin-degrading gut commensals on the epithelial TJ barrier function, the influence of 10 selected mucin-degrading gut commensals (2 *Parabacteroides* spp., 6 *Bacteroides* spp., 1 *B. dentium* and 1 A. *muciniphilia*) on TEER value of filter grown Caco-2 cell monolayers was tested. Cell monolayers without bacterial incubation were considered as a control group. Changes in the TEER value after co-culture were used as a readout parameter for potential effects on intestinal epithelial TJ barrier function. As shown in Figure 4A, 2 *Parabacteroides* spp., 4 *Bacteroides* spp., and *A. muciniphilia* significantly increased the TEER value of Caco-2 cell monolayers by around 10% after 12-hour coincubation. However, two *Bacteroide* species (*B. intestinihominis* and *B. ovatus*) had no significant effects on the TEER value over the 48 hours of co-incubation (Figure 4B). In contrast, *B. dentium* and the control strain *L. rhamnosus* ATCC 53103 and *E. coli* NCTC 9001 significantly decreased the TEER value after co-incubation (Figure 4B). Together, these results suggest most of the test mucin-degrading gut commensals could enhance intestinal epitheal TJ barrier function.

The TJ proteins are the primary determinants of intestinal epithelial TJ barrier function (20). Therefore, we examined if the regulation of TJ proteins mediated the enhancement of the epithelial barrier function by the gut commensals. The transcription of TJ protein genes, including Zo-1, Occludin, Claudin-1, Caludin-2 and E-cadherin, was determined by measuring the mRNA level through qRT-PCR (Figure 5). we found the effect of mucin-degrading gut commensals on the mRNA expression of TJ protein genes is bacteria-specific. After co-incubation with Caco-2 cell monolayer, *B. thetaiotaomicron* significantly upregulated the mRNA level of Zo-1 (Figure 5A), Claudin-1(Figure 5C) and E-cadherin (Figure 5E). At the same time, *P. distasonis* significantly increased the Occludin (Figure 5B) while *B. salyersiae* significantly upregulated Claudin-1 (Figure 5C) and E-cadherin (Figure 5E). In contrast, the control strain *E. coli* NCTC 9001 significantly downregulated the Occludin mRNA level (Figure 5B). *P. johnsonii* had on significant impact on the mRNA expression of TJ protein genes. In addition, all the selected gut commensals and *E. coli* NCTC 9001 did not affect the expression of Claudin-2 mRNA (Figure 5D).

Protective effect of gut commensals on the intestinal barrier challenged by an inflammatory stimulus

Inflammatory cytokines like IL-1β have been demonstrated to cause inflammation in intestinal epithelial cells and compromise intestinal epithelial TJ barrier function (21). We hypothesized that the selected gut commensals that exhibited an enhanced barrier function as shown above could protect the intestinal epithelial cell barrier against disruption induced by IL-1β. As shown in Figure 6, the addition of IL-1β at physiological concentration (10 ng/mL) to Caco-2 cell monolayers caused a significant and time-dependent drop in the TEER value, decreasing to 81.7% after 12 hours and 77.2% after 24 h when compared to control (100%). The presence of *P. distasonis* prevented the decrease of TEER induced by IL-1β, with the corresponding relative TEER value increased to ~106%. However, the other gut commensals, including *P. johnsonii*, *P. distasonis*, *B. salyersiae*, *B. thetaiotaomicron* and *A. muciniphila* only partially attenuated the decrease of TEER value induced by IL-1β.

Mucin-degrading gut commensals restore IL-1 β induced dysregulation of TJ proteins and immune response

To investigate whether the changes in transepithelial resistance on epithelial cell monolayers following IL-1β and gut commensals treatments were associated with regulation of TJ proteins, the mRNA expression of the TJ protein genes Zo-1, Occludin, Claudin-1, Claudin-2 and E-cadherin were quantified

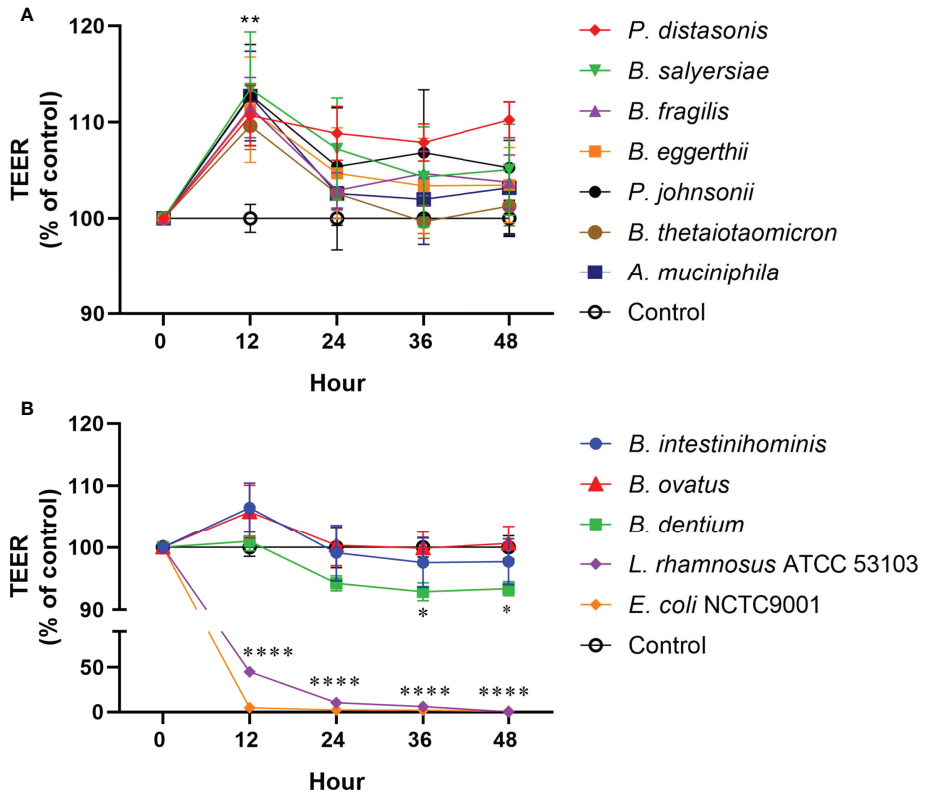

FIGURE 4
Effect of mucin-degrading gut commensals on the development of the TEER of Caco-2 monolayer. Changes in the TEER values of the Caco-2 cell monolayers co-cultured with bacteria were monitored over 48 h experiment. Cell monolayers without bacteria incubation were used as the control group and the TEER values were normalized to 100%. *p < 0.05, **p < 0.01, ****p < 0.0001 treated groups vs. control group. Error bars represent the standard deviation of the mean values from at least three replicates. Significance was determined by one-way ANOVA analysis.

by qRT-PCR analysis. The stimulation of Caco-2 cell monolayers with inflammatory cytokines IL-1 β did not affect the mRNA expression Zo-1(Figure 7A), Occludin (Figure 7B) and Claudin-1(Figure 7C), however, it caused a significant increase in Claudin-2 (2.66 ± 0.44 fold, $p<0.01$) (Figure 7D) and a significant decrease in E-cadherin (0.51 ± 0.18 fold, $p<0.05$) (Figure 7E). In addition, there was a small decrease trend in the Occludin (0.82 ± 0.10 fold, $p>0.05$) (Figure 7B), but it did not reach statistical significance. After co-incubation with *P. distasonis* and *B. thetaiotaomicron* for 24 hours, a significantly increased in Occludin was observed compared to IL-1β group (Figure 7B). Furthermore, *P. distasonis* ameliorated the IL-1β-induced increase in Claudin-2 (Figure 7D) and decrease in E-cadherin (Figure 7E). In addition, we observed co-incubation with *A. muciniphila* significantly increased the mRNA level of Zo-1 (Figure 7A), although IL-1β did not affect Zo-1. Neither gut commensals nor IL-1β treatment caused a significant effect on the mRNA expression of Claudin-1 (Figure 7C).

Concurrently, the effect of these three gut commensals on the immune response was monitored as well. IL-1β (10 ng/mL) significantly increased the mRNA expression of NFKBIA gene (Figure S4A) and NF-kB downstream pro-inflammatory cytokine gene IL-8 (Figure S4B). The presence of *P. distasonis* and *A. muciniphila* significantly inhibited the IL-1β induced increase of NFKBIA and IL-8, suggesting a inhibition of NF-κB pathway by these two gut commensals. However, co-culture with *B. thetaiotaomicron* did not cause significant change in NFKBIA and IL-8.

Gut commensals restore IL-1 β induced TJ protein localization disruption

The expression and localization of the TJ proteins Zo-1, Occludin, Claudin-2, and E-cadherin were assessed by immunofluorescence staining. In control Caco-2 monolayers, Zo-1, Occludin, and E-cadherin staining formed a honeycomb-type structure outlining the cellular junctions (Figure 8). IL-1β did not cause an obvious change on Zo-1(Figure 8A). However, it caused a disturbance in the junctional localization of Occludin (Figure 8B) and E-cadherin (Figure 8C). The faint immunofluorescence signal of Occludin staining at cell

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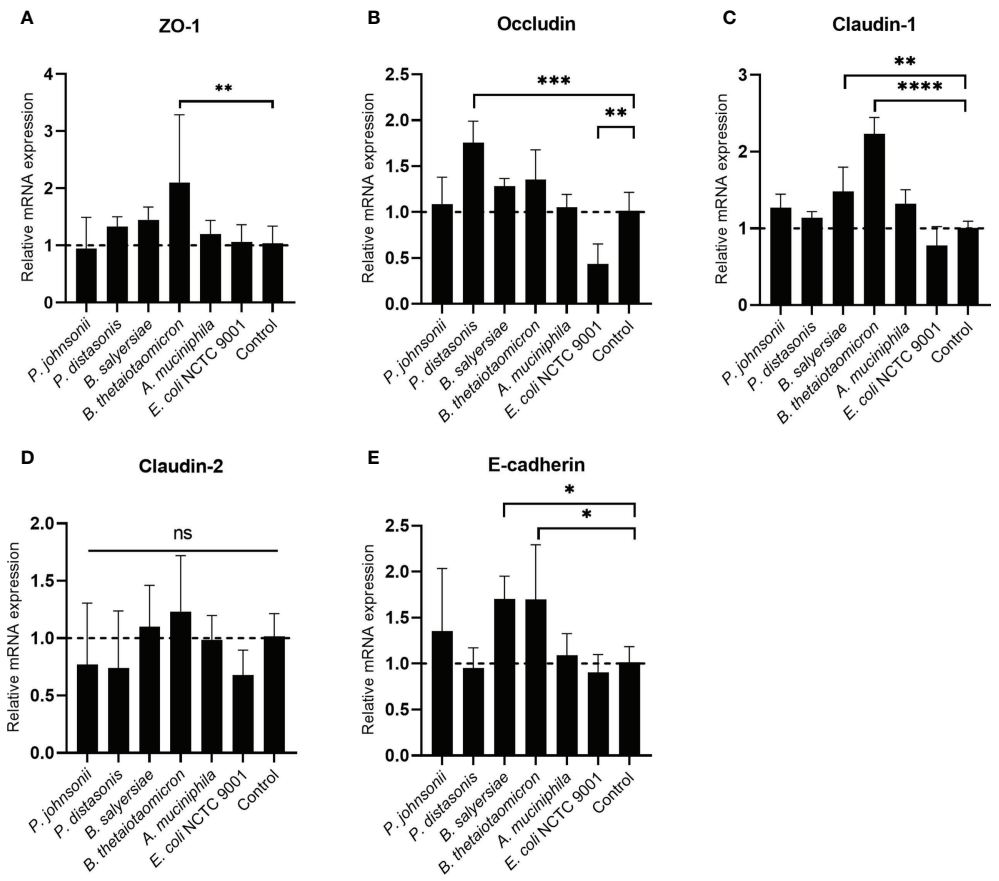

FIGURE 5
Impact of selected mucin-degrading gut commensals on the mRNA expression of TJ protein genes. Developed Caco-2 cell monolayers were co-cultured with gut commensals 24 h. The mRNA level of TJ protein Zo-1 **(A)**, Occludin **(B)** Claudin-1 **(C)**, E-cadherin **(D)** and Claduin-2 **(E)** were detected by qRT-PCR. Error bars represent the standard deviation of the mean values from at least three replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ treated groups vs. control group, ns: no significant. Significance was determined by one-way ANOVA followed by Dunnett's test for multiple comparisons.

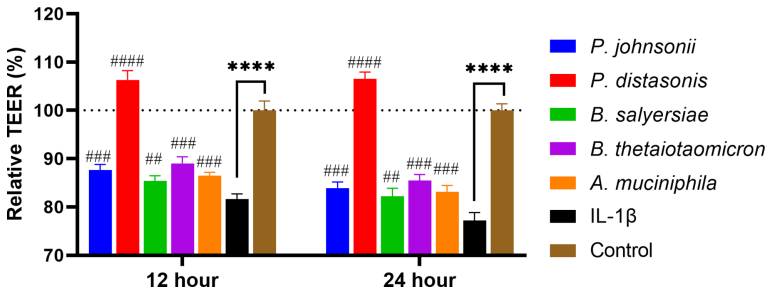

FIGURE 6
Selected mucin-degrading gut commensals attenuated the drop of TEER in Caco-2 cell monolayers induced by IL-1β. Caco-2 cell monolayers treated with gut commensals were followed by stimulation by IL-1β (10 ng/mL). The TEER value was measured at 12 h and 24 h time points. ****p < 0.0001 IL-1β vs. control. ##p < 0.01, ###p < 0.001, ####p < 0.0001 IL-1β vs. treated groups. Cells without any treatment were used as a control. Error bars represent the standard deviation of the mean values from at least three replicates. Significance was determined by one-way ANOVA followed by Dunnett's test for multiple comparisons.

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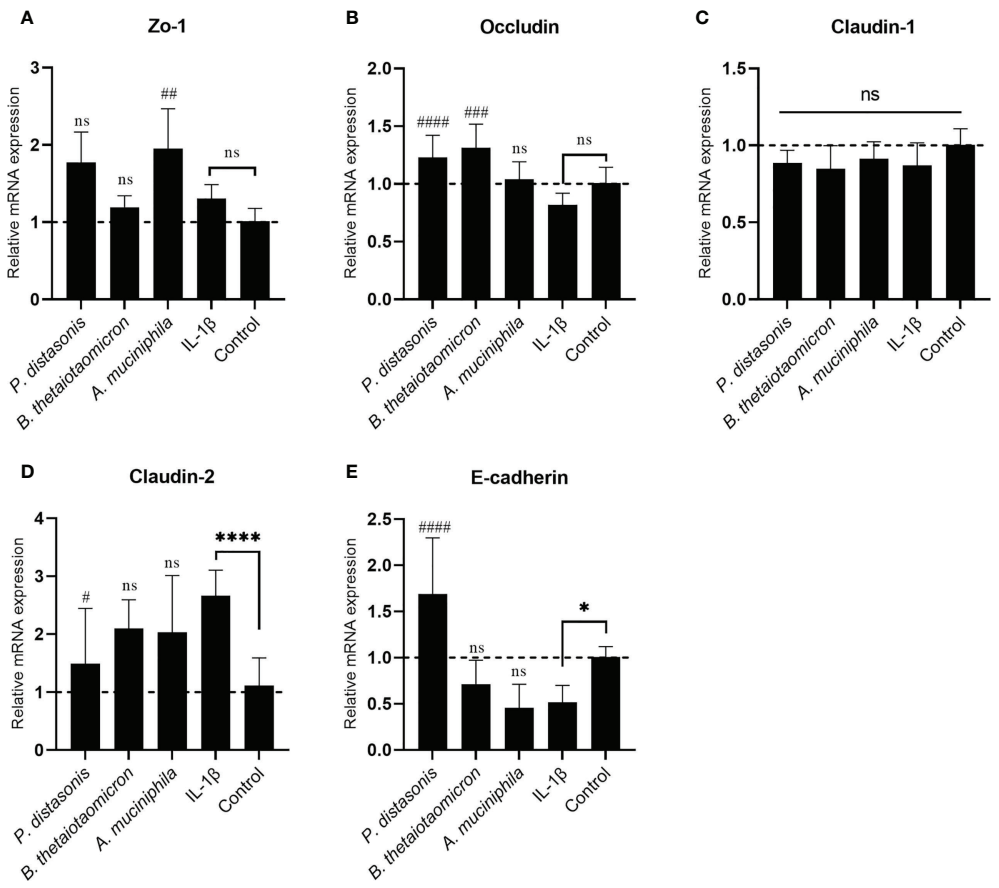

FIGURE 7
Co-culture with *P. distasonis, B. thetaiotaomicron* and *A. muciniphila* restored the IL-1β induced dysregulation of intestinal TJ protein genes' mRNA expression. Caco-2 cell monolayers co-incubated with gut commensals were followed by stimulation by IL-1β (10 ng/mL). Cells without any treatment were used as a control. The mRNA expression of TJ protein Zo-1 **(A)**, Occludin **(B)**, Claudin-1 **(C)**, Claudin-2 **(D)** and E-cadherin **(E)** were measured by qPCR. Cells without any treatment were used as a control. *p < 0.05, ****p < 0.0001 IL-1β vs. control, ns: no significant. #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 IL-1β vs. treated groups. Error bars represent the standard deviation of the mean values from at least three replicates. Significance was determined by one-way ANOVA followed by Dunnett's test for multiple comparisons.

junctions indicated the synthesis of TJ protein was affected, and the irregular and distorted junctional location of E-cadherin suggested the TJ disruption. Co-incubation with *A. muciniphila*, *B. thetaiotaomicron* and *P. distasonis* restored the Occludin and E-cadherin at junctional localization areas (Figures 8A, B).

Moreover, Claudin-2 detection in control Caco-2 cell monolayers (Figure 8D) was low. However, an increased Claudin 2 signals was observed after IL-1β stimulation. The presence of *P. distasonis* remarkably prevented the IL-1β stimulated increase in the expression of Claudin-2 (Figure 8D). These results indicated that the intestinal epithelial TJ proteins were impaired after by the inflammatory cytokines, and supplementation of *P. distasonis, A. muciniphila* and *B. thetaiotaomicron* could completely or partly restore it.

Discussion

Mucin degradation by the gut microbiota plays an essential role in the dynamic and homeostasis of mucus barrier. However, few publications well defined the proportion of this bacterial population with gut microbiota. Hoskins and Boulding (1981) estimated that an average 1% of the total faecal bacteria in healthy subjects were able to degrade the mucin glycoproteins based on the "most probable number (MPN)" method. However, *in silico* analysis of the genomes of gut microbiota revealed that up to 86% of the human gut microbiota encode genes for cleavage of mucin glycans and 89% encoding genes for the metabolism of the released monosaccharides, suggesting that the mucin-degrading bacteria in the human gut microbiota may

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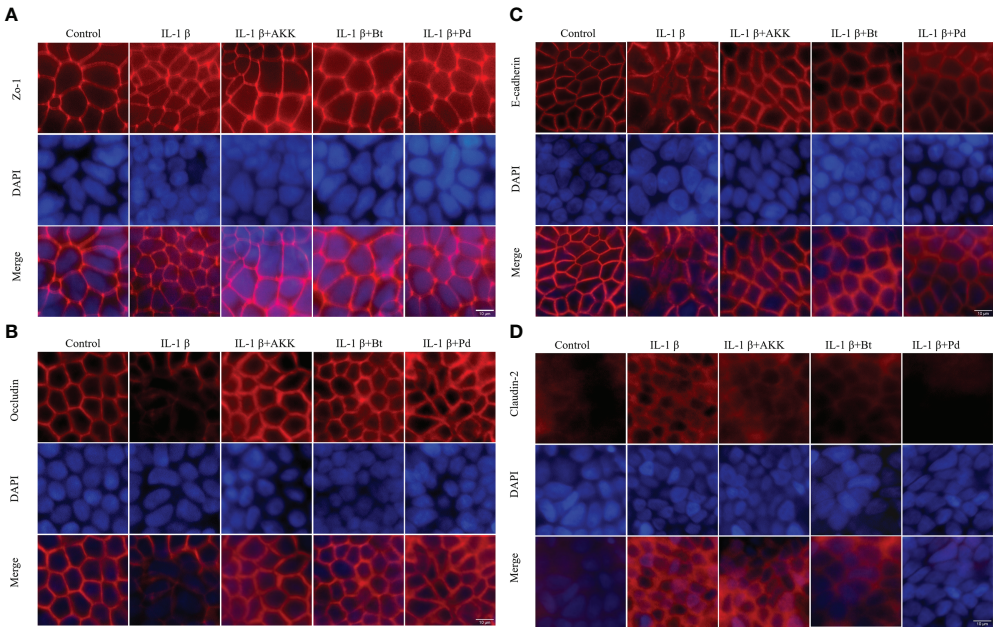

FIGURE 8
Protective effect of *P. distasonis* (Pd), *A. muciniphila* (AKK) and *B. thetaiotaomicron* (Bt) on the immunofluorescence localization of TJ proteins in Caco-2 cell monolayers challenged by IL-1β. Caco-2 cell monolayers were treated with IL-1β and co-incubated with gut commensals for 24 h. TJ protein Zo-1 **(A)**, Occludin **(B)**, E-cadherin **(C)**, and Claudin-2 **(D)** were stained with the respective antibodies (red), and nuclei were DAPI stained (blue) and imaged by confocal microscopy. Images are of 100 × magnification. Scale Bar = 10 μm.

have been underappreciated (22). In this study, through a targeted culture-based 16S rRNA gene sequencing, we showed 21.1% of the OTUs, representing 73.3% of family-level taxonomic groups, from faecal samples were able to grow on the MM media with porcine mucin as the sole carbon sources. *In vitro* monoculture screening of 38 bacterial species also found many potential, previously uncharacterized mucin degraders. Together, these results demonstrated that the mucin-degrading bacteria in the gut microbiota is more abundant than previously thought.

Mucin-degrading gut commensals are expected to interact intimately with the host epithelial immune system due to their close proximity to the gut epithelium and immune cells (23). However, the bacteria-cell co-culture results showed these isolated gut commensals (8 *Bacteroides* spp., 2 *Parabacteroides spp*, *Akkermanisa muciniphila* and *Bifidobacterial dentium*) elicit small amount mRNA expression of inflammatory cytokines IL-8 and TNF-α, suggesting the mucin-degrading gut commensals were tolerated by the host epithelial cells. Low or non-immunogenicity is important for gut commensals to escape the clearance by host immune response and reside in the GI tract (24). This is especially important in consideration of the mucus-associated gut commensals are in close proximity to host IEC.

Apart from low immunogenicity, we found all the selected mucin-degrading gut commensals could supress the IL-8 and TNF-α stimulated by *E. coli* at various degree, suggesting a potential of anti-inflammatory effects of these gut commensals. In recent years, a number of studies reported the anti-inflammatory property of bacteria and their potential application in the treatment of gut inflammation diseases like IBD (25). However, most of the related studies focus on probiotics like *Lactobacillus* and *Bifidobacterium* (26) and specific individual gut commensals like *Faecalibacterium prausnitzii* (27). The present study first described the anti-inflammatory property that seems common among mucin-degrading gut commensals, although this study does not explore whether there is a connection between anti-inflammatory property and mucin-degrading ability. In addition, we found the anti-inflammatory effect was more evident at the competition model than in prevention condition, suggesting the anti-inflammation needs the presence of gut commensals. Probiotics have been reported to exert the anti-inflammation effect by interference with pathogen binding to IEC (28). Therefore, we postulated that the anti-inflammatory property of the gut commensals tested above may be attributed to the same mechanism. However, the result revealed that mucin-degrading gut commensals have no significant antagonizing effect on *E. coli* adhesion to HT-29 cells, indicating the anti-inflammation property of the mucin-degrading gut commensals tested here is through other mechanisms. Further study of two intracellular signaling

pathways, NF-kB pathway and MAPK pathway showed the isolated mucin-degrading gut commensals could significantly suppressed the NF-kB pathway by downregulation of the *E. coli* induced NFKBIA. However, no inhibitory effect on the MAPK pathway was observed. These results suggest that the commensals exert the anti-inflammatory effect by suppressing the NF-kB pathway but not the MAPK pathway. It also explains why the gut commensals inhibited only part of induced pro-inflammatory cytokines.

We further investigated the impact of these mucin-degrading bacteria on the intestinal epithelial barrier through a Caco-2 cell model. We thought this study is essential as mucin degradation by bacteria is considered a pathogenicity factor as excess mucin degradation disrupts the mucosal barrier (29). Impaired or compromised intestinal barrier function allows bacteria-derived molecules such as Lipopolysaccharides (LPS) into the mucosa and then cause intestinal and even systematic inflammatory responses (1). However, the results showed seven of the tested mucin-degrading gut commensals (2 *Parabacteroides* spp., 4 *Bacteroides* spp. and *A. muciniphilia*) significantly increased the TEER value of Caco-2 cell monolayers by around 10% after 12-hour co-incubation, suggesting an enhancement of the barrier function of the epithelial monolayer. The beneficial effect of probiotics such as *Lactobacillus* and *Bifidobacterium* on the intestinal epithelial barrier function have been investigated widely (30). Although the exact mechanism by which the bacteria exert this beneficial effect is not well defined, publications reveal that certain probiotic (e.g *Bifidobacterium* and *Lactobacillus* spp.) promote epithelial barrier function through regulation of TJ proteins (31, 32). To test if the mucin-degrading gut commensals isolated in this study act as the exact mechanism, mRNA expression of TJ protein genes was measured after co-culture. Indeed, the mRNA expression of TJ protein genes, including Zo-1, Occludin, Claudin-1 and E-cadherin, were upregulated after co-culture with the gut commensals. For example, *B. thetaiotaomicron* upregulated the mRNA level of Zo-1, Claudin-1 and E-cadherin while *P. distasonis* significantly increased the mRNA level of Occludin. This indicates the regulation of TJ proteins is bacterial species-specific. In addition, commensal species *P. johnsonii* and *A. muciniphila* did not affect mRNA expression of all TJ protein genes test, although it increases the TEER value of cell monolayer. TEER of the cell monolayers cultured on Transwell inserts reflects the tight junctions formed between the epithelail cells. However, TJ proteins belong to a big family and contain many isoforms (33). Therefore, we speculated the TEER increase by *P. johnsonii* and *A. muciniphila* maybe duo to the regulation of TJ proteins other than the tested in this study.

For some probiotics, the realization of their beneficial effects relies upon prior disruption of TJ homeostasis. For example, *E. coli* Nissle 1917 (EcN) did not cause a significant change to the intestinal epithelial barrier function of T84 human intestinal epithelial cells after co-incubation (34). However, EcN supplementation restored the barrier permeability disrupted by enteropathogenic *E. coli* (EPEC). In addition, a commercial probiotic formulation called VSL#3 demonstrated its beneficial effects in a DSS- induced mouse colitis mode (35). Therefore, IL-1β, a pro-inflammatory cytokine, was introduced in this study to simulate an inflamed cell monolayer with a compromised barrier function. IL-1β has been demonstrated with the ability to cause an increase in intestinal epithelial TJ permeability at a physiological concentration (10 ng/mL) (21). Indeed, the addition of IL-1β to the cell monolayer caused a significant drop of TEER value. Furthermore, a decrease in the mRNA expression of TJ protein Occludin and E-cadherin and an increase in Claudin-2 were observed. However, the supplementation of mucin-degrading gut commensals (*P. distasonis*, *P. johnsonii*, *B. salyersiae*, *B. thetaiotaomicron* and *A. muciniphila*) entirely or partially attenuated the IL-1β induced decrease in TEER value. *A. muciniphila* is a known mucin-degrading bacterium and many publications demonstred that this bacterium has a positive effect on intestinal barrier integrity (36). Consistently, *A. muniniphila* here partially restored the IL-1β induced TEER drop and disrupted TJ proteins Occludin and E-cadherin. Notably, we found *B. thetaiotaomicron* and *P. distasonis* test here exert similar protective effect against the stimulation of IL-1β. Particularly, supplementation of *P. distasonis* completely prevented the IL-1β induced TEER drop and disruption of TJ proteins. These observations indicated that increased Occludin, E-cadherin and decreased Claudin-2 expression may contribute to the ability of the gut commensal species to strengthen the epithelial barrier. The genus *Bacteroides* contains the most predominant species of Bacteroidetes order in the human intestinal tract. The health-promoting properties of the species within this genus have been recognized relatively recently with *B. fragilis* being the best studied representative (37). However, results here demonstrate for the first time the barrier-enhancing potential for other *Bacteroides* and *Parabacteroides* spp.

Previous studies demonstrated that IL-1β induced disruption of intestinal TJ barrier function is mediated by the activation of several signaling pathways, such as NF-KB pathway (21). Consistently, the inhibition of IL-1β activated NF-KB pathway by *P. distasonis* and *A. muciniphila* was also observed in this study. Thus, inhibition of the NF-KB activation, resulting in TEER enhancement and restoration of TJ proteins maybe a mechanism mediated the barrier-enhancing potential for the gut commensals in this study. However, *B. thetaiotaomicron* showed no inhibitory effect on IL-1β activated NF-KB pathway, suggesting other signaling pathways involved in regulating TJ proteins.

In contrast to the beneficial effects, two *Bacteroide* species (*B. intestinihominis* and *B. ovatus*) had no significant effects on the TEER value of Caco-2. Notably, one isolate *B. dentium*, together with control strains *L. rhamnosus* ATCC 53103 and pathogenic *E. coli* NCTC 9001, significantly decreased the TEER value after co-culture. Although the exact mechanism by which the isolate *B. dentium* and *L. rhamnosus* ATCC 53103 decrease the TEER is not investigated in this study, the color of cell culture media (DMEM) was observed to become yellow after co-culture, suggesting an

acidified environment for the cell monolayers. This result also demonstrated that caution must be taken when interpreting data from *in vitro* models.

In addition, the potential pathogenicity of *Bacteroides* spp. cannot be ignored as the health-promoting characteristics of *Bacteroides* are strictly strain-dependent (38). For example, some enterotoxigenic *B. fragilis* can cause disease such as bacteremia when they have escaped their normal habitats (39). Therefore, further studies are needed to well define the mechanism by which epithelail barrier function is enhanced and the potential risk factors.

In summary, we found the gut commensals with mucin-degrading ability were widely distributed in the human gut microbiota. These gut commensals were tolerated by the epithelial cells and incited low or little level of pro-inflammatory immune response. In contrast, they suppress the inflammatory response induced by *E. coli* through downregulation of NF-KB pathway. Moreover, a total of 7 out of the 10 test gut commensals enhanced the epithelial TJ barrier function, as showed by increased TEER value and upregulated mRNA expression of TJ protein genes. Notably, *P. distasonis* completely protected the epithelial barrier from the challenge of IL-1β by restoring the expression and distribution TJ proteins, while *A. muciniphila* and *B. thetaiotaomicron* could partially restore the disrupted epithelial TJ barrier.

Our findings here provide evidence for the anti-inflammatory and epithelial barrier-enhancing property of mucin-degrading gut commensals, especially *Bacteroides* spp. and *Parabacterodies* spp. These findings enable human gut commensals, the unconventional probiotics with specific phenotype and function, to be promising and new therapeutics in the future for inflammatory bowel disease and other inflammatory disorders.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA635743.

Ethics statement

The studies involving human participants were reviewed and approved by Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (reference number 2016.707). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, MI; performance of experiment, MP and NB; data analysis, MP; writing-original draft preparation, MP; writing-review and editing, MP, NB, and MI; supervision, MI; funding acquisition, MI. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1021094/full#supplementary-material

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