Original Article

Metagenomic analysis of rats with diarrhea treated with mixed probiotics: response to consecutive and alternate-hour supplementation

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Background: Diarrhea is the leading contributory factor of sickness and mortality among children under five and an economic burden for families. This study aimed to investigate the effects of mixed probiotics supplementation at different times (consecutive and alternate-hour) on intestinal microecology in Sprague-Dawley (SD) rats with acute diarrhea.

Methods: A total of 40 SD rats were randomly assigned to four groups, including the control group, model group, probiotic group A, and probiotic group B. An acute diarrhea model was induced by administration of 5% dextran sulfate sodium. Rats in probiotic group A and probiotic group B were fed with *Clostridium butyricum* (*C. butyricum*), *Bifidobacterium infantis* (*B. infantis*), and *Saccharomyces boulardii* (*S. boulardii*) for a total of 7 days. Probiotic group A was fed with all probiotics simultaneously. Probiotic group B was fed with *C. butyricum* and *B. infantis* simultaneously, and then after a 2-hour interval, with *S. boulardii*. Metagenomic next-generation sequencing was used to analyze the fecal samples from every rat. The metagenomic sequencing used in this experiment was used to evaluate the effect of probiotics on the composition as well as function of the gut microbiota in order to gain a deeper comprehension of probiotic-host interactions on health and disease.

Results: The structure of the gut microbiota in probiotic group A showed significant changes. Compared to the model group, the abundance of some beneficial bacteria had increased, including *Actinobacteria* (P=0.048), *Lactobacillus* (P=0.050), and *Lactobacillus johnsonii* (P=0.042), and many opportunistic pathogenic bacteria has decreased, such as *Ruminococcus* (P=0.001). Compared to the control group, the abundance of some beneficial bacteria had increased, including *Fusobacteria* (P=0.02) and *Phascolarium* (P=0.002), and there was a reduction in the abundance of many opportunistic pathogenic bacteria such as *Roseburia* (P=0.03), *Lachnoclosterium* (P=0.009), and *Oscillibacter*_sp_1–3 (P=0.002). In addition, metagenomic analysis showed that as well as an up-regulation of glycoside hydrolase expression, amino acid and inorganic ion transport, and metabolism-related pathways, there was a down-regulation of cell motility.

Conclusions: Simultaneous administration of probiotics may have more positive implications in improving the gut microbiota of acute diarrhea rats.

Keywords: Probiotics; gut microbiome; metagenomic; Sprague-Dawley rats (SD rats); diarrhea

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Introduction

Pediatric diarrhea is a gastrointestinal disease caused by a variety of factors and is characterized by increased frequency and changes in the stools (1). Despite a steady decline in childhood diarrhea deaths over the past three decades, diarrhea continues to be the leading contributory factor of sickness and mortality among children under five (2). Therefore, it is necessary to intervene and treat infantile diarrhea as early as possible. Lai *et al.* found that there were more *Proteobacteria* counts and lower *Firmicutes*, *Actinobacteriota*, and *Bacteroidetes* populations in the stool of children with diarrhea (3). Regardless of the cause of acute diarrhea, the main methods of treatment are rehydration therapy, dietary therapy, and medication, of which medication includes probiotics (4). Clinical studies have found that a variety of probiotics have high safety and effectiveness for the treatment of pediatric diarrhea, and probiotics are strain- and dose-dependent (5-7). Despite the increasing clinical use of probiotics, the mechanism of action of probiotics on specific diseases, especially the effect on pre-existing flora, and the interactions between different probiotics are still not fully understood. Therefore, it is worthwhile to further investigate how probiotics can improve diarrhea. Three probiotics [*Clostridium butyricum* (C. *butyricum*), *Bifidobacterium longum* (*B. longum*) subspecies *infantis*, *Saccharomyces boulardii* (*S. boulardii*)] have been shown to have the potential to reduce the duration of diarrhea and hospitalization in children (8,9). With the advancement of efficient bioinformatics tools and high level of throughput metagenomic technologies, the scientific understanding of probiotics' impact on gut microbiota has grown (10). Metagenomic studies help elucidate probiotics and human-microbe interactions (11). Therefore, macrogenomics can be used to evaluate the effect of probiotics on the composition as well as function of the gut microbiota in order to gain a deeper comprehension of probiotic-host interactions on health and disease (12).

He *et al.* reported that the combined application of *Lactobacillus reuteri*, *Bacillus coagulans*, *B. longum*, and *C. butyricum* has a synergistic effect of restoring intestinal microecological balance (13). Tompkins *et al.* found that non-enteric-coated bacterial probiotics should not be taken with or just before a fatty meal (14). However, few studies have reported on the different dosing times of probiotic complexes. Our preliminary research results reported functional data, including the general condition of rats (mental state, diet, water intake, activity response, fur color, weight change, fecal characteristics), and serum cytokine levels [C-reactive protein (CRP), procalcitonin (PCT), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α)], rat liver function [alanine aminotransferase (ALT), aspartate transaminase (AST), albumin], histopathological changes in rat colon mucosa (colon length, colon histological score). The results showed that *C. butyricum*, *B. infantis*, and *S. boulardii* significantly improved the clinical symptoms of diarrhea in rats and alleviated body weight loss, reduced serum inflammation in diarrhea and had a certain degree of hepatoprotection, attenuated the shortening of the length of the colon induced by dextran sulfate sodium (DSS), and promoted the restoration of the structural integrity of the colon. Although these results were somewhat as expected, the two modalities (consecutive and alternate-hour supplementation) were similar in terms of their clinical outcomes, and there was no significant difference in the clinical results (15). The goal of this study was to ascertain whether administering probiotics separately at different periods (simultaneous *vs.* 2-hour intervals) would have a possible beneficial effect on intestinal microecology and to investigate the effects of the two combinations on the composition and function of the intestinal microbial

Highlight box

Key findings

- Consecutive supplementation of probiotics had a positive impact on improving the intestinal homeostasis of acute diarrhea in rats compared with alternate-hour supplementation of probiotics.

What is known and what is new?

- Probiotics have a beneficial effect on improving diarrhea.
- The goal of this study was to ascertain whether administering probiotics separately at different periods (simultaneous *vs.* 2-hour intervals) would have a possible beneficial effect on intestinal microecology and to investigate the effects of the two combinations on the composition and function of the intestinal microbial community using second-generation sequencing of the metagenome.

What is the implication, and what should change now?

- The present study, based on metagenomics sequencing analysis, found that the difference between simultaneous administration of probiotics was more significant than spaced administration of probiotics in terms of both intestinal flora and metabolic pathways. This finding theoretically suggests that the simultaneous administration group should have had better clinical efficacy, but this was not the case; more in-depth basic and clinical studies are required. This study helps to elucidate the mechanism of action of probiotics in improving diarrhea to a certain extent.

community using second-generation sequencing of the metagenome. We present this article in accordance with the ARRIVE reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-24-129/rc).

Methods

Animals and sample collection

Female Sprague-Dawley (SD) rats, aged 5 weeks, and with body weight of 145.03±0.54 g (mean ± standard error of the mean) were acquired from SPF (Beijing) Biotechnology Co., Ltd., with a temperature of 25±1 ℃, air humidity of 15–20%, good ventilation, and 12 hours of light and 12 hours of dark circulation every day. A total of 40 rats were randomly divided into cages and fed with standard rat feed. The water was changed regularly every day, and the rats were free to move and eat throughout the experiment, housed in pathogen-free facilities for 5 days to adapt to the circumstances before the experiment (16). A computer-based random sequence generator was used to number the rats, and they were then randomly assigned to four experimental groups in a completely randomized design. The control rats were kept normally without any intervention, and 30 remaining rats in the model, probiotic A, and probiotic B groups were given 5% DSS drinking water to induce an acute diarrhea model. This experiment was conducted as a double-blind trial. For each animal, three different investigators were involved as follows: a first investigator administered the treatment based on the randomization table. This investigator was the only person aware of the treatment group allocation. A second investigator was responsible for the experimental procedure. Finally, a third investigator (also unaware of treatment) conducted data measurement. A protocol was prepared before the study without registration.

After modeling using the above method, the model group was given 0.9% saline by gavage, 2 mL each time, twice daily. Probiotic group A was given 1 mL of 1.35% combined *C. butyricum* and *Bifidobacterium* powders solution (270 mg/kg/day; drug batch number: S20020014; Shandong Kexing Bioproducts Co., Ltd., Jinan, China) and 0.45% *S. boulardii* sachets solution 1 mL (90 mg/kg/day; drug batch number: S20020014; Laboratoires Biocodex, Paris, France) by gavage at the same time, twice daily. Probiotics group B was first given 1 mL of 1.35% combined *C. butyricum* and *Bifidobacterium* powders solution by gavage, followed by 1 mL of 0.45% *S. boulardii* sachets solution by gavage 2 hours later, twice daily. The experiment was conducted for 7 consecutive days. The rest of the time, the rats in each group were allowed to eat and drink freely. We will refer to the control group as KB, the model group as MX, the probiotic group A as GY, and the probiotic group B as JG. During the 7-day experiment, the general condition of the rats (spirit, water intake, diet, activity response, and coat color), fecal traits, and body weight changes were recorded every day. A total of 40 rats were used in the experiment. These groups originally numbered 10 animals. The KB rats generally performed well, and none of the 30 rats in the MX, GY, and JG died throughout the entire modeling period. During the modeling process, the rats showing signs of mental fatigue, decreased activity, weight loss, and loose stools (without bloody stools), indicated successful modeling. Therefore, when the above symptoms appeared, no welfare measures were given to the animals. If the rats experienced hemafecia or died prematurely, they were excluded. These rats were successfully modeled. During the experimental process, adverse events were not observed and no animal reached any of the humane endpoints. Therefore, on the 7th day of the experiment, none of them were removed from the study; each group of rats was euthanized using cervical dislocation method, and cardiac puncture and centrifugation were used to collect fresh blood for serum samples for serum immunological index measurement. The feces of rats in each group were collected and then frozen and stored in a –80 ℃ refrigerator. Metagenomic next-generation sequencing was used to analyze the fecal samples (*Figure 1*). Animal experiments were conducted at Hubei Experimental Animal Research Center (within Hubei Disease Control and Prevention Center). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hubei Disease Control and Prevention Center and approved by the Animal Ethics Committee of Hubei Disease Control and Prevention Center (No. 202420035).

Measurement of immunological indicators

When the experiment had concluded, rats in each group were executed separately using the cervical dislocation method, fresh blood was collected by cardiac puncture, and serum samples were collected by centrifugation. Enzyme-linked immunosorbent assay (ELISA) kits for rats were used for the measurement of immunoglobulin G (IgG), rat immunoglobulin M (IgM), and rat immunoglobulin A (IgA). A Rayto RT-6100 Enzyme Analyzer (Rayto, Shenzhen,

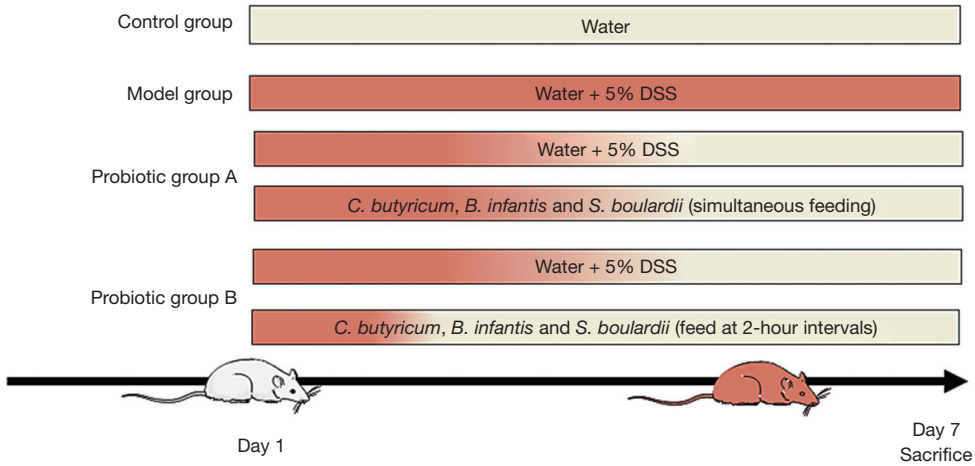

Figure 1 Rats were randomly assigned to four groups: control group, model group, probiotic group A and probiotic group B. 5% DSS-induced rats with diarrhea were orally administrated with *Clostridium butyricum* (*C. butyricum*), *Bifidobacterium infantis* (*B. infantis*), and *Saccharomyces boulardii* (S. *boulardii*) at the same time or at 2-hour interval. The rats were given normal drinking water throughout the experiment. DSS, dextran sodium sulfate.

China) was used to determine the levels of IgG, IgM, and IgA in serum according to the manufacturer's instructions.

DNA extraction

The DNeasy® PowerSoil® Pro Kit (QIAGEN, Germantown, MD, USA) was used for extracting DNA from fecal samples. DNA extraction was performed by centrifugation column method. The quality of DNA was checked using agarose gel electrophoresis. The concentration was detected by the Qubit 3.0 Analysis Kit (Thermo Fisher Scientific, Waltham, MA, USA), all the operations were carried out concerning the method of the instruction manual. Then, the samples were stored at –20 ℃ until use.

Sequencing of metagenomics and quality assurance

Library construction was performed using NEXTFLEX Rapid DNA-Seq Kit (Revvity, Waltham, MA, USA), with the following specific process: (I) joint linking; (II) use magnetic beads to screen and remove self-connected fragments of the connector; (III) enrichment of library templates using polymerase chain reaction (PCR) amplification; (IV) the magnetic beads were used to recover PCR products and obtain the final library. The sequencing platform Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) was used for metagenomic sequencing.

Strict quality assurance of the data was carried out by performing the following filtering methods: (I) eliminating junction containing reads; (II) elimination of low-quality reads, including those where more than 10% of the N was fired; removal of reads with more than 50% of the bases of the entire reads with quality value Q≤10, Following the aforementioned quality control steps, high-quality clean data was expressed in FASTQ format. Next, the reads of all the samples were merged and assembled by splicing using the splicing software MEGAHIT or IDBA-UD (17,18) founded on the de Brujin graph's principles, and based on the overlap relationship between kmer. Using the de Brujin graph, the contigs were formed, and the screened contigs above 800 bp were counted and used for subsequent analyses (Table S1).

Using the splicing software MEGAHIT based on the principle of successful de Bruijn graphs (19) (https://github.com/voutcn/megahit) (Version 1.1.2), the optimized sequence was spliced and assembled. Based on genome assembly, the prediction analysis of genes was carried out, and the predicted genes were subjected to gene set construction as well as functional annotation analysis. (I) Open reading frame (ORF) prediction of the spliced contigs sequences was performed using Prodigal (20) (http://metagene.cb.k.u-tokyo.ac.jp/) software and converted into sequences of amino acid; (II) using CD-HIT (http://metagene.cb.k.u-tokyo.ac.jp/) software, the ORF prediction findings of each sample and hybrid assembly were subjected to de-redundancy to provide the first gene catalogue of non-redundant (genes are the nucleic acid sequences

encoded by successive non-redundant genes), and clustered by default with 95% identity and 90% coverage, and the representative sequence was determined to be the longest; (III) the clean readings from each sample were compared individually (95% identity) with the non-redundant gene set using bowtie2 (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml) software, and the abundance information of the genes in the associated samples were counted; (IV) the number of reads on the comparison and the gene length were used to compute the abundance information of each gene in each sample (21).

Taxonomy and functional annotation

The length of the gene catalogue was counted using Python (https://www.python.org/) and then analyzed for species abundance: (I) a comparison of genes with functional databases was performed using the DIAMOND (22) software to compare unigenes with bacterial (bacteria), fungal (fungi), archaeal (archaea), and viral (viruses) sequences sampled from the National Center for Biotechnology Information (NCBI)'s NR (Version: 2021.11) database (blastp, evalue ≤1e−5) (23); (II) filtering of the comparison results: those with an evalue ≤ minimal evalue*10 were selected for additional evaluation out of all the comparison results for each sequence; (III) after the filtering process, since there may be more than one comparison for each sequence, the comparison results were selected for subsequent analyses; its sequence could yield several comparison results as well as information on multiple species classifications, ensuring its biological significance. The classification level prior to the emergence of the first branch was utilized as the species annotation information of the sequence, and the lowest common ancestor (LCA) algorithm (applied to the systematic classification of MEGAN software) was adopted.

Each functional database was compared with non-redundant genes using the DIAMOND software, and annotations with evalue <1e−5 were taken to filter the proteins with the greatest sequence similarity, thus obtaining the functional annotation information. (I) Filtering of the comparison results: for the comparison results of each sequence, the comparison result with the highest score (one HSP >60 bits) was selected for the subsequent analysis (24); (II) based on the comparison outcomes, the relative abundance of various functional tiers was determined (each functional tier's relative abundance was calculated as the total of the relative abundance of the genes associated with that functional tier) (25-27), in which the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was divided into five tiers (28), the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database was divided into three tiers (29), and the Carbohydrate-Active Enzyme (CAZy) database was divided into three tiers (30); (III) a table demonstrating the number of genes in each sample at each taxonomic level was created using the functional annotation results and gene abundance tables. For each function, the number of genes in a sample was equal to the number of genes for which the abundance among the genes annotated for that function was not zero.

Statistical analyses

The data were statistically analyzed using SPSS software, version 27.0.1 (IBM Corp., Armonk, NY, USA), and comparison between groups was performed with the *t*-test or Kruskal-Wallis test. Principal coordinate analysis (PCoA) was performed based on Bray-Curtis distance and the principal coordinate combination with the highest contribution rate was selected for graphical display. Differential species were detected between different groups using rank sum test and dimensionality reduction was implemented through linear discriminant analysis (LDA) to evaluate the impact of differential species, in order to screen for species with significant differences between groups as potential biomarkers. Correlation analysis was performed using Spearman's analysis, with $P<0.05$ considered statistically significant, and graphs were generated using Origin 9.0 (OriginLab Corp., Northampton, MA, USA).

Results

Immunological changes after probiotics administration

Serum immunoglobulins (IgA, IgG, IgM) were slightly increased in JG compared to GY, but serum immunoglobulins (IgA, IgG, and IgM) were elevated in both GY and JG compared to KB and there was no significant difference between the two groups; additionally, there was a significant difference between the two groups and a significant increase in serum immunoglobulins (IgA, IgG, and IgM) compared to the model group (*Figure 2*, *Table 1*). In conclusion, the improvement of intestinal homeostasis by the combination of probiotics may be related to the ability to regulate serum immunoglobulins; however, there was no significant difference in the

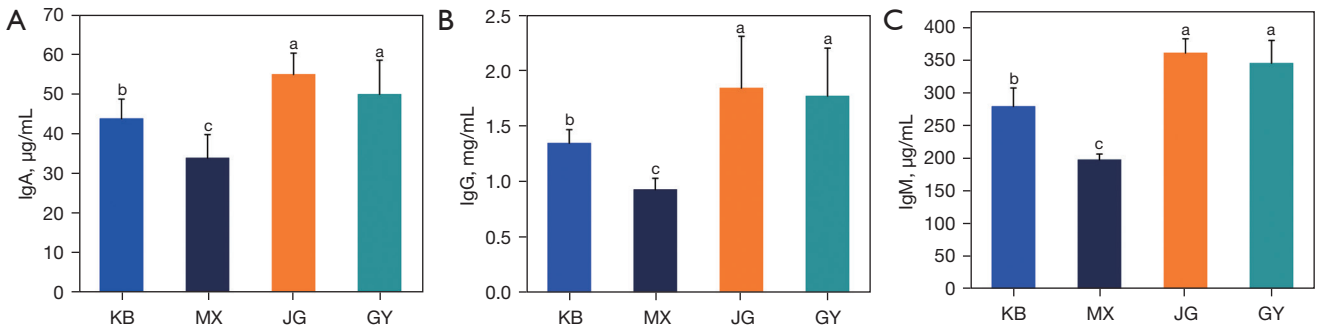

Figure 2 Comparison of the four groups of serum immunoglobulins after probiotics administration. (A) IgA values (described as μg/mL feces) from fecal samples of the four groups collected on day 7. Different groups with different colors. Probiotic group A (GY) and probiotic group B (JG) generated fecal samples with substantially greater levels of total IgA than did the model group (MX) and control group (KB). (B) IgG values (described as mg/mL feces) from fecal samples of the four groups collected on day 7. Different groups with different colors. Probiotic group A (GY) and probiotic group B (JG) generated fecal samples with substantially greater levels of total IgG than did the model group (MX) and control group (KB). (C) IgM values (described as μg/mL feces) from fecal samples of the four groups collected on day 7. Different groups with different colors. Probiotic group A (GY) and probiotic group B (JG) generated fecal samples with substantially greater levels of total IgM than did the model group (MX) and control group (KB). Data were presented as mean ± standard error of the mean (n=40); statistically significant variations were found when compared to KB and MX. Differences were marked with letters a, b, and c. The data marked with different letters indicate that the differences were statistically significant. The same letters represented no statistically significant differences. Control group: fed with water; model group: fed with 5% DSS to induce acute diarrhea; probiotic group A: acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B: acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. Ig, immunoglobulin; DSS, dextran sodium sulfate.

modulation of immunoglobulins between simultaneous and interval administration of probiotics.

Diversity modulation of gut microbiota by probiotics

Fecal samples were collected at the end of this experiment (day 7) to compare the changes in the alpha diversity of intestinal bacteria among the four groups, and the Shannon diversity index, Simpson index, and inverse Simpson (invsimpson) index were chosen to assess the bacterial diversity of the different groups (*Figure 3*, *Table 2*). The results showed that different associations of probiotics could not cause changes in Shannon index (H=4.03, P=0.26), Simpson index (H=4.68, P=0.20), and invsimpson index (H=4.68, P=0.20), and the groups did not differ significantly from one another.

At the conclusion of the experiment, the intestinal flora of the four groups of rats did not differ statistically significantly: phylum level (F=1.143, P=0.35), class level (F=1.474, P=0.20), order level (F=1.471, P=0.24), family level (F=1.265, P=0.23), genus level (F=1.717, P=0.07), species level (F=1.670, P=0.057), according to PCoA based on Bray-Curtis distance (*Figure 4*, *Table 3*). These results indicate that neither the simultaneous administration of probiotics nor the administration of probiotics at 2-hour intervals had a significant effect on the intestinal bacterial structure of the rats.

Changes in gut microbiota

At the level of phylum, the abundance of *Fusobacteria* was decreased (t=2.110, P=0.049) in JG compared to GY. Compared to KB, the abundance of *Actinobacteria* (t=2.483, P=0.02), *Fusobacteria* (t=2.714, P=0.02) was increased in GY, whereas that of *Fibrobacteres* (t=−2.656, P=0.02), *Euryarchaeota* (t=−4.132, P<0.001), and *Cyanobacteria* (t=−4.311, P<0.001) decreased. In JG, *Fibrobacteres* (t=−2.789, P=0.01), *Euryarchaeota* (t=−3.866, P=0.001), and *Cyanobacteria* (t=−3.435, P=0.003) decreased in abundance, and *Actinobacteria* (t=1.810, P=0.09) increased in abundance. *Cyanobacteria* (t=−3.014, P=0.007) reduced in comparison to MX in GY (*Figure 5*, *Table 4*).

At the level of genus, the relative abundance of *Akkermansia* (t=1.597, P=0.13) was increased in GY compared to JG. The abundance of *Lactobacillus* (t=3.053, P=0.01) and *Akkermansia* (t=2.125, P=0.06) was increased

Table 1 Analysis of serum immunological indices in the four groups of rats

Group	IgA (μg/mL)	IgG (mg/mL)	IgM (μg/mL)
Probiotic group A (GY) (%)	49.882±8.594	1.766±0.430	344.940±34.892
Probiotic group B (JG) (%)	54.891±5.367	1.838±0.465	360.603±21.767
Control group (KB) (%)	43.740±4.941	1.342±0.122	278.928±28.244
Model group (MX) (%)	33.858±5.875	0.926±0.102	197.474±8.760
t value			
KB *vs.* GY	1.936	2.855	0.708
KB *vs.* JG	4.717	3.105	6.998
GY *vs.* JG	−1.483	−0.341	−1.143
MX *vs.* GY	4.789	5.715	12.334
MX *vs.* JG	8.115	5.760	21.868
KB *vs.* MX	4.071	8.306	8.711
P value			
KB *vs.* GY	0.07	0.02	<0.001
KB *vs.* JG	<0.001	0.01	<0.001
GY *vs.* JG	0.16	0.74	0.27
MX *vs.* GY	<0.001	<0.001	<0.001
MX *vs.* JG	<0.001	<0.001	<0.001
KB *vs.* MX	<0.001	<0.001	<0.001

Data are presented as mean ± standard error of the mean. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. Ig, immunoglobulin; DSS, dextran sodium sulfate.

in GY compared to the KB, whereas *Oscillibacter* (t=−3.371, P=0.003), *Roseburia* (t=−2.447, P=0.03), and *Lachnoclostridium* (t=−2.929, P=0.009) decreased in abundance. JG had increased abundance of *Lactobacillus* (t=3.404, P=0.007), whereas the abundance of *Prevotella* (t=−2.963, P=0.02), *Oscillibacter* (t=−2.471, P=0.02), and *Roseburia* (t=−3.177, P=0.008) decreased. Compared with MX, *Ruminococcus*'s relative abundance in GY and JG (t=−4.079, P=0.001; t=−2.982, P=0.01) decreased; the decrease in GY was more obvious, and *Lactobacillus* (t=2.186, P=0.050) had increased in abundance in GY (*Figure 6*, *Table 5*).

At the level of species, the relative abundance of *Oscillibacter* sp_1-3 (t=−2.513, P=0.02) and *Bacteroidales bacterium*_55_9 (t=−3.179, P=0.009) decreased in JG in contrast to KB. The decrease in these two species was more significant in GY [*Oscillibacter* sp_1-3 (t=−3.575, P=0.002) and the decrease in *Bacteroidales bacterium*_55_9 (t=−3.404, P=0.007)] in GY. *Phascolarctobacterium succinatutens* increased in abundance in JG (t=3.055, P=0.007), yet the increase was more pronounced in GY (t=3.933, P=0.002), and the difference between JG and GY was statistically significant. The abundance of *Lactobacillus johnsonii* was increased when comparing GY to KB (t=3.067, P=0.01). Compared with MX, the abundance of *Lactobacillus johnsonii* in GY increased (t=2.283, P=0.042) (*Figure 7*, *Table 6*).

LDA effect size analysis

In order to screen for biomarkers with significant differences between groups, the difference species between different groups were first detected through rank sum test, and LDA was used to reduce dimensionality and evaluate the impact of different species, resulting in the LDA score. The enriched microbiota of GY included

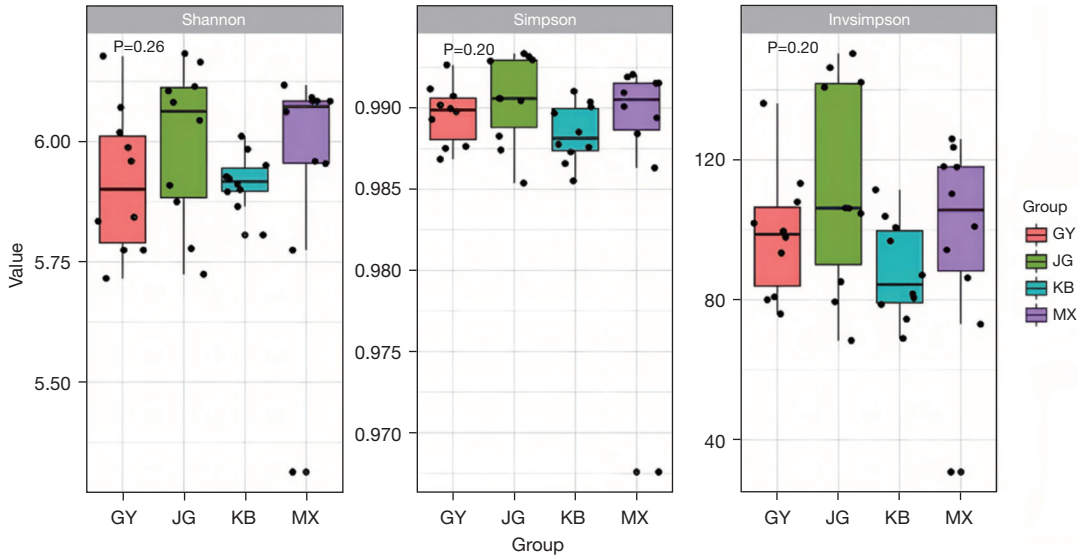

Figure 3 Gut microbial alpha diversity of the four groups on day 7 (Shannon index; Simpson index; invsimpson index). No statistically significant differences between groups. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. Invsimpson, inverse Simpson; DSS, dextran sodium sulfate.

Table 2 Changes in alpha diversity of intestinal flora in the four groups of rats

Group	Shannon index	Simpson index	Invsimpson index
Probiotic group A	5.901 (5.775, 6.032)	0.990 (0.988, 0.991)	98.782 (80.659, 109.309)
Probiotic group B	6.063 (5.851, 6.127)	0.991 (0.988, 0.993)	106.220 (83.770, 143.188)
Control group	5.917 (5.888, 5.959)	0.988 (0.987, 0.990)	84.399 (77.656, 101.461)
Model group	6.073 (5.909, 6.086)	0.990 (0.988, 0.992)	105.645 (83.000, 119.440)
H value	4.032	4.677	4.677
P value	0.26	0.20	0.20

Data are presented as median (interquartile range). Control group: fed with water; model group: fed with 5% DSS to induce acute diarrhea; probiotic group A: acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B: acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. Invsimpson, inverse Simpson.

Prevotella_sp_CAG604, Phascolarctobacterium_succinatutens, Prevotella_copri, Prevotella_sp_AM42_24, Akkermansia_muciniphila, Clostridium_sp_CAG678, Coriobacteriia_bacerium, Prevotella_sp_CAG1058, Prevotella_sp_CAG1185, Blautia_argi, Prevotella_sp_CAG1124, and Anaeromassilibacillus_sp_An172. The enriched microbiota of JG was Roseburia_sp_CAG309. The enriched microbiota of MX was Blautia_sp_CAG257, Ruminococcus_sp_Marseille_P6503, and Blautia_sp_Marseille_P3201T. The enriched microbiota of KB was Oscillibacter_sp_1-3, Acetatifactor_muris, Butyricicoccus_sp_1XD8_22, Roseburia_sp_CAG303, Oscillibacter_sp_PC13, Anaerotruncus_sp_G32012, and Oscillibacter_sp_CAG155 (LDA threshold >3.0) (*Figure 8*).

Functional gene alteration

In the eggNOG metabolic pathways, three metabolic

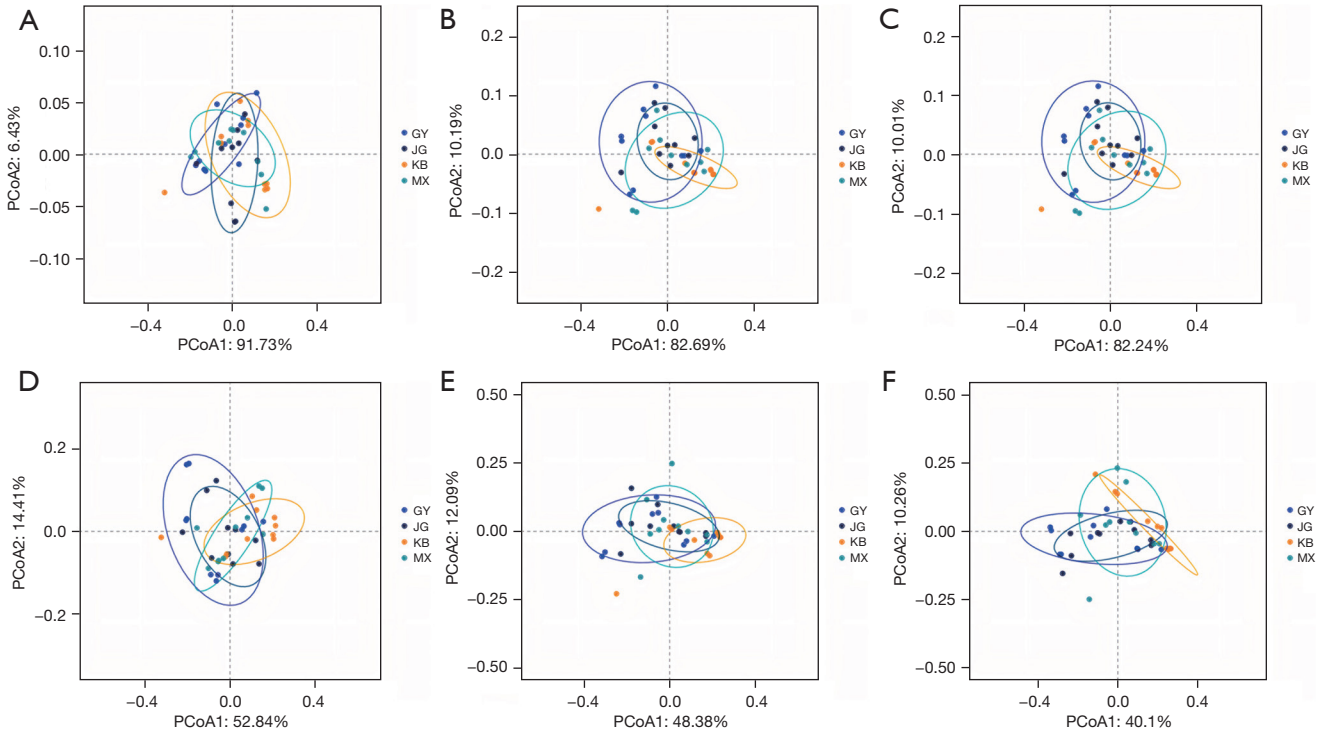

Figure 4 Comparison of the four groups of diversity modulation of gut microbiota after probiotics administration. PCoA of the four groups at the phylum level (A), class level (B), order level (C), family level (D), genus level (E) and species level (F). The Bray-Curtis distance of species abundance served as the basis for the PCoA. Samples from different groups are represented by dots of different colors. The species composition structure of the samples is increasingly similar the closer the samples are spaced apart in the graph. The difference between the four groups produced by the adonis analysis is shown by the F value and P value on the PCoA score graphs. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. PCoA, principal coordinate analysis; DSS, dextran sodium sulfate.

pathways with significant differences were found in GY compared with KB, in which P: Inorganic ion transport and metabolism pathway (t=3.787, P=0.04), E: Amino acid transport and metabolism pathway (t=3.507, P=0.005) were increased in abundance in GY group, whereas K: Transcription pathway (t=–2.923, P=0.04) was decreased in abundance in GY compared to KB. Two metabolic pathways with significant differences were found in JG compared to KB, namely E: Amino acid transport and metabolism pathway (t=4.172, P=0.003), P: Inorganic ion transport and metabolism pathway (t=4.420, P=0.03); both metabolic pathways were higher in JG. In KEGG metabolic pathways, compared to KB, in GY, there were four significantly different KEGG pathways, three of which: one carbon pool by folate (PATH: ko00670) (t=4.612, P=0.02), porphyrin and chlorophyll metabolism (PATH: ko00860) (t=5.785, P=0.047), and selenocompound metabolism (PATH: ko00450) (t=3.781, P=0.03) were increased in abundance in GY, whereas cell motility (t=–5.528, P=0.01) was decreased in abundance in GY in contrast to KB. In the metabolic pathways of CAZy, one metabolic pathway, the GH8 (glycoside hydrolase family 8) metabolic pathway, was found to be significantly different in GY compared with KB (t=4.155, P=0.02) (*Figures 9-11*, *Table* 7).

Correlations between taxa and pathways

Utilizing Spearman correlation analysis, the association

Table 3 Adonis analysis of β-diversity

Taxonomy	R^2	F value	P value
Phylum level	0.087	1.143	0.35
Class level	0.109	1.474	0.20
Order level	0.109	1.471	0.24
Family level	0.095	1.265	0.23
Genus level	0.125	1.717	0.07
Species level	0.122	1.670	0.057

between gut microbiota and metabolic pathways was investigated, which may indicate potential associations between microorganisms and function. Some potentially beneficial bacteria such as *Phascolarctobacterium* spp. (r=0.903, r=0.782, r=0.915, r=−0.964) and *Phascolarctobacterium succinatutens* (r=0.903, r=0.903, r=0.976, r=−0.952) were positively correlated with the expression of glycoside hydrolases, amino acid, and inorganic ion transport metabolism, and negatively correlated with cell motility. Some potentially harmful bacteria such as *Spirochaetes* (r=0.867, r=−0.842, r=−0.733, r=−0.903), *Fibrobacteres* (r=0.745, r=−0.818, r=−0.733, r=−0.842), and *Cyanobacteria* (r=0.842, r=−0.806, r=−0.855, r=−0.964), the genus *Lachnoclostridium* (r=0.927, r=−0.879, r=−0.782, r=−0.867), the genus *Aspergillus* (Flavonifractor) (r=0.964, r=−0.879, r=−0.782, r=−0.879), *Anaerotruncus* (r=0.952, r=−0.879, r=−0.770, r=−0.879), *Acetatifactor* (r=0.939, r=−0.927, r=−0.733, r=−0.891), *Acetalibacter* (r=0.927, r=−0.855, r=−0.733, r=−0.879), *Oscillibacter*_sp_1-3 (r=0.939, r=−0.842, r=−0.745, r=−0.867), *Butyricoccus*_sp_1XD8-22 (r=0.964, r=−0.879, r=−0.782, r=−0.879) were positively correlated with cell motility, whereas the expression of glycoside hydrolases, and amino acid and inorganic ion transport metabolism were negatively correlated (*Figure 12*). Taken together, these results suggest that the homeostasis of the intestinal flora may be related to the expression of glycoside hydrolases and the amino acid and inorganic ion transport metabolism pathways, suggesting that these factors may work as a whole to ameliorate acute diarrhea.

Discussion

The etiology of diarrhea in children is complex and related to age, diet, climate, living environment, socio-economic development, and genetics (10). Regardless of its cause, diarrhea itself can lead to an imbalance in the intestinal flora. Although the results of many randomized controlled trials suggest that probiotics can be used for the treatment of diarrhea (31), the exact mechanism by which probiotics can treat diarrhea is still unclear. In the present study, we assessed the modulation of the intestinal microbiota of rats by different temporal associations with probiotics, using metagenomics next-generation sequencing technology. Probiotics have been demonstrated in earlier research to enhance host health by modifying the intestinal microbiome (32). Our results suggest that giving multi-strain probiotics at intervals does not better improve the gut microbiota, whereas giving multi-strain probiotics has a more significant alteration of the gut flora and is more conducive to restoring a healthy gut microbiota.

At the phylum level, the relative abundance of *Actinobacteriota* was increased in GY compared with KB and MX, which may be related to the alleviation of diarrhea by probiotics. *Actinobacteriota* is a gram-positive bacterium, with high guanine-cytosine (GC) content, and one of the largest bacterial phyla. Together, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Clostridium*, and *Proteobacteria* dominate the gut microbiota (33). It has been reported that compared to healthy children, children with diabetes have a higher prevalence of *Actinobacteriota*, and *Firmicutes* is significantly reduced (34). In our study, the relative abundance of *Fusobacterium* was higher in GY than in JG and the difference was statistically significant, indicating that simultaneous administration of probiotics is more helpful in restoring a healthy gut than spaced use of probiotics. The results of the study by Suchodolsk showed that in inflammatory bowel disease (IBD) in dogs and cats, *Fusobacterium* was reduced, especially *Clostridium* clusters XIVa, and IV (i.e., *Lachnospiraceae*, *Ruminococcaceae*, *Faecalibacterium* spp.), indicating that these bacterial species are significant producers of short-chain fatty acids and might be involved in maintaining gut health (35).

At the genus level, the relative abundance of *Ruminococcus* was reduced in GY compared to the model group and the difference was statistically significant. The relative abundance of *Ruminococcus* was also reduced relatively in JG. *Ruminococcus* was one of the first stomach bacteria to be discovered; the *Ruminococcus* genus includes both beneficial and harmful bacteria, for example, *Ruminococcus gnavus* is considered a harmful bacterium, and Nishino *et al.* demonstrated that the abundance of *Ruminococcus* in patients with Crohn's disease compared to patients with

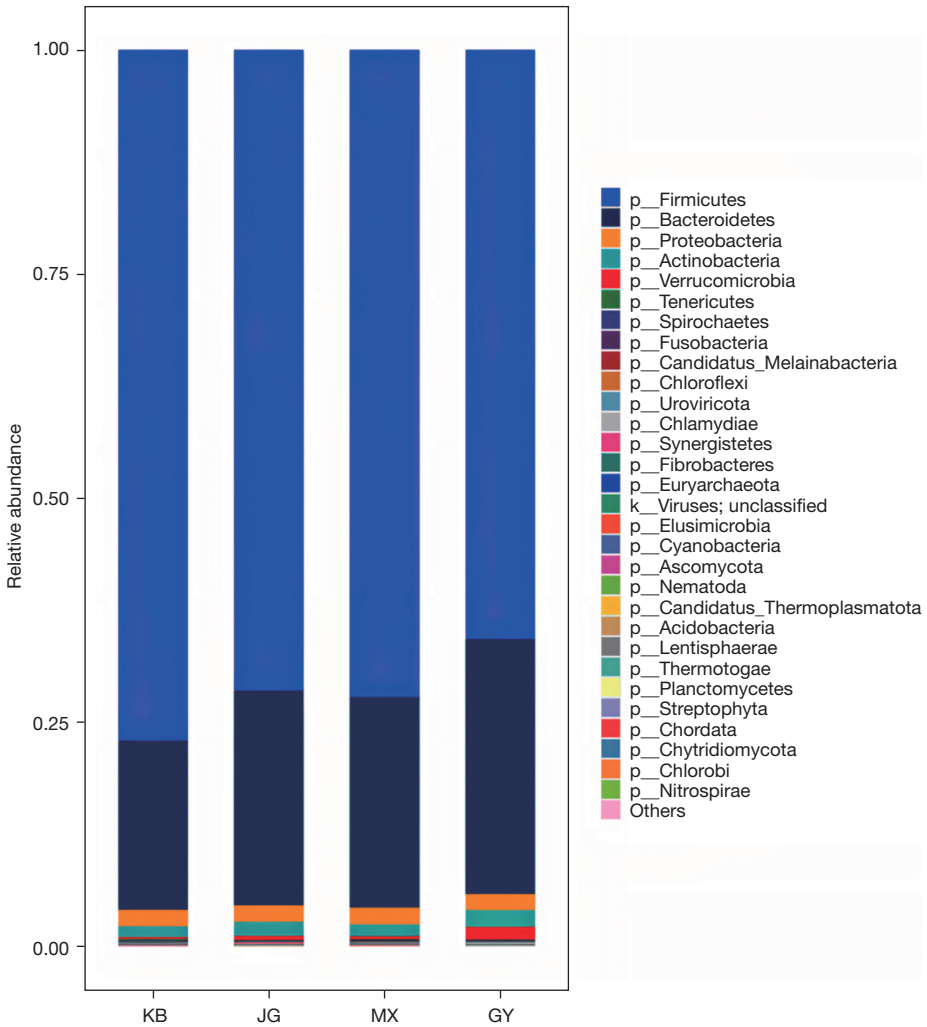

Figure 5 Differences in gut microbial composition between the four groups: KB, JG, MX, and GY. Fecal samples were collected on the 7th day. In the caption, each hue in the bar graph denotes a corresponding taxon group at the phylum level. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. DSS, dextran sodium sulfate.

non-IBD was significantly increased (36). Crost *et al.* showed that elevated relative abundance of *Ruminococcus gnavus* correlated with symptom severity in patients with Crohn's disease and found that *Ruminococcus* produce a pro-inflammatory polysaccharide, glucomannan, which can induce TNF-α secretion from dendritic cells in a toll-like receptor 4 (TLR4)-dependent manner (37), which, in combination with clinical manifestations and other results, led to the speculation that the GY may have had a higher reduction in harmful *Ruminococcus* bacteria.

The relative abundance of *Lactobacillus* was increased in both GY and JG compared to KB, with a greater increase in GY. It has been reported that, in intestinal microbiota research, *Lactobacillus* is the most prominent probiotic among the lactic acid bacteria (38). The probiotic *Lactobacillus* can adhere to the surface of host intestinal epithelial cells through the surface S-layer proteins, and competitively inhibit the invasion and colonization of intestinal pathogenic microorganisms (39), in addition to *Lactobacillus* inhibiting the pro-inflammatory cytokines (40).

Table 4 Analysis of fecal samples from the four groups of rats at the phylum level of intestinal flora

Group	*Fusobacteria*	*Actinobacteria*	*Fibrobacteres*	*Euryarchaeota*	*Cyanobacteria*
Probiotic group A (GY) (%)	0.10±0.03	1.85±0.72	0.03±0.01	0.02±0.01	0.02±0.01
Probiotic group B (JG) (%)	0.07±0.03	1.55±0.48	0.03±0.01	0.02±0.01	0.02±0.00
Control group (KB) (%)	0.07±0.01	1.16±0.50	0.04±0.01	0.03±0.01	0.03±0.00
Model group (MX) (%)	0.08±0.02	1.29±0.33	0.04±0.01	0.03±0.01	0.02±0.01
t value					
KB *vs.* GY	2.714	2.483	–2.656	–4.132	–4.311
KB *vs.* JG	0.121	1.810	–2.789	–3.866	–3.435
GY *vs.* JG	2.110	1.076	–0.329	–0.455	–1.443
MX *vs.* GY	1.934	2.192	–1.542	–1.510	–3.014
MX *vs.* JG	–0.498	1.398	–1.420	–1.130	–1.921
KB *vs.* MX	–0.828	–0.727	0.978	2.441	1.208
P value					
KB *vs.* GY	0.02	0.02	0.02	<0.001	<0.001
KB *vs.* JG	0.91	0.09	0.01	0.001	0.003
GY *vs.* JG	0.049	0.30	0.75	0.66	0.17
MX *vs.* GY	0.07	0.048	0.14	0.15	0.007
MX *vs.* JG	0.62	0.18	0.17	0.27	0.07
KB *vs.* MX	0.42	0.48	0.34	0.03	0.24

Data are presented as mean ± standard error of the mean. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. DSS, dextran sodium sulfate.

Lactobacilli are Gram-positive anaerobic or slightly aerobic rod-shaped bacteria. The relative abundance of *Lachnoclostridium* was reduced in both GY and JG compared to the control group, but the reduction was more prominent in GY, which was consistent with the study of Chen *et al.* (41), who showed that patients with ulcerative colitis had high levels of potentially pathogenic bacteria such as *Lachnoclostridium* detected in their feces and had less severe disease in remission. In contrast, Kang *et al.* showed that microbial diversity was reduced in fecal samples from patients with sarcopenia and probable sarcopenia, including *Lachnoclostridium*. Interestingly, the relative abundance was increased in irritable bowel lesions and colorectal tumors, and the relative increased abundance in liver steatosis and metabolic diseases (42); however, the relative abundance was decreased in autoimmune diseases (43); possible mechanisms need to be further investigated. The mean abundance of *Akkermansia* was increased in GY compared to KB. Chelakkot *et al.* demonstrated that *Akkermansia muciniphila* was more prevalent in the feces of individuals with ulcerative colitis and Crohn's disease, compared to healthy individuals. Possible mechanisms need to be further investigated, which may be related to the extracellular vesicles of *Akkermansia muciniphila* (AmEVs) reducing intestinal permeability by regulating the tight junctions in mice (44).

At the species level, the relative abundance of *Oscillibacter*_sp_1-3 decreased in both GY and JG compared to the control group, with a more pronounced decrease in GY. Zhang *et al.* demonstrated that fasting blood glucose was reduced in the offspring of mice fed a diet rich in fat chow and given inulin, a probiotic, as compared to the

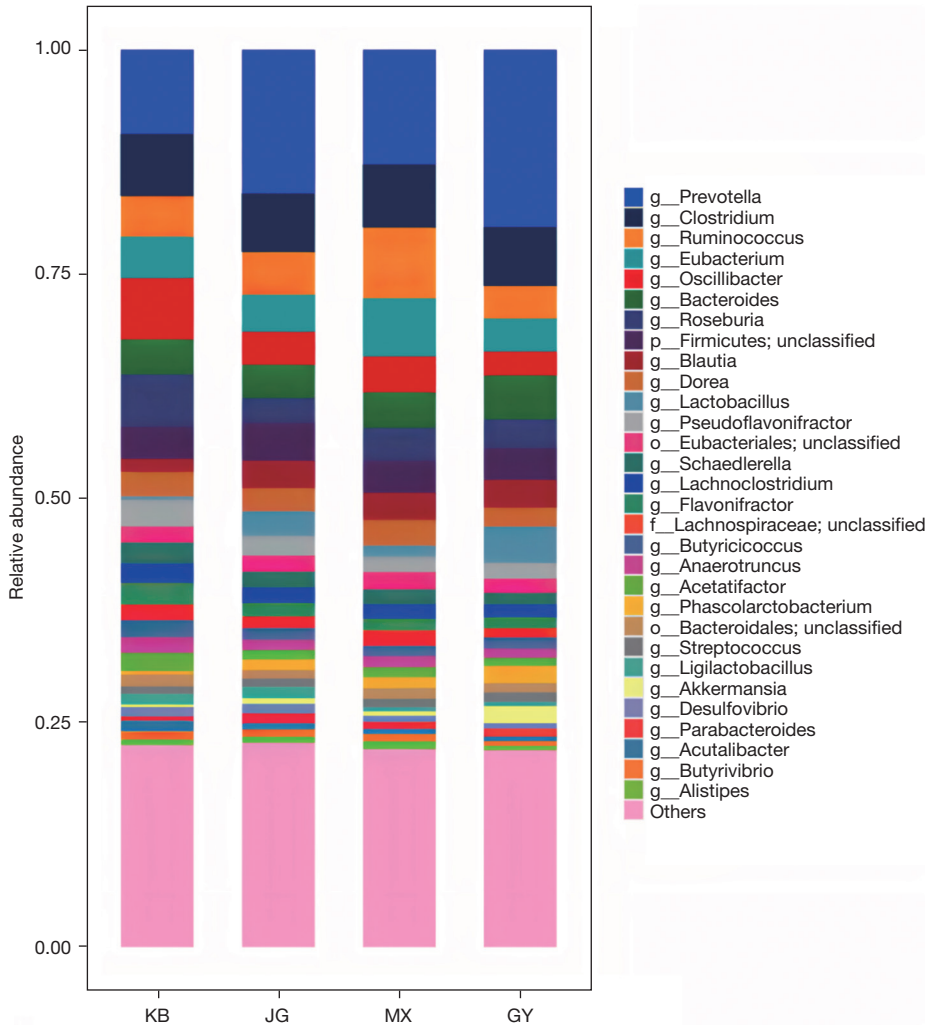

Figure 6 Differences in gut microbial composition between the four groups: KB, JG, MX, and GY. Fecal samples were collected on the 7th day. In the caption, each hue in the bar graph denotes a corresponding taxon group at the genus level. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour intervals. DSS, dextran sodium sulfate.

progeny of mice fed high-fat chow alone. The area under the blood glucose curve of the glucose tolerance test was reduced, and it also improved intestinal dysbiosis. It was observed that the abundance of *Oscillibacter*_sp_1-3 in the cecum contents of the offspring of mice fed a high-fat diet and given inulin was significantly lower than that of the group fed a high-fat diet fed (45), which is in agreement with our findings. Little is known about the physiological effects of *Oscillibacter*_sp_1-3, yet it was observed that *Oscillibacter*_sp_1-3 was negatively correlated with proximal colonic barrier function score. This may be due to other gut microbial metabolites altering the abundance of *Oscillibacter*_sp_1-3 strains, or it is possible that *Oscillibacter*_sp_1-3 directly regulate the components that maintain the integrity of gut barrier function (46). The abundance of *Lactobacillus johnsonii* was increased in GY compared to KB and MX, and the difference was statistically significant. *Lactobacillus johnsonii* belongs to the *Lactobacillus* family.

Table 5 Analysis of fecal samples from the four groups of rats at the genus level of intestinal flora

Group	*Akkermansia*	*Prevotella*	*Lactobacillus*	*Oscillibacter*	*Roseburia*	*Lachnoclostridium*	*Ruminococcus*
Probiotic group A (GY) (%)	1.95±2.43	0.01±0.00	3.91±3.59	2.85±2.61	3.25±1.81	1.41±0.52	3.66±1.43
Probiotic group B (JG) (%)	0.64±0.97	0.01±0.01	2.65±2.02	3.82±2.85	2.80±1.07	1.77±0.59	4.75±1.49
Control group (KB) (%)	0.31±0.36	0.11±0.11	0.41±0.49	6.96±2.83	5.88±2.87	2.20±0.67	4.50±2.38
Model group (MX) (%)	0.48±0.63	0.06±0.12	1.25±1.41	4.04±2.07	3.67±2.08	1.59±0.31	7.82±2.89
t value							
KB *vs.* GY	2.125	–3.001	3.053	–3.371	–2.447	–2.929	–0.952
KB *vs.* JG	1.002	–2.963	3.404	–2.471	–3.177	–1.509	0.288
GY *vs.* JG	1.597	–0.669	0.967	–0.794	0.677	–1.428	–1.672
MX *vs.* GY	1.856	–1.351	2.186	–1.125	–0.476	–0.945	–4.079
MX *vs.* JG	0.414	–1.318	1.806	–0.193	–1.171	0.830	–2.982
KB *vs.* MX	–0.775	1.011	–1.752	2.635	1.974	2.590	–2.806
P value							
KB *vs.* GY	0.06	0.02	0.01	0.003	0.03	0.009	0.35
KB *vs.* JG	0.33	0.02	0.007	0.02	0.008	0.15	0.78
GY *vs.* JG	0.13	0.51	0.35	0.44	0.51	0.17	0.11
MX *vs.* GY	0.08	0.21	0.050	0.28	0.64	0.36	0.001
MX *vs.* JG	0.68	0.22	0.09	0.85	0.26	0.42	0.01
KB *vs.* MX	0.45	0.33	0.10	0.02	0.06	0.02	0.01

Data are presented as mean ± standard error of the mean. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval.

Its common mechanism of action as a potential anti-inflammatory bacterium in different diseases includes the adjustment of immunological response, the interaction with the intestinal flora, and the improvement of barrier function (47). Jia *et al.* showed that *Lactobacillus johnsonii* colonization was reduced in colitis mice, and they found that *Lactobacillus johnsonii* could relieve colitis through the TLR1/2-STAT3 pathway to promote the activation of interleukin 10 (IL-10) in $CD206^{+}$ macrophages (48). Thus, *Lactobacillus johnsonii* may serve as an immunomodulating anti-inflammatory therapeutic target for colitis. The abundance of *Phascolarctobacterium succinatutens* was reduced in JG compared to GY, and the difference was statistically significant. Huang *et al.* showed that spraying a complex probiotic fermentation solution in the living environment of piglets changed the structure and diversity of the intestinal flora of the piglets and increased some of the abundance of beneficial bacteria, such as *Phascolarctobacterium succinatutens* (49).

We also observed relatively insignificant changes in functional genes compared to changes in intestinal microbiota, which is consistent with The Human Microbiome Project Consortium. The abundance of functional genes in gut microbes is more stable than the community structure (50). The application of probiotics also caused functional changes, with a decrease in cell motility in GY compared to KB. According to Xu *et al.* (51), cell motility was lower in fecal samples from diarrheic dogs in the probiotic-treated group compared to the control group, and these results suggest a relationship between cell motility and bacterial movement in liquid and its diffusion on the surface. Thus, increasing bacterial

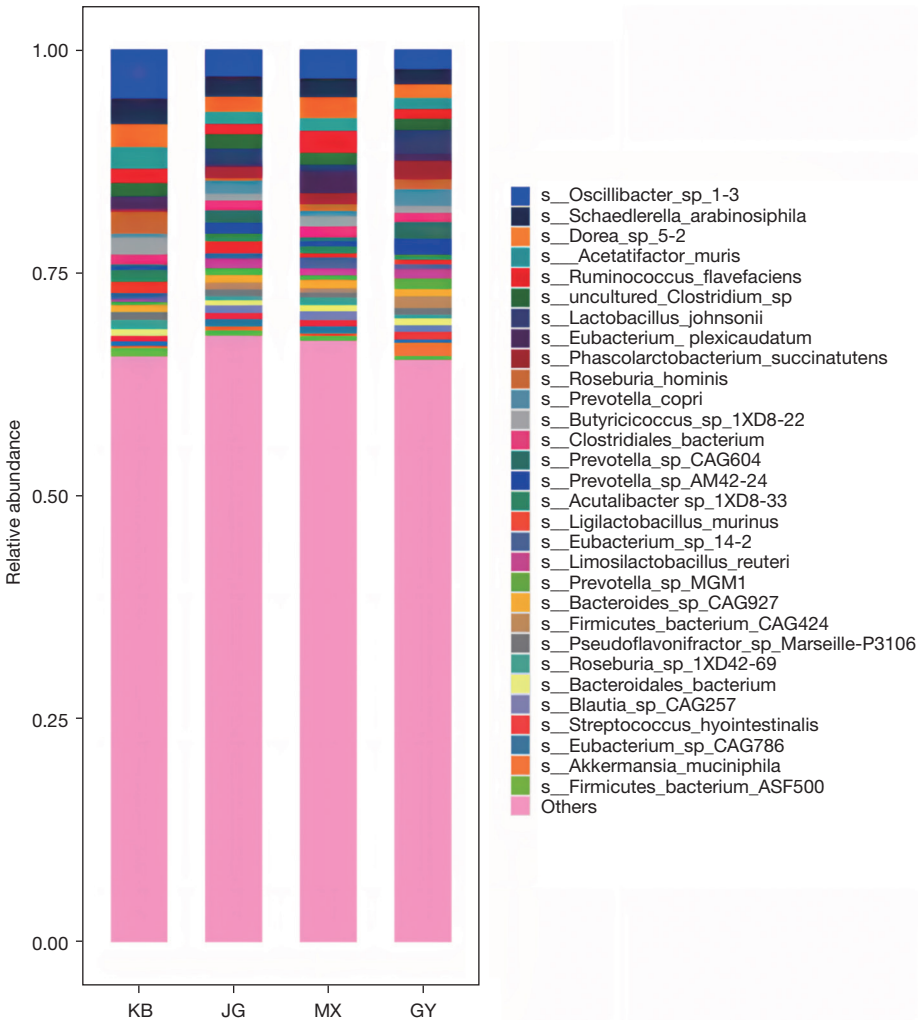

Figure 7 Differences in gut microbial composition between the four groups: KB, JG, MX, and GY. Fecal samples were collected on the 7th day. In the caption, each hue in the bar graph denotes a corresponding taxon group at the species level. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. DSS, dextran sodium sulfate.

cell motility may increase bacterial virulence (52). Furthermore, we found that there was a negative correlation between cell motility and a few enhanced beneficial microorganisms, such as *Acidaminococcaceae* and *Phascolarctobacterium*. Glycoside hydrolase expression was up-regulated in GY as compared to KB and the relative abundance of *Prevotella* was increased in JG. In the study by Aakko *et al.* (53), it was observed that exo-beta-(1,4)-xylanase, xylan-1,4-β-xylosidase, alph-1-arabinofuranosidase, and several other CAZy belonging to the glycosyl hydrolases family were associated with high abundance of *Prevotella* in the intestinal microbiota, whereas in subjects with low abundance of *Prevotella* among the microbiota, the population did not have *Prevotella*-derived CAZy, which is consistent with our findings. This suggests the possibility of determining the metabolic capacity of the microbiota at a certain level by macrogenomics CAZy. In addition, the application of probiotics also enhanced some microbial metabolic pathways, including amino acid and inorganic ion transporter metabolism, which may contribute

Table 6 Analysis of fecal samples from the four groups of rats at the species level of intestinal flora

Group	*Oscillibacter*_sp_1–3	*Bacteroidales bacterium*_55_9	*Phascolarctobacterium succinatutens*	*Lactobacillus johnsonii*
Probiotic group A (GY) (%)	2.24±2.02	0.01±0.00	1.96±1.13	2.60±2.47
Probiotic group B (JG) (%)	3.05±2.30	0.01±0.00	1.15±0.54	1.54±1.20
Control group (KB) (%)	5.52±2.09	0.02±0.02	0.38±0.59	0.20±0.24
Model group (MX) (%)	3.22±1.60	0.01±0.01	1.19±0.76	0.70±0.93
t value				
KB *vs.* GY	–3.575	–3.404	3.933	3.067
KB *vs.* JG	–2.513	–3.179	3.055	3.478
GY *vs.* JG	–0.838	–0.389	2.049	1.226
MX *vs.* GY	–1.208	–1.585	1.787	2.283
MX *vs.* JG	–0.193	–1.322	–0.143	1.755
KB *vs.* MX	2.768	1.879	–2.679	–1.644
P value				
KB *vs.* GY	0.002	0.007	0.002	0.01
KB *vs.* JG	0.02	0.009	0.007	0.006
GY *vs.* JG	0.41	0.70	0.06	0.24
MX *vs.* GY	0.24	0.14	0.09	0.042
MX *vs.* JG	0.85	0.21	0.89	0.10
KB *vs.* MX	0.01	0.08	0.02	0.13

Data are presented as mean ± standard error of the mean. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum, Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. DSS, dextran sodium sulfate.

to the production of more amino acids and inorganic ions for the maintenance of host intestinal homeostasis. The up-regulation of these pathways was positively correlated with beneficial bacteria such as *Acidaminococcaceae*, and negatively correlated with potentially harmful bacteria such as the *Spirochaetes*, *Fibrobacteres*, and *Cyanobacteria*. These results suggest that the beneficial mechanism of probiotics may be related to the transport metabolism of amino acids and inorganic ions, as well as the enrichment of related genes that convert ingested dietary fiber and amino acids into host available nutrients (54). This can generate new amino acids, peptides, and secondary metabolites to combat diarrhea (55).

Of course, there are also shortcomings in this study, the most obvious of which is the relatively small sample size (10 rats per group), which may have led to the appearance of some false negatives or false positives. In this study, we used SD rats as test subjects to ensure homogeneity, but the same shortcoming exists, namely, the relatively low resolution of taxonomic mapping of the rat gut microbiota. Although a small number of species can be recognized in the rat gut microbiota, the lowest taxonomic unit of the rat gut microbiota with a high level of confidence is the genus (13). Few bacteria with reference genomes have been isolated from the rat gut and are available for public database access, and the rat gut microbiota is not well understood. Therefore, it is crucial to improve the availability of reference genomes of rat gut microorganisms to improve quasi-population classification in rat microbiome

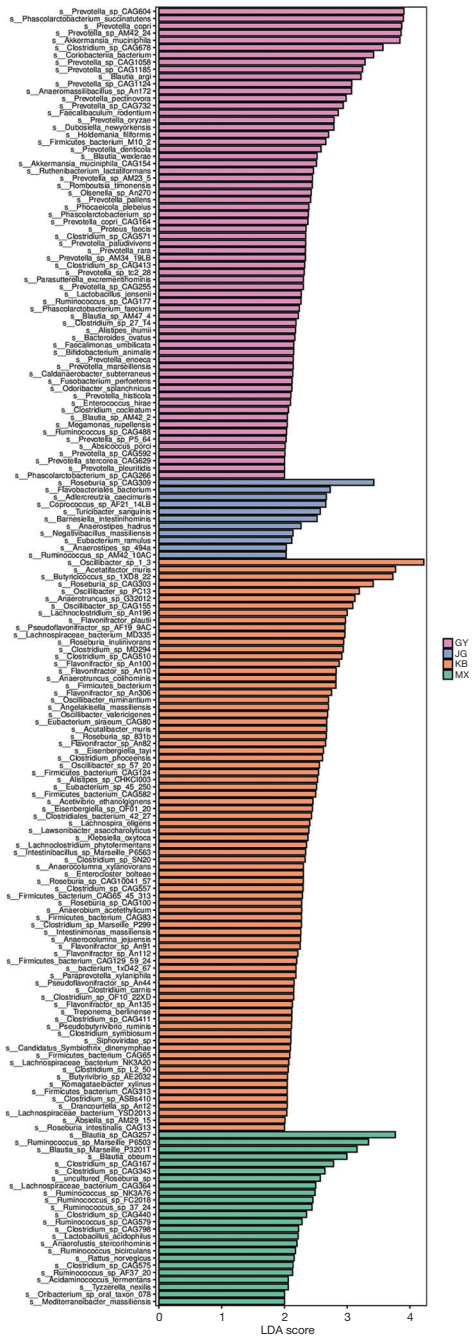

Figure 8 LefSe analysis of intestinal microbiota between the four groups. LDA score threshold >3. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with Clostridium butyricum, Bifidobacterium infantis, and Saccharomyces boulardii at the same time; probiotics group B (JG): acute diarrhea model fed with Clostridium butyricum, Bifidobacterium infantis, and Saccharomyces boulardii at 2-hour interval. LefSe, linear discriminant analysis Effect Size; LDA, linear discriminant analysis; DSS, dextran sodium sulfate.

studies (56). The results of this study need to be confirmed by further clinical trials, which will require additional large-scale clinical trials using multi-omics and will need to consider inter-individual differences (dietary habits, age, gender, weight, and other physiological characteristics of the children).

In addition, the effect of probiotics has a significant dose-dependent effect (5). Wu *et al.* showed that the incidence of antibiotic-induced intestinal dysbiosis was significantly lower in the group of double-dose probiotic preparation compared to the group of conventional-dose probiotic preparation (57). Konstantis *et al.* found that a single probiotic in a low dose and a short course of treatment might be more effective in improving the symptoms and quality of life of irritable bowel syndrome more effectively (58), which warrants future studies on the quantitative-effectiveness relationship of probiotics.

Conclusions

The present study analyzed the effects of different time-linked probiotics on the intestinal microecology of rats with acute diarrhea based on metagenomics sequencing. The results showed that no significant changes were observed in microbial diversity after 7 days of simultaneous administration of probiotics; however, the composition and function of the intestinal microbiota underwent obvious changes, with an increase in the relative abundance of some beneficial species and a decrease in potentially harmful species, especially the alteration of the intestinal flora of the rats in GY. In addition, up-regulation of glycoside hydrolase expression and pathways related to the transport metabolism of amino acids and inorganic ions, and down-regulation of cell motility were also observed in GY. Although our previous studies have shown comparable clinical efficacy between simultaneous and spaced administration of probiotics (15), the present study, based on metagenomics sequencing analysis, found that the difference between simultaneous administration of probiotics was more significant than spaced administration of probiotics in terms of both intestinal flora and metabolic pathways. This theoretically suggests that GY should have had better clinical efficacy, but this was not the case and this calls for more in-depth basic and clinical studies. Continuous supplementation of probiotics may have a positive effect on improving intestinal homeostasis in rats with acute diarrhea. This study to some extent helps to elucidate the possible mechanisms by which probiotics can improve diarrhea.

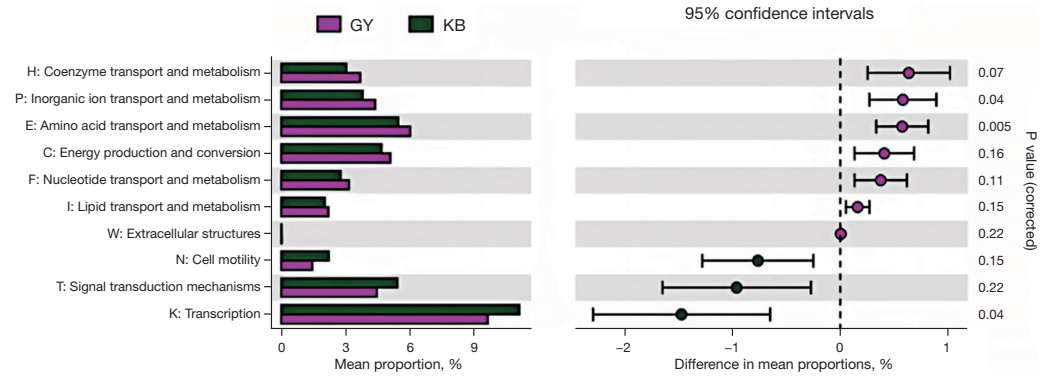

Figure 9 Modifications to the gut microbiota's functional profile in rats with diarrhea between probiotic group A (GY) and control group (KB) in eggNOG pathway. The red column represents GY; the green column represents KB. Control group (KB): fed with water; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time. eggNOG, evolutionary genealogy of genes: Non-supervised Orthologous Groups; DSS, dextran sodium sulfate.

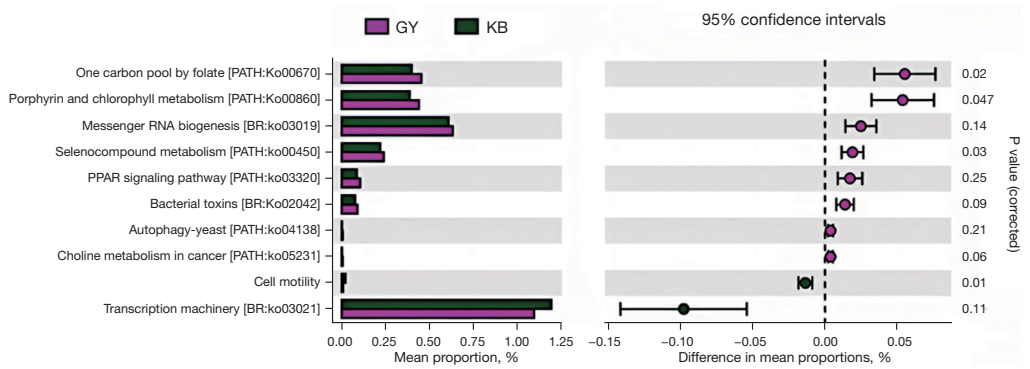

Figure 10 Modifications to the gut microbiota's functional profile in rats with diarrhea between probiotic group A (GY) and control group (KB) in KEGG pathway. The red column represented GY group; the green column represents KB. Control group (KB): fed with water; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time. KEGG, Kyoto Encyclopedia of Genes and Genomes; DSS, dextran sodium sulfate.

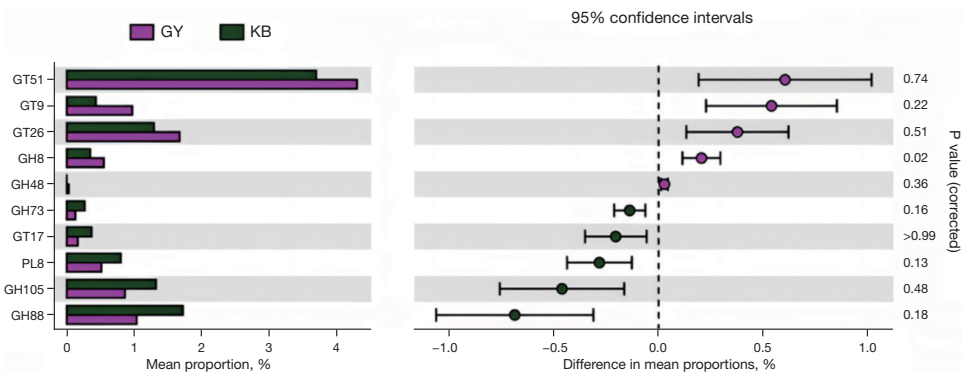

Figure 11 Modifications to the gut microbiota's functional profile in rats with diarrhea between probiotic group A (GY) and control group (KB) in CAZy pathway. The red column represented GY group; the green column represents KB. Control group (KB): fed with water; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time. CAZy, Carbohydrate-Active Enzyme.

Table 7 Analysis of metabolic pathways in fecal samples from the four groups of rats

Group	KEGG				CAZy	eggNOG		
	One carbon pool by folate (PATH: ko00670)	Porphyrin and chlorophyll metabolism (PATH: ko00860)	Selenocompound metabolism (PATH: ko00450)	Cell motility	Glycoside Hydrolase Family 8	P: Inorganic ion transport and metabolism	E: Amino acid transport and metabolism	K: Transcription
Probiotic group A (GY) (%)	3,480.958±339.169	3,368.791±242.132	1,836.698±161.113	70.910±42.239	66.071±19.247	44,725.499±3,380.265	32,495.243±3,715.999	71,371.947±5,781.980
Probiotic group B (JG) (%)	3,366.241±235.794	3,457.640±369.448	1,813.249±114.329	91.934±34.663	57.848±15.714	44,766.557±2,727.302	31,956.782±2,508.184	75,372.276±4,187.976
Control group (KB) (%)	2,892.185±218.959	2,801.323±193.877	1,599.867±115.234	158.832±27.312	38.394±8.563	39,973.806±2,078.370	27,968.824±1,687.457	79,803.788±7,057.325
Model group (MX) (%)	3,245.163±351.657	3,375.826±524.712	1,836.698±161.113	104.494±41.881	48.135±15.765	42,202.205±2,747.064	30,020.183±2,856.570	75,193.231±6,510.051
t value								
KB *vs.* GY	4.612	5.785	3.781	−5.528	4.155	3.787	3.507	−2.923
KB *vs.* JG	4.659	4.974	4.157	−4.794	3.438	4.420	4.172	−1.708
GY *vs.* JG	0.878	−0.636	0.375	−1.217	1.046	−0.030	0.380	−1.772
MX *vs.* GY	1.604	−0.038	2.266	−1.785	2.280	1.832	1.670	−1.388
MX *vs.* JG	0.967	0.403	2.267	−0.731	1.380	2.095	1.611	0.073
KB *vs.* MX	−2.895	−3.248	−1.472	3.437	−1.717	−2.046	−1.955	1.519
Adjusted P value								
KB *vs.* GY	0.02	0.047	0.03	0.01	0.02	0.04	0.005	0.04
KB *vs.* JG	0.24	>0.99	0.19	0.01	0.48	0.03	0.003	0.12
GY *vs.* JG	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
MX *vs.* GY	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
MX *vs.* JG	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
KB *vs.* MX	>0.99	>0.99	>0.99	0.62	>0.99	0.86	0.44	0.99

Data are presented as mean ± standard error of the mean. Control group (KB): fed with water; model group (MX): fed with 5% DSS to induce acute diarrhea; probiotic group A (GY): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at the same time; probiotics group B (JG): acute diarrhea model fed with *Clostridium butyricum*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* at 2-hour interval. KEGG, Kyoto Encyclopedia of Genes and Genomes; CAZy, Carbohydrate-Active Enzymes database; eggNOG, evolutionary genealogy of genes: Non-supervised Orthologous Groups.

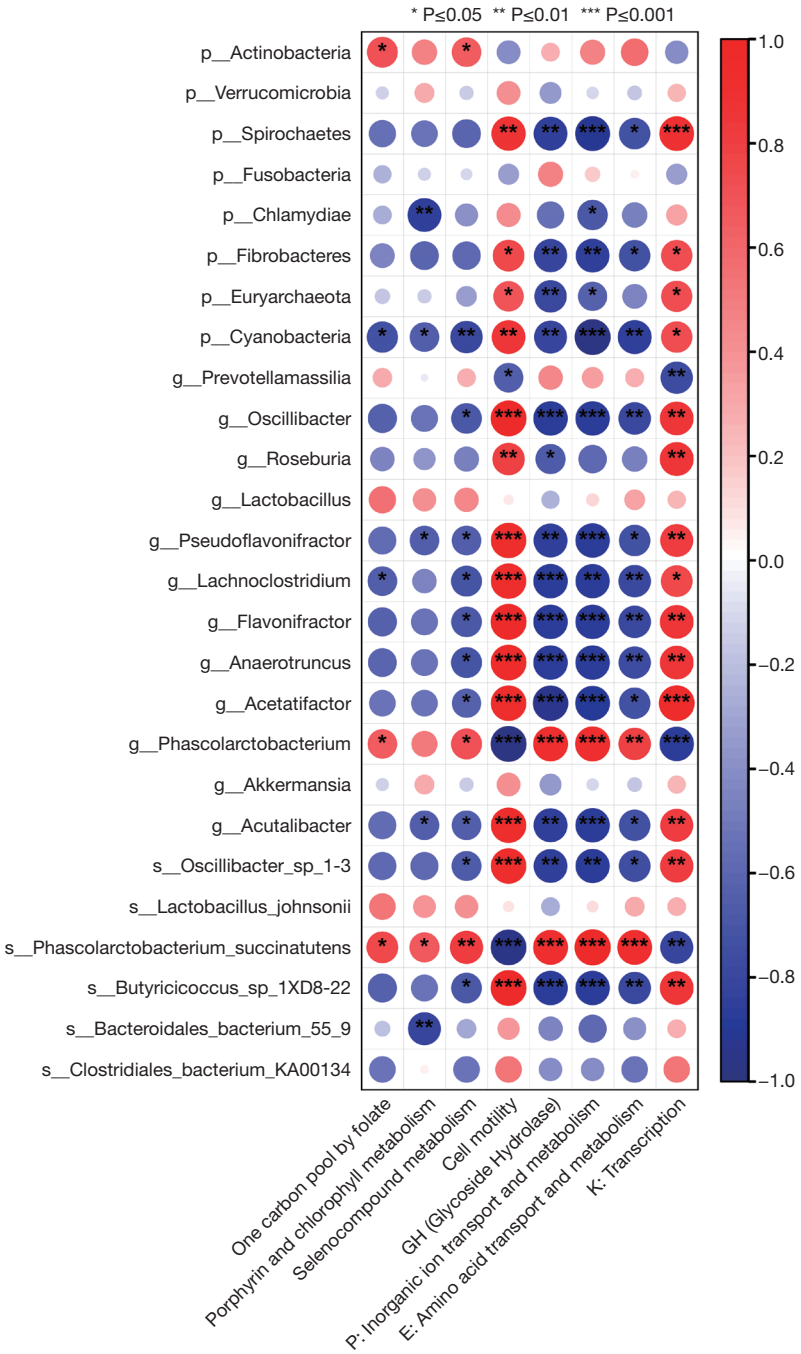

Figure 12 Spearman correlation heat map of intestinal microbiota and functional gene categories. The color scheme symbolizes the Spearman's rho, ranking between −1 and 1. A value larger than zero implies a positive association, and vice versa. The color and size of circles show the correlation strength as presented by the color scheme.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-129/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-129/coif). All authors report receiving metagenomic next-generation sequencing technology from Wuhan Igenebook Biotechnology Co., Ltd. and Yundi Biotechnology Co., Ltd. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hubei Disease Control and Prevention Center and approved by the Animal Ethics Committee of Hubei Disease Control and Prevention Center (No. 202420035).

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