Randomized controlled trial comparing the impacts of *Saccharomyces boulardii* and *Lactobacillus rhamnosus* OF44 on intestinal flora in cerebral palsy rats: insights into inflammation biomarkers and depression-like behaviors

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Background: Cerebral palsy (CP) is a unique neurological disorder which adversely affects motion. Cytokines and gut microbial composition contribute to CP and other diseases, such as reproductive tract inflammation and bone loss. Importantly, *Saccharomyces boulardii* (*S. boulardii*) reduces the degree of inflammation and improves overall health status. As our previous study showed that *Lactobacillus rhamnosus* (*L. rhamnosus*) OF44, a selected strain of gut bacteria originally used to treat reproductive tract inflammation and bone loss, has effects similar to that of *S. boulardii*, we decided to use *L. rhamnosus* OF44 on CP rats. Validation of the effects of *L. rhamnosus* OF44 on CP adds to its confirmed effects in treating osteoporosis and reproductive tract microbiota disorders, increasing its potential as a probiotic. The purpose of this was to ascertain whether *L. rhamnosus* OF44 can alleviate the symptoms of CP.

Methods: CP rat models were created through left carotid artery ligation. Following this, 100-day old CP rats were exposed to *L. rhamnosus* OF44, *S. boulardii*, or normal saline gastric gavage daily for 28 days. Grouping of the rats is determined randomly. Before and after the gavage, behavioral experiments were conducted and the inflammation levels assessed via measurements of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) inflammatory markers. The efficacy of the outcome is measured by performing statistical analysis like the *t*-test on the data to see its significance. Additionally, variations inside gut microbiome were evaluated via 16S ribosomal RNA sequencing.

Results: Before intervention, CP rats failed to exhibit depression-like behavior (P=0.6). *L. rhamnosus* OF44 treatment significantly reduced the level of IL-6 (P=4.8e–05), *S. boulardii* treatment significantly reduced the level of TNF-α (P=0.04). In addition, both treatments altered the composition and complexity of the gut microbiome.

Conclusions: Our results indicated that *L. rhamnosus* OF44 has potential in alleviating inflammation and altering the gut microbial composition in CP, and that it has the potential to clinically treat CP. There are some limitations of this study. For example, dietary differences and their effects on gastrointestinal dysfunction are not considered in this study, and only two behavioral experiments were used.

Introduction

Cerebral palsy (CP) is a unique type of neurological disorder that degrades an individual's ability to move and maintain equilibrium and posture (1). CP is commonly triggered via brain injuries or malformation before, during, or after birth (2). Symptoms include impaired motor skills and coordination, muscle tightness or spasticity, difficulty with speech and communication, and impaired balance and coordination. Different gut microbiomes have different effects on general health, as well as diseases. For example, there is evidence of prebiotics being able to reduce the amount of cytokines in humans as well as animals; however, only a few studies suggest that prebiotics can enhance immune system functions (3). As for patients with CP, studies suggest that excessive *Prevotella* may be a biomarker for CP (4), and *Prevotella* is related to inflammation (5), suggesting that distinctive gut microbiota of CP patients contribute to their high levels of cytokines. Though CP has negative effects on both the patients and their caregivers (6), there currently exists no curative treatment for CP; however, therapy and treatment have been shown to alleviate the symptoms, including acceptance and commitment therapy, action observations, and bimanual training, among about 20 other treatments (4,7). Although a comprehensive understanding of diseases helps to improve treatment regimen, the etiology and pathophysiology of CP remain largely unknown despite considerable research efforts. Recent evidence suggests that gut microbiota contributes to the progression of CP (8), but the exact mechanisms and pathways remain largely elusive.

Lactobacillus rhamnosus (*L. rhamnosus*) OF44 is a selected strain of human gut bacteria. Gut microbiota has important effects in the general health of human, and it plays a role in the development of many kinds of diseases, for example, cancer (9) and neurological disorders (10). *L. rhamnosus* OF44 was originally used for the therapy and prevention of diseases triggered via reproductive tract dysbiosis or bone loss (11). In a study on *L. rhamnosus* OF44 as an intervention for bone loss, it was found that this strain exerted immunomodulatory effects, similar to many other strains of *Lactobacillus* (12-17). Since both brain disorders and metabolic immune diseases are related to inflammation (18), we attempted to use *L. rhamnosus* OF44 to modify the gut microbiota of CP rats and assess its impact on CP.

We hypothesized that the manipulation of the gut microbiota by *L. rhamnosus* OF44 can improve the general conditions of CP. Differences in cytokine levels and gut microbiota composition have been observed between rats with CP and healthy ones. It has also been reported that the administration of *Saccharomyces boulardii* (*S. boulardii*) can improve bowel status, reduce depression-like behavior, lower the hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, and modify the gut microbiome of hemiplegic spastic CP rats. However, despite the identified effects of *S. boulardii*, its unpredictable and less pronounced impact on gut microbiota of CP mice suggests it may not be the ideal microbe for CP treatment (19). Additionally, given the proven efficacy of *L. rhamnosus* OF44 in treating and preventing osteoporosis and reproductive tract disorders, which both involves inflammation and changes

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Highlight box

Key findings
- *Lactobacillus rhamnosus* (*L. rhamnosus*) OF44 intervention reduces the inflammation level of cerebral palsy (CP) rats. The intervention also has the potential to change the gut microbial composition and metabolic functions of CP rats.

What is known and what is new?
- *Saccharomyces boulardii* (*S. boulardii*) intervention can alleviate the depressive state, reduce inflammation, and alter the gut microbial of rats with CP.
- *L. rhamnosus* OF44, a gut microbial strain, demonstrated the same affects as those of *S. boulardii* in reducing inflammation levels.

What is the implication, and what should change now?
- Our findings indicate that the use of probiotics can alleviate inflammation in CP rats and that these probiotics can potentially be used to treat CP in humans.

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in microbiota (20,21), exploring its role in CP opens avenues for investigating other potentially more effective treatments for CP, as well as the full clinical potential of *L. rhamnosus* OF44. To this end, we used a rat model of CP and behavioral experiments, enzyme-linked immunosorbent assay (ELISA), and 16S ribosomal RNA (rRNA) sequencing to compare the motor symptoms, depressive states, cytokine levels, and gut microbiomes among groups administered with different interventions. Comparison of the results obtained from the control groups and those treated with *S. boulardii* could provide a comprehensive evaluation of the efficacy of *L. rhamnosus* OF44 intervention in CP. The current animal study is crucial in addressing the research question as it delves into the efficacy of *L. rhamnosus* OF44 in the context of CP by elucidating the distinct impact of *L. rhamnosus* OF44 on inflammation, behavior, and gut microbiota. The proposed outcomes serve as meaningful indicators of the potential effectiveness of *L. rhamnosus* OF44 for CP because various factors contributing to the development of CP, for instance, gut microbiota and inflammation, are addressed. Collectively, our findings contribute novel insights into the potential function of the gut microbiota in CP and may further lead to the innovation of therapeutic strategies for the management or prevention of this disorder. We present this article in accordance with the ARRIVE reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-23-566/rc).

Methods

We employed a randomized parallel controlled trial design with simple randomization, using 18 CP model rats and 6 healthy rats as experimental subjects. We estimated the number of rats used with references of other studies in the same area (8,19). Simple randomization ensures that each rat has an equal chance of being assigned to each group. This minimizes the bias of selection in the experiment. By choosing to estimate sample size with accordance to other studies on CP and gut microbiota, our study is aligned with existing research, and the validity of our study is enhanced. Blood and stool samples are collected before the intervention (0 day), 1 week (7 days), 2 weeks (14 days), 3 weeks (21 days), and 4 weeks after the intervention (28 days). On day 0, three CP rats (1 in group A, 1 in group B, and 1 in group C) were euthanized, along with six control rats. The contents in the ceca were gathered and frozen at –80 ℃, while the colon tissues

(cleared of contents) and brain tissues were fixed in formalin at room temperature for preservation. On day 14, six CP rats (2 in group A, 2 in group B, and 2 in group C) were euthanized, with the same procedures being performed as those on day 0. Blinding procedures were implemented to minimize potential bias in the experiment. Personnel who were involved in data collection, outcome assessments, and data analysis were kept unaware of the group assignments. A protocol was prepared before the study without registration.

Animals

All animals used in the experiment were acquired from Zhuhai Bestest Biotechnology Co., Ltd. (Zhuhai, China), and kept in a specific pathogen-free animal laboratory by Shenzhen TOP Biotechnology Co., Ltd. (Shenzhen, China). The animal study protocol was approved by the Institutional Review Board of Department of Pediatrics of Longgang District Maternity & Child Healthcare Hospital of Shenzhen City (ethical approval number: LGFYYXLLLQ-2020-002), in compliance with the Chinese national standards GB14925 and GB14922, as well as the standard operating procedures of Shenzhen TOP Biotechnology Co., Ltd., for the care and use of animals. During the study period, all rats were subjected to uniform management measures, such as drug adjustment, nutritional intervention, and standardized lifestyle. At 100 days of age, 12 male and 12 female specific pathogen-free grade Sprague Dawley (SD) rats were assigned to a CP group (n=18) or a control group (n=6) at random. These rats were all kept inside a feeding space at 22±1 ℃ and 50–70% moisture under a single 12-hour light-dark cycle to avoid confounding variables.

Modeling of CP

CP was modeled in neonatal rats through left carotid artery ligation, and 3- to 7-day-old rats were randomly grouped. The surgical procedure involved anesthetizing the 7-day-old neonatal rats with isoflurane, fixing them in a supine position, and disinfecting the skin with 75% alcohol. A midline incision was created in the neck, the left carotid artery was isolated using vascular clamps, double-ligation with No. 7 sterile sutures, and cutting off of the vessels. A gelatin sponge was used for hemostasis during surgery, which was followed by wound closure and re-disinfection of the skin. The entire surgical procedure was completed within 10 minutes.

After surgery, rats were subjected to rewarming inside a water bath at a temperature of 37 ℃. They were allowed to rest in a cage for 2–4 hours before being placed into a sealed hypoxia chamber maintained at a 37 ℃ temperature, which was filled with a gas mixture containing 92% nitrogen and 8% oxygen (flow rate 0.5 L/min; humidity 70%±5%). Rats had reached 100 days of age when the experiment was started. During the 1-hour hypoxic period, observation was made at least every 30 minutes to monitor respiratory distress. If respiratory distress was observed, the rat was immediately transferred to a warming box. After modeling surgery, the rats were kept warm and fed milk.

L. rhamnosus OF44 and S. boulardii preparation

The rats in the model group were split into three groups at random: group A, group B, and group C. To generate data acquired from rats to humans, dosages of gavage are adjusted for rats according to the different surface areas of the two species. Rats in the group A were administered *L. rhamnosus* [10^9 colony-forming units (CFU)/day; total 0.2 mL], rats in group B were administered *S. boulardii* (10^7 CFU/day; total 0.2 mL), and rats in group C were given normal saline as placebo. In addition, there was a control group of 6 healthy rats that were euthanized on day 0 of the experiment after blood and stool samples were obtained. *L. rhamnosus* OF44 was prepared by adding the frozen *L. rhamnosus* OF44 bacterial solution to MRS liquid medium. The frozen *L. rhamnosus* OF44 solution was cultured at 37 ℃ for 24 hours and then centrifuged at 8,000 g for roughly 3 min, with the subsequent supernatant being discarded. The remaining cell pellet was rinsed with sterile phosphate-buffered saline (PBS) three times. Finally, the remaining *L. rhamnosus* OF44 was diluted with sterile PBS to obtain a bacterial suspension of $1×10^9$ CFU/mL. After adaptive feeding, the rats were orally gavaged with bacterial solution, at a dose of $1×10^9$ CFU per rat per day, for a total of 4 weeks (28 days). *S. boulardii* was prepared by adding commercial *S. boulardii* sachets into a solution at a concentration of 0.05 g/mL ($1×10^7$ CFU/mL). After adaptive feeding, the rats were orally gavaged with bacterial solution at a dose of $1×10^7$ CFU per rat per day for a total of 4 weeks (28 days).

Behavioral evaluation of the models

Two behavioral tests were implemented in this study for behavioral evaluation of the rat models: the Y-maze experiment and the tail suspension test. The Y-maze test involves a three-arm maze apparatus in the shape of a "Y", in which each arm is identical in size and shape. In our study, a nontransparent organic plastic sheet was used to create the shape of the maze, which consisted of three arms (35 cm in length, 10 cm in width, and 20 cm in height) at angles of 120º between each arm. The rat was placed at the end of 1 arm and allowed to freely roam the maze. The sequence by which the rats entered each arm was recorded.

The tail suspension test is a common and rapid means of evaluating the depressive state of model animals. In this study, it was used to determine whether there was a difference in the depressive states between CP rats and healthy rats. In the tail suspension test, the rat is hung by its tail, which prevents it from escaping and causes it to enter a unique state of immobility that indicates a depressive state. The experiment records the time of immobility as indicator of the depressive state. The rat is suspended 20–25 cm away from the working table, with the posterior one-third of its tail fixed on the bracket with adhesive tape. In our study, the rat was considered immobile only when it was completely motionless for 6, and the immobility time was recorded by an experimenter who was unaware of the study's purpose in 6 minutes.

Measurement of serum inflammatory cytokines

Plastic tubes were used to collect blood samples, from which the serum was obtained through centrifugation at 3,000 g for 10 minutes at a temperature of 4 ℃. The obtained serum was conserved at –80 ℃ for subsequent analysis. To minimize the impact of the circadian rhythm of the HPA axis on the results, all samples within each group were gathered in the same time window (from 10:00 AM to 12:00 AM). Concentrations of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) inside the serum samples were measured via commercial ELISA kits (product codes: MM-0047R1, MM-0190R1, MM-0175R2, MM-0180R2) by Jiangsu Meimian Industrial Co., Ltd. according to the producer's instructions. Plates were read by 1 ELISA reader at a 450-nm wavelength. The sensitivity of the kit for IL-1β was 0.1 pg/mL, while that for the IL-6, IL-8, and TNF-α kits was 1.0 pg/mL.

16S rRNA sequencing of the rats

Sample collection
On days 0, 7, 14, and 21, 2 fresh fecal specimens were

gathered from each rat using a 1.5-mL Eppendorf (EP) tube and frozen at –80 ℃ instantly until examination.

DNA extraction and polymerase chain reaction (PCR) amplification

Microbial community genomic DNA was extracted from the fecal specimens using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) with TaKaRa Premix Taq Version 2.0 (Takara Bio, Shiga, Japan) according to the manufacturers' instructions. The extracted DNA was examined on 1% agarose gel. Additionally, the DNA concentration and purity were examined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 16S rRNA sequencing was performed to identify bacterial diversity using the 515F and 806R primers targeting the V4 region. In addition, 528F and 706R primers targeting the V4 region of the 18S rRNA gene were adopted to characterize the diversity of the eukaryotic microbes, while ITS5-1737F and ITS2-2043R primers targeting the ITS1 region were used to characterize the fungal diversity. Furthermore, regions 16S V3–V4/16S V4–V5, archaeal 16S V4–V5, 18S V5, and ITS2 were amplified. Functional gene-specific primers were also implemented in our analysis.

PCR was completed using a BioRad S1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification for the 16S rRNA gene was performed as follows: initial denaturation at 94 ℃ for 5 minutes, 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 52 ℃ for 30 s, extension at 72 ℃ for 30 s, and a single extension at 72 ℃ for 10 minutes finishing at 4 ℃. The PCR mixtures comprised 25 µL of 2× Premix Taq, excessive deoxynucleotide triphosphates (dNTPs), 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 0.4 µL of TransStart FastPfu DNA Polymerase, 50 ng of template DNA, and up to 50 µL of double-distilled water (ddH$_2$O). PCRs were implemented in triplicate. The product of PCR was derived from a 1% agarose gel and purified via the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek) and quantified via Quantus Fluorometer (Promega, Madison, WI, USA).

Illumina Nova 6000 sequencing

Purified amplicons were gathered in equimolar concentrations and paired-end sequenced (2×300) on an Illumina Nova 6000 platform (Illumina, San Diego, CA, USA) according to standard protocols of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Processing of sequencing data

For the analysis of 16S rRNA gene sequencing figures, fastp software (version 0.23.2; https://github.com/OpenGene/fastp) was used to acquire clean reads and to remove primers based on the barcode information. Then, the *fastq_mergepairs* command in USearch (v.10; http://www.drive5.com/usearch/) was adopted with the objective of merging the paired-end clean reads with a minimal overlap, a length of 16 bp, and a maximal mismatch of 5 bp in the overlap region. Raw tags were filtered using *fastp* software to generate clean tags. Operational taxonomic units (OTUs) were generated at a 97% similarity cutoff via UPARSE (version 7.1; http://drive5.com/uparse/), and chimeric sequences were deleted. The Majorbio Cloud Platform (www.majorbio.com) was employed for data analysis. Barcodes and primers were used to distinguish samples, and sequence directions were regulated through exact barcode matching and 2-nucleotide mismatch in the primer matching.

Statistical analysis

Analysis of behavioral experiments and serum inflammatory cytokine levels

The original data of the Y-maze test is converted to alteration rates before statistical analysis. Then, the data of both the tail suspension test and the Y-maze test are evaluated using *t*-tests in Excel. The serum inflammatory cytokine optical density values are first regressed on linear functions for concentration using the equations provided in the instructions of the experimental kits. Then, *t*-tests are used to compare the concentrations of the cytokines among different treatment groups, and across the timeline.

Analysis of sequencing data

Alpha diversity and Beta diversity are used to analyze the sequencing data. They are two indicators of the diversity of an ecosystem, in this case the gut microbial of rats. Three indicators are used to assess alpha diversity, which are Chao index, Shannon diversity, and Simpson diversity. Chao index assesses diversity based on whether a species exist in the sample. Shannon diversity is based on the uniqueness of a species, and Simpson diversity is based on the total number of individuals of a species. In contrast to alpha diversity, beta diversity assesses the dissimilarities among samples. Heatmap is used to present beta diversity, where
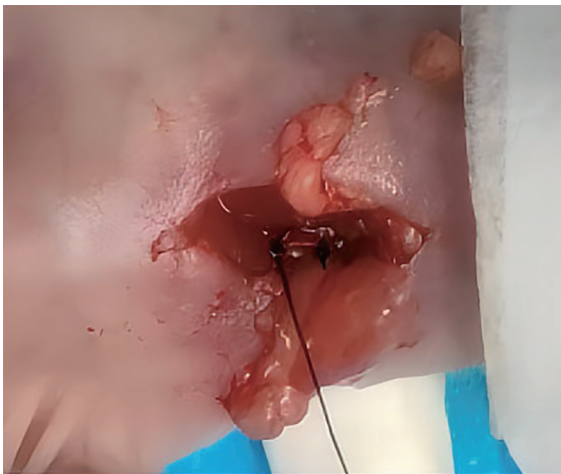
Figure 1 Right common carotid artery ligation.

different colors represent the similarities and differences among samples.

Results

Establishment of the CP rat model

To establish the CP rat model, we employed right common carotid artery ligation (22). This ligation induced ischemia in the rat, which caused neuronal death and glial cell reaction, mimicking the process of CP in humans. To support the validity of the model, we captured and recorded the surgical procedure and provided clear photos of the ligated right neck region of the rats (*Figure 1*).

These models exhibited significant motor and cognitive impairments consistent with symptoms of human CP. These data provide strong evidence for our study, indicating that we successfully established a rat model of CP.

### *Behavioral experiment analysis*

Tail suspension experiments have been used to indicate when the emotional state of mice with CP has become depressive (19). We aimed to investigate whether the emotional state of rats is also affected by CP, so we conducted tail suspension experiments on rats before the probiotic intervention was initiated. The tail suspension test is an extensively used method for evaluating anxiety and depression in rodents (23). The tail suspension test was conducted on day 0 of the intervention to assess the emotional states of rats. In this test, the rats were suspended

by their tails for a certain period of time, and their immobility time was measured as an indicator of depression. We hypothesized that the CP rats would show more immobility time than would the healthy rats, reflecting their higher level of depression, as in CP mice (19). However, the tail suspension test did not result in a statistically significant between the depressive states of healthy and CP rats. Further studies are needed to explore possible explanations for these results.

After the probiotic intervention concluded, we verified whether the intervention had an impact on the spatial memory and learning of rats, which are among the main functions affected by CP, as reported in children (24,25). We thus conducted the Y-maze test on the last day of the intervention to assess the spatial memory and learning in rats. Both traits are highly correlated with CP. The test provides information on a mouse's ability to recognize and remember new environments, which is useful in evaluating neurological impairments associated with CP (26). The Y-maze test was chosen for two reasons: (I) to inspect the CP-related movement conditions in intervened rats; and (II) to avoid repeating the tail suspension test since rats have the memory of previously conducted tests, which could affect the results. The test results suggested that after the intervention, the correct conversion rates of all three groups of CP rats were lower than those of healthy rats. The correct conversion rates of the group A rats were the lowest. In addition, the correct conversion rates of group C rats were higher than those of both the group A rats and group B rats. However, no statistical significance was observed (*Figure 2*).

L. rhamnosus OF44 and S. boulardii exerted anti-inflammatory effects in CP rats

A previous study has suggested that neurological diseases such as CP lead to increased inflammation levels (27). Inflammation has been shown to cause increase in oxidative stress and disruption in the blood-brain barrier, both of which are common impairments in CP (28). Given that high inflammation levels can manifest as an increase in cytokines, such as IL (29), we tested whether interventions with *L. rhamnosus* OF44 and *S. boulardii* could affect the production of cytokines and thus reflect the inflammation level in rats with CP. We isolated the serum from the blood samples and evaluated the amount of cytokines, specifically that of IL-1β, IL-6, IL-8, and TNF-α, to assess the effects of *L. rhamnosus* OF44 and *S. boulardii* on the induction of inflammation. We found there to be a significant increase
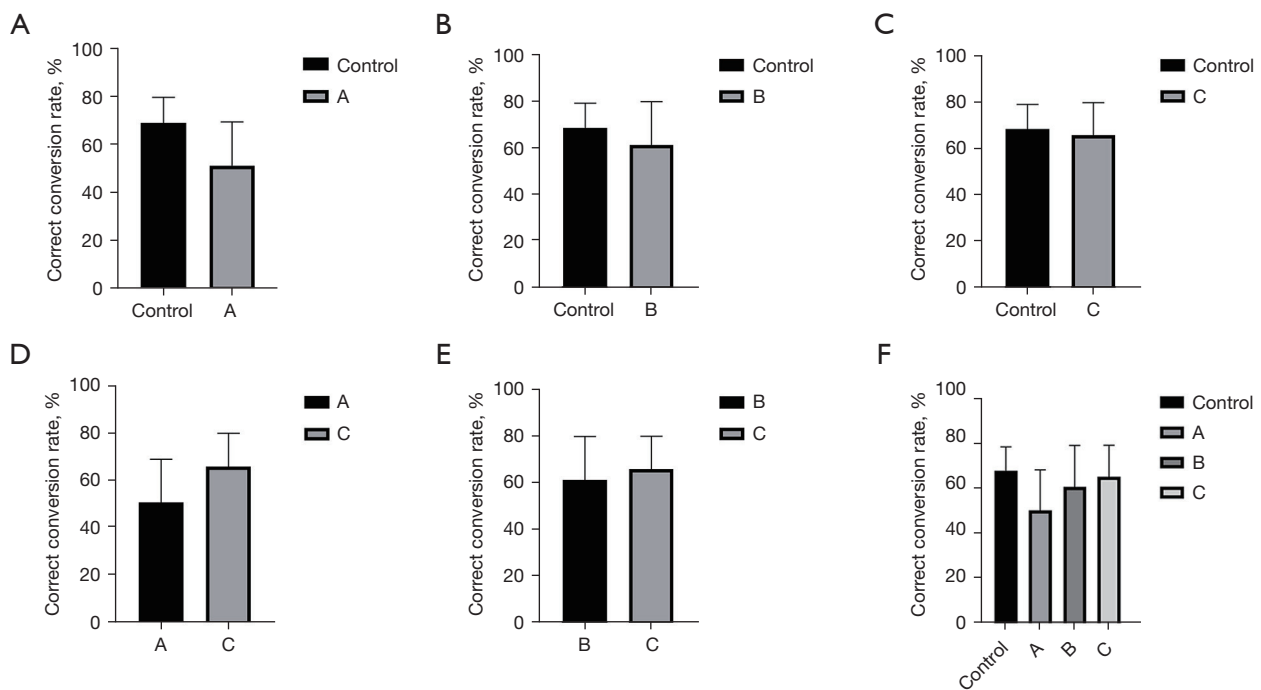
Figure 2 Y-maze test conversion rates of the different groups. (A) Comparison of the correct conversion rate between the control group and group A; (B) comparison of the correct conversion rate between the control group and group B; (C) comparison of the correct conversion rate between the control group and group C; (D) comparison of the correct conversion rate between group A and group C; (E) comparison of the correct conversion rate between group B and group C; (F) comparison of the correct conversion rate among the four groups. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; control, healthy control group. CP, cerebral palsy.

in the IL-1β level in group C rats on day 28 compared with day 14 (P<0.05). This indicated that the IL-1β level was increased in rats experiencing CP without intervention over time. Furthermore, the levels of IL-1β were not significantly different among different groups or across different time spans of groups A and B (*Figure 3*).

Compared with those in group C, IL-6 concentrations were reduced significantly in group A (P<0.01) on day 21 of the intervention. In addition, on day 28 of the intervention, there was a significant rise in the IL-6 level in rats from group A in comparison with those of days 0, 7, and 14. We observed similar results in the group B rats: on day 28 of the intervention, there was also a significant rise in the IL-6 level in rats from group B in comparison with the levels on days 0, 7, and 14. No other significant changes were found. This suggested that interventions with *L. rhamnosus* OF44 had some effect in reducing the level of IL-6. In comparison, *S. boulardii* showed less impact on reducing the amount of IL-6 in CP rats. Furthermore, the IL-6 level in group C rats showed a significant increase during the course

of the intervention, which points to an increase in the IL-6 level over time in rats with CP. The results also thus imply that the interventions with both *L. rhamnosus* OF44 and *S. boulardii* may potentially slow the increase of IL-6 levels (*Figure 4*).

There was a significant difference in the IL-8 level between the control group and CP groups before the intervention (P<0.05). This indicated that CP rats had higher IL-8 levels than did healthy rats, supporting the idea of an increased inflammation level in rats with CP. In addition, a significant difference was observed in the IL-8 levels between group A and group B rats on day 21 of intervention (P<0.05). The level of IL-8 in group B rats was obviously lower than that in group A rats, suggesting that *S. boulardii* intervention was more effective in lowering IL-8 levels than was *L. rhamnosus* OF44 intervention. Moreover, for group A rats, on day 21 and day 28 of the intervention, the IL-8 levels were significantly different from those of days 0, 7, and 14 (P<0.05), decreasing as time elapsed. This suggested that intervention with *L. rhamnosus* OF44
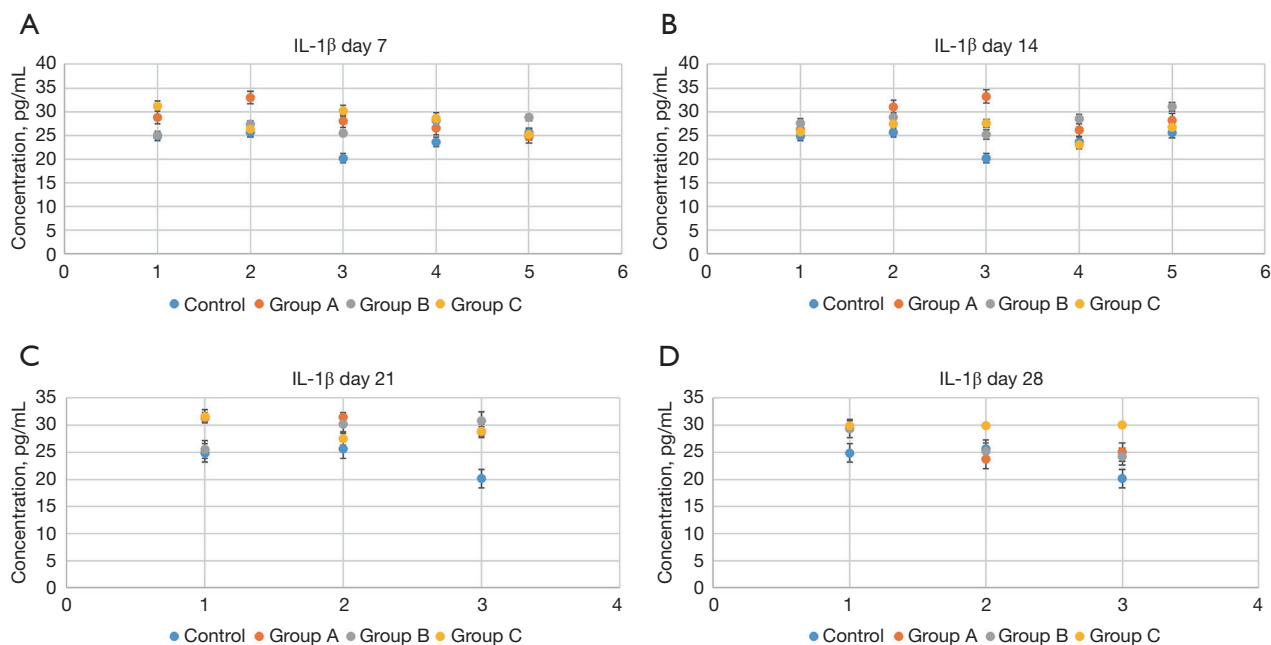
Figure 3 The level of IL-1β in the CP rats. (A) The level of IL-1β in different groups on day 7; (B) the level of IL-1β in different groups on day 14; (C) the level of IL-1β in different groups on day 21; (D) the level of IL-1β in different groups on day 28. The level of IL-1β in the control group is from day 0 in all four graphs since the healthy control rats were all euthanized on day 0 of the intervention and only one set of samples is taken. The numbers in the x-axis represent different rats. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; control, healthy control group. CP, cerebral palsy; IL-1β, interleukin-1β.

was also effective in lowering IL-8 levels, and its effects were more stable and long lasting. Additionally, the IL-8 level in group B rats was significantly different between day 0 and day 7; between day 21 and days 0, 7, and 14; between day 0 and day 28; and between day 28 and day 21. These results suggested that overall, the intervention of *S. boulardii* reduced the IL-8 levels in rats, but the intervention seemed to be less stable than that of the *L. rhamnosus* OF44 intervention (*Figure 5*).

On day 14 of the intervention, group B rats exhibited an evident decline in TNF-α concentration compared to group C rats (P<0.05). This suggests that the *S. boulardii* intervention had the ability to decrease the TNF-α level in CP rats. In addition, on day 21 of the intervention, the TNF-α level in the group A rats was obviously less than that of the group B rats (P<0.05). This implies that on day 21, *L. rhamnosus* OF44 exhibited a stronger ability to alleviate inflammation than did *S. boulardii*. In group A, the TNF-α levels on day 21 and day 28 were markedly reduced compared with those on day 0 (P<0.01). This indicates that *L. rhamnosus* OF44 intervention had some effect in reducing

the amount of TNF-α. Furthermore, in group B, the TNF-α levels on day 14 and day 28 were obviously reduced compared to those on day 0 (P<0.01). This suggested that *S. boulardii* also has the ability to reduce TNF-α levels. Finally, compared with days 0, 7, and 14 of the intervention, the TNF-α level in group C rats reduced significantly on day 28 (P<0.05). This was an unexpected result, which might have occurred due to the limited number of rat models that we used in this study (*Figure 6*).

16S rRNA sequencing analysis

Alpha diversity

Chao index: there was no obvious distinction in the Chao indicator among the three groups. However, intervention was associated with a significant increase in the Chao index of gut microbiota of all three groups of CP rats (*Figure 7A*).

The Shannon and Simpson diversity indices exhibited similar trends. At baseline, the healthy rats had a lower Shannon diversity index score than did the CP rats. After 1 week, the CP rats had a significant increase in Shannon
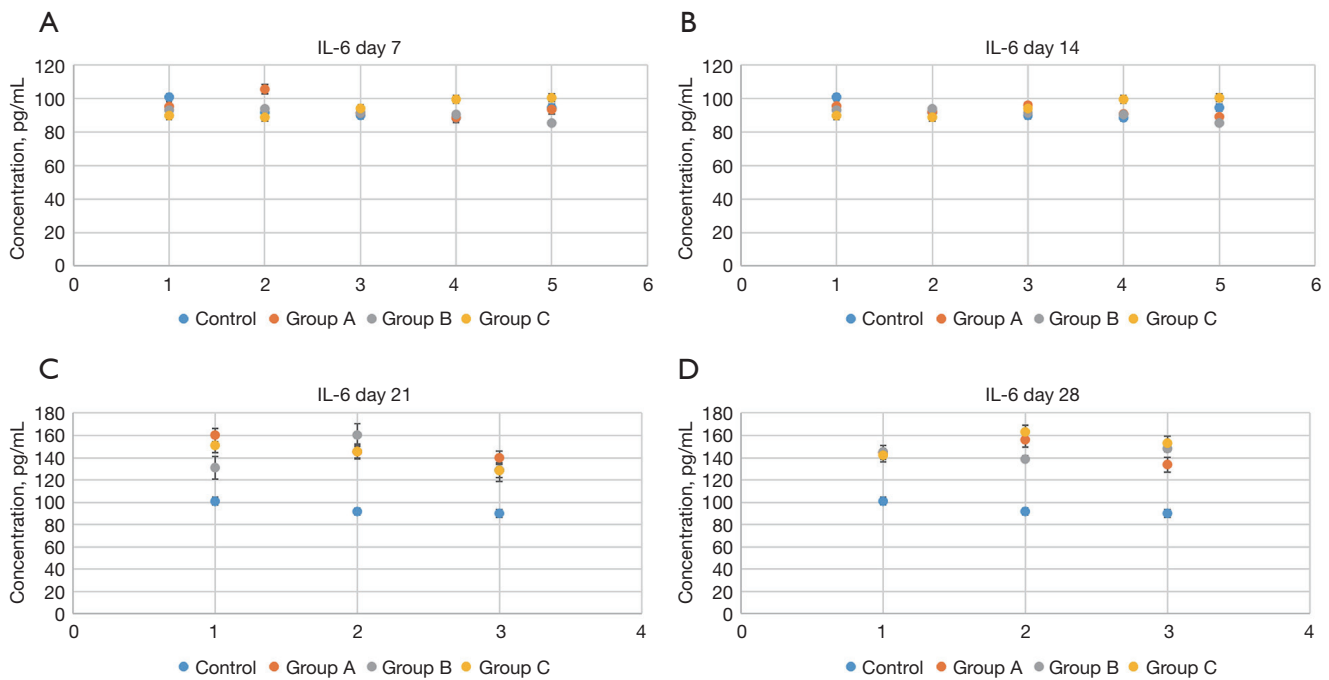
Figure 4 The level of IL-6 in the CP rats. (A) The level of IL-6 in different groups on day 7; (B) the level of IL-6 in different groups on day 14; (C) the level of IL-6 in different groups on day 21; (D) the level of IL-6 in different groups on day 28. The level of IL-6 in the control group is from day 0 in all four graphs since the healthy control rats were all euthanized on day 0 of the intervention and only one set of samples was taken. The numbers in the x-axis represent different rats. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; control, healthy control group. CP, cerebral palsy; IL-6, interleukin-6.

diversity index score. However, no obvious distinction was observed in the three CP groups. After 2 weeks, the group C rats exhibited a rising trend in Shannon diversity index score, while the scores of the group A and B rats remained relatively stable. After 3 weeks, the group C rats experienced a significant drop in Shannon diversity index score, but no obvious difference was detected across the three groups. After 4 weeks, the group C rats further declined in Shannon diversity index score. Additionally, no obvious difference was found between the three groups (*Figure 7B*).

Simpson diversity: the healthy rats had a lower Simpson diversity index score than did the CP rats at baseline. No obvious differences in Simpson diversity index score were noted across the three groups of CP rats at any time point (*Figure 7C*).

In contrast to our previous study, the alpha diversity results did not show that healthy rats had a more favorable gut microbiota or that CP rats received improved gut microbiota diversity following intervention (8).

Beta diversity

UniFrac: the dissection on the weighted UniFrac heatmap revealed the differences in gut microbiota composition between healthy rats and rats in group C at 2 and 3 weeks' intervention. Additionally, after 1 week's intervention, there was a noticeable difference in gut microbiota composition between groups A and B compared to the control group. Moreover, the gut microbiota composition of the group C rats at 1 week of intervention differed from that of the group B rats at 2 weeks of intervention. After 2 weeks of intervention, the difference in gut microbiota composition between groups A or B and the control group decreased but was still significant, and there was also a considerable difference between group A or B rats and group C rats in this regard. These findings indicate the following: (I) the gut microbiota composition between healthy rats and untreated CP rats is different, as observed in the UniFrac heatmap analysis; (II) the intervention with *L. rhamnosus* OF44 and *S. boulardii* showed a tendency to alter the gut microbiota
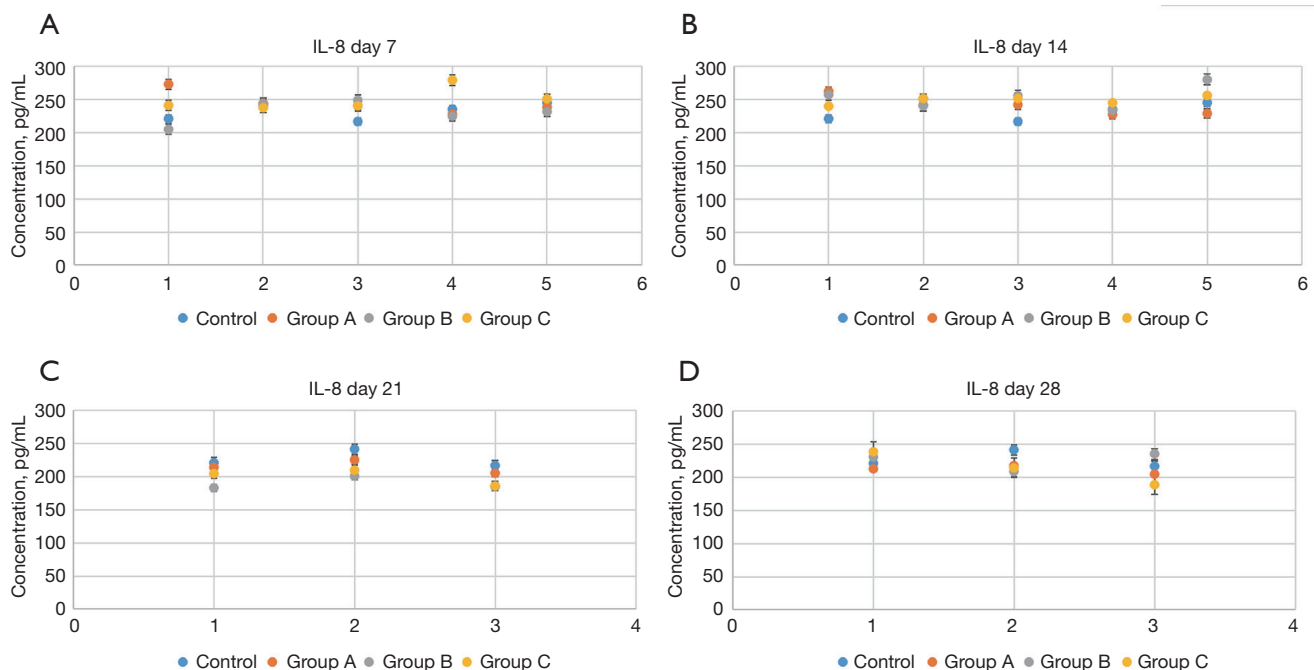
Figure 5 The level of IL-8 in the CP rats. (A) The level of IL-8 in different groups on day 7; (B) the level of IL-8 in different groups on day 14; (C) the level of IL-8 in different groups on day 21; (D) the level of IL-8 in different groups on day 28. The level of IL-8 in the control group is from day 0 in all four graphs since the healthy control rats were all euthanized on day 0 of the intervention and only one set of samples was taken. The numbers in the x-axis represent different rats. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; control, healthy control group. CP, cerebral palsy; IL-8, interleukin-8.

composition in rats, but more pronounced results may require a longer duration of intervention (*Figure 8*).

Principal co-ordinates analysis (PCoA) indicated that there existed no significant difference among the three CP groups while there is significant difference between the three CP groups and the control group. Meanwhile, there were both similarities and differences between the microbiota composition of the healthy rats and that of the CP rats. In terms of intervention time, there was no noticeable difference in the PCoA images of the four groups within 4 weeks' intervention (*Figure 9*).

Nonmetric multidimensional scaling (NMDS) analysis revealed a significant difference in composition of gut microbiota between healthy rats and rats with CP. However, no apparent difference was observed among the other groups. After the rat CP model was created, the gut microbiota of these rats was found to be completely different from that the control group. However, the groups treated with *S. boulardii* and physiological saline were not significantly different in this regard (*Figure 10A*). In addition, the gut microbiota of

rats treated with *L. rhamnosus* OF44 gradually changed from the early stage to the later stage (*Figure 10B*).

The statistical analysis of significant differences in gut microbiota

Linear discriminant analysis effect size (LEfSe) of species population characterized the genus abundance of each specimen at the level of genus. At that genus level, the feature taxa in the group A rats [linear discriminant analysis (LDA) >2] were *Lachnospiraceae* NK4A136 group, *Desulfovibrio*, and *Lachnoclostridium*. The feature taxa in the group B rats (LDA >2) were *Roseburia*, *Lachnospiraceae* UCG-006, and *Anaerovoracaceae*. The feature taxa in the group C rats (LDA >2) were *Helicobacteraceae*, *Campylobacteria*, *Helicobacter*, *Campilobacterota*, and GCA 900066575. Feature taxa in the control group rats (LDA >2) were *Erysipelotrichales*, *Dubosiella*, *Coriobacteria*, *Actinobacteriota*, *Enterorhabdus*, and *Coriobacteriaceae* UCG-002 (*Figure 11*).

The rank-sum test was used to test hypotheses concerning

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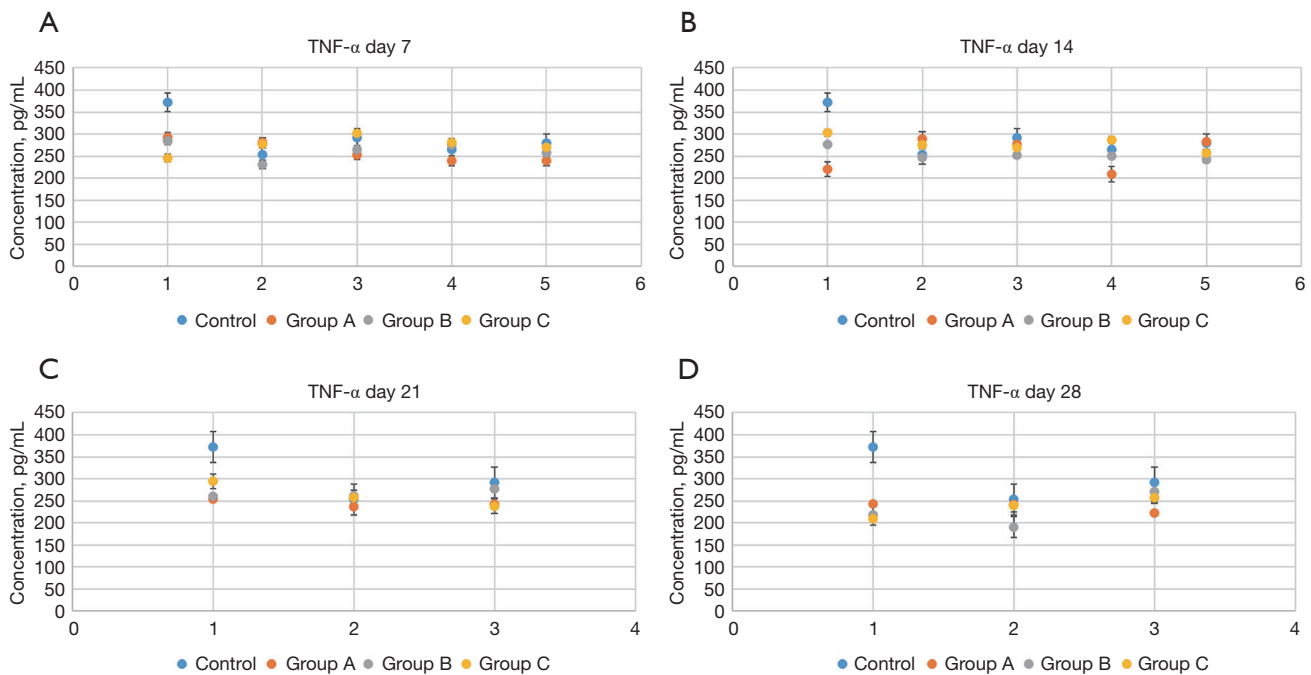
Figure 6 The level of TNF-α in the CP rats. (A) The level of TNF-α in different groups on day 7; (B) the level of TNF-α in different groups on day 14; (C) the level of TNF-α in different groups on day 21; (D) the level of TNF-α in different groups on day 28. The level of TNF-α in the control group is from day 0 in all four graphs since the healthy control rats were all euthanized on day 0 of the intervention and only one set of samples was taken. The numbers in the x-axis represent different rats. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; control, healthy control group. CP, cerebral palsy; TNF-α, tumor necrosis factor alpha.

the differences in microbial composition. *Table 1* shows the genera that differed significantly across the samples from the different groups (P<0.05, Kruskal-Wallis test).

At the level of phylum, the groups showed differences in *Lachnospiraceae* NK4A136 group, *Desulfovibrio*, *Roseburia*, *Treponema*, *Clostridia* UCG-014, (*Eubacterium*) xylanophilum group, *Alistipes*, *Prevotellaceae* NK3B31 group, *Bacteroides*, *Alloprevotella*, *Dubosiella*, *Helicobacter*, *Prevotella*, *Lachnospiraceae* UCG-006, *Escherichia-Shigella*, (*Eubacterium*) *siraeum* group, *Monoglobus*, (*Eubacterium*) *ruminantium* group, *Candidatus* Saccharimonas, *Turicibacter* UCG-009, *Romboutsia*, *Colidextribacter*, *Elusimicrobium*, *Lachnoclostridium*, *Enterorhabdus* UCG-007, *Rikenellaceae* RC9 gut group, *Coriobacteriaceae* UCG-002, GCA-900066575, (*Eubacterium*) nodatum group, *Lachnospiraceae* ND3007 group, *Paludicola*, *Intestinimonas*, *Papillibacter*, *Lactococcus*, *Muribaculum*, *Fournierella*, Family XIII UCG-001, *Peptococcus*, *Mycoplasma*, *Butyricimonas*, *Lachnospiraceae* NK4B4 group, *Tyzzerella*, *Marvinbryantia*, *Rothia*, *Harryflintia*, *Acetitomaculum*, *Anaerofustis*, (*Eubacterium*) brachy group, *Collinsella*,

Parvibacter, *Sporobacter*, *Clostridium sensu stricto* 1 UCG-008, *Subdoligranulum*, *Proteus*, *Saccharimonadales*, *Paraprevotella*, *Mucispirillum*, *Oligella*, *Faecalitalea*, *Fusicatenibacter* UCG-004, *Aureimonas*, *Eubacterium hallii* group, *Shinella*, *Devosia*, and *Comamonas* (*Table 1*).

Functional predictions

The PICRUSt (phylogenetic detection of communities through reconstructing unobserved states) technique was used to predict the functional composition of the microbiome according to the 16S rRNA sequencing results. This involved mapping the species composition information from 16S sequencing to a prebuilt table of species functional gene composition, allowing for the prediction of functional outcomes. LEfSe and the Mann-Whitney test were used to estimate the effect sizes of each component metabolic pathway (MetaCyc_pathway), Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway, and KEGG orthology (KO) function on the observed gut microbial
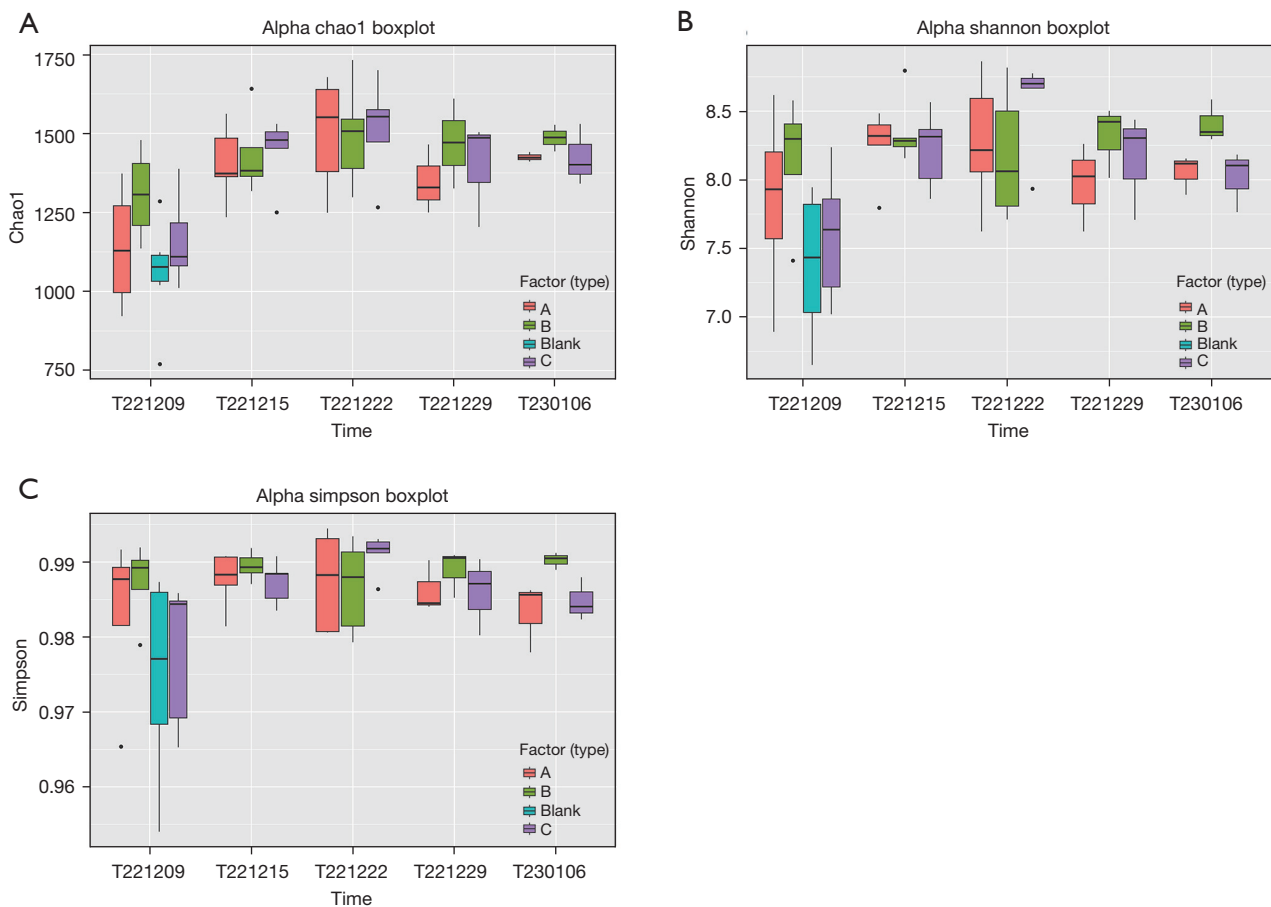
Figure 7 Changes in the gut microbiota in four groups through time. The horizontal coordinate represents time points. Each time point contains the groups of different intervention methods. The vertical coordinate refers to value of the chao1 diversity indicator of the different groups. The box plot shows five statistics (minimal, first quartile, median, third quartile and maximal; i.e., five lines from bottom to top), with the outliers labeled with "o". (A) Chao1 index. (B) Shannon index. (C) Simpson index. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group. T221209 means Dec. 9th, 2022, or day 0. T221215 means Dec. 15th, 2022, or day 7. T221222 means Dec. 22nd, 2022, or day 14. T221229 means Dec. 29th, 2022, or day 21. T230106 means Jan. 6th, 2023, or Day 28.

differences. This approach aimed to identify metabolic pathways that significantly contributed to the differentiation between sample groups (with a default filtering condition of LDA >2). In the LefSe analysis conducted, no differential metabolic pathways were found in the predicted enzyme commission number (EC) and KO functional databases. However, in the KEGG metabolic pathway analysis, three differential pathways were identified for the different time points of intervention in the group A rats. These pathways were P441-PWY, GLYCOLYSIS-E-D, and PWY-6588. The detailed results are as follows:

During the 1-week intervention in group A rats with CP,

the P441-PWY pathway showed a significant downregulation compared to other time points. However, after 4 weeks of intervention, both the Entner-Doudoroff (ED) glycolysis pathway (GLYCOLYSIS-E-D) pathway and PWY-6588 pathway were significantly upregulated. This suggests that the intervention effects of *L. rhamnosus* OF44 are dependent on intervention time. The P441-PWY pathway is involved in the metabolism of neuroamino acids (30), and thus might have been inhibited or damaged. This indicated that during the 4-week intervention, the condition of CP in rats might have still been worsening. The GLYCOLYSIS-E-D pathway is involved in intracellular glycolysis and the alanine pathway,
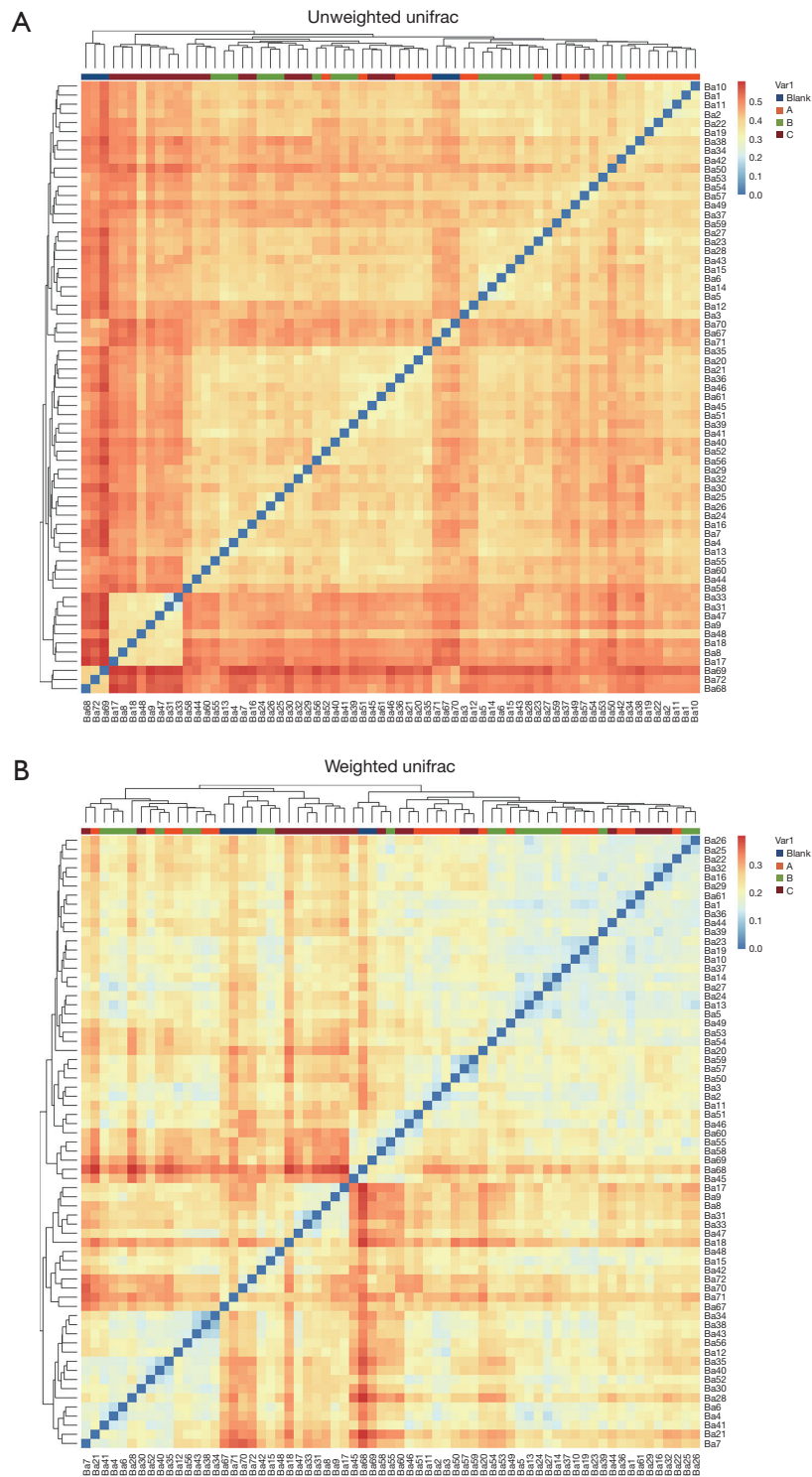
Figure 8 Changes in gut microbiota of the rats are shown by the beta diversity heatmap. Based on the statistical results of the differences of each specimen, cluster dissection on samples was implemented. Additionally, the difference between samples was calculated to estimate the similarity of species composition in the different specimens. If the specimens are closer, their species compositions are more similar. (A) Unweighted heatmap. (B) Weighted heatmap. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group.
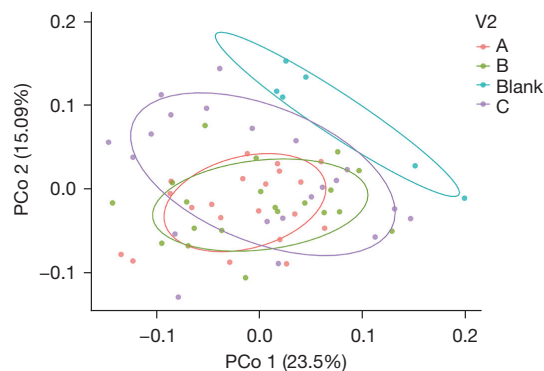
Figure 9 Gut microbial compositions of rats in the different groups. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group. PCo 1, principle coordinate 1; PCo 2, principle coordinate 2.

and glycolysis is a metabolic pathway that breaks down glucose into pyruvate and lactate while producing a certain amount of adenosine triphosphate (ATP). The alanine pathway facilitates the further metabolism of pyruvate into ethanol and acetic acid (31), while the PWY-6588 facilitates the fermentation of pyruvate into acetone (32) and serves as an alternative pathway to glycolysis (*Figure 12*).

Furthermore, group A and group C showed differences in certain metabolic pathways, including P441-PWY, GLYCOLYSIS-E-D, and PWY-5505. The detailed results are as follows:

In addition to the upregulation of the P441-PWY pathway and GLYCOLYSIS-E-D pathway in group C rats, the PWY-5505 pathway was downregulated in group A rats. The PWY-5505 pathway, also known as the methylmalate pathway, is involved in the synthesis and breakdown of methylmalate in organisms (33). Methylmalate is an important intermediate metabolite that participates in various biochemical reactions, such as the tricarboxylic acid (TCA) cycle, glucose metabolism, and fatty acid metabolism (34) (*Figure 13*).

In addition, at week 4 of intervention, group C rats exhibited two differential pathways, PWY0_162 and PWY_7196 (*Figure 14*).

Discussion

Despite another study suggesting that CP affects the emotional states and tail suspension test results of rats (19), our study found there to revealed no obvious difference between the depressive states of rats with and without CP.
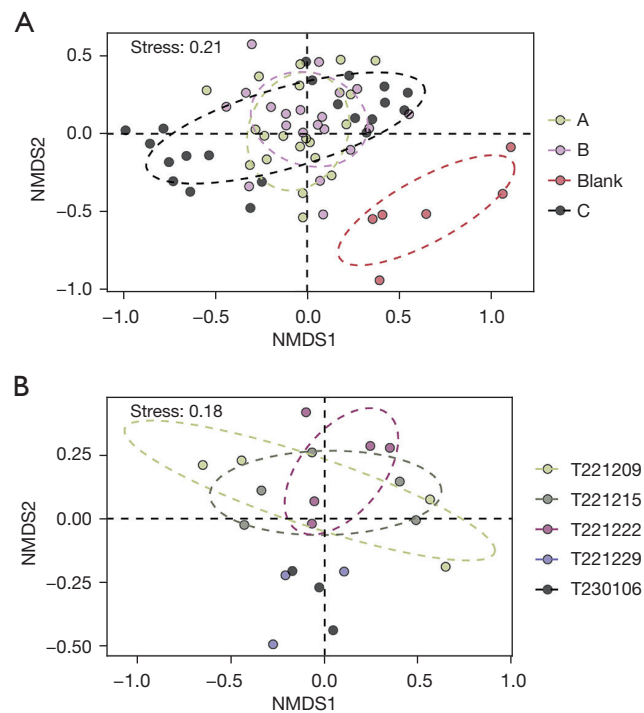
Figure 10 NMDS analysis results at different time points for the different groups and feeding methods. (A) Groups of rats with CP: group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group. (B) NMDS analysis of the *Lactobacillus rhamnosus* treatment group at different time points. T221209 means Dec. 9th, 2022, or day 0. T221215 means Dec. 15th, 2022, or day 7. T221222 means Dec. 22nd, 2022, or day 14. T221229 means Dec. 29th, 2022, or day 21. T230106 means Jan. 6th, 2023, or day 28. NMDS, non-metric multidimensional scaling; CP, cerebral palsy.

This may be due to the limited number of rats used in this study. At the end of the intervention, we employed another type of behavioral experiment—the Y-maze test—to evaluate the spatial motion ability of rats. Our hypothesis was that the correct conversion rate of CP rats would be lower than that of healthy rats since due to the reduced spatial memory and learning capacity in CP rats (24,25). Additionally, we expected that intervention with probiotics would increase the correct conversion rate to a level between that of CP rats without intervention and that of healthy rats. The results suggested that the correct conversion rate of CP rats was indeed lower than that of healthy rats. However, contrary to our expectations, intervention with probiotics seemed to lower the correct conversion rate to a level lower

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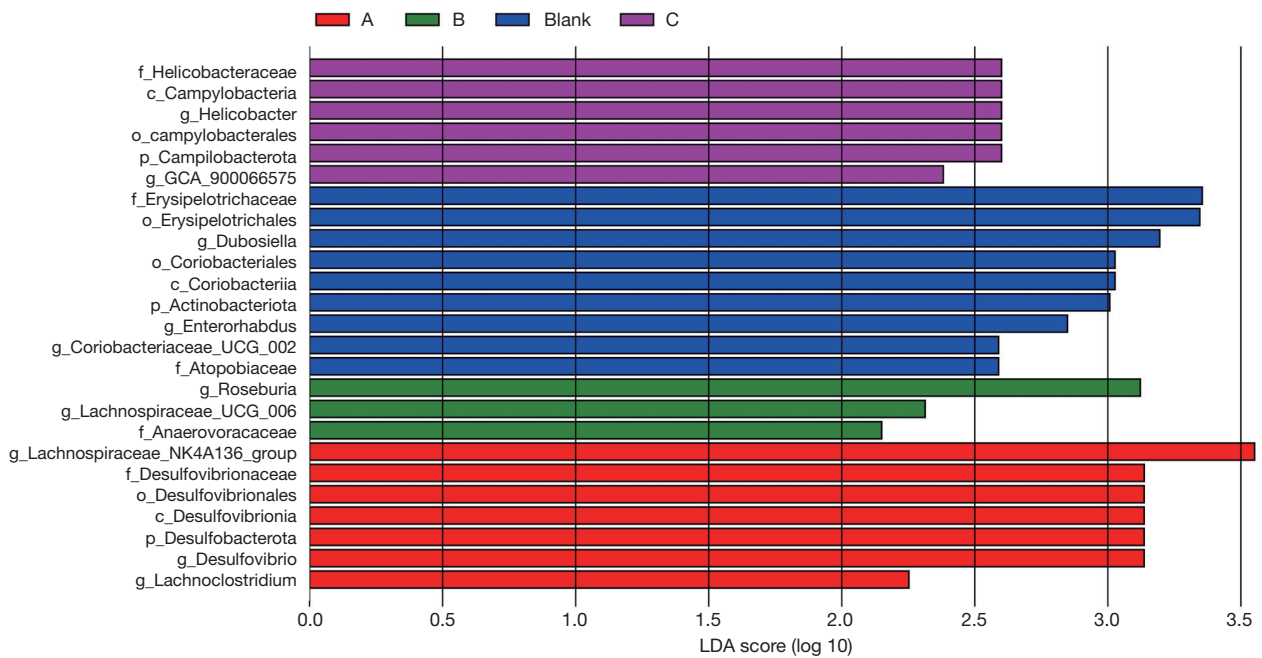
Figure 11 The feature taxa in the different groups. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group. GCA, genome catalogue of Archaea; LDA, linear discriminant analysis; UCG, unclassified bacteria.

Table 1 A portion of feature taxa at the genus level in different groups

Taxon name	Mean				P value	FDR
	A	B	Blank	C		
g_Lachnospiraceae_NK4A136_group	0.058498	0.029340	0.010203	0.028502	0.001871	0.019747
g_Desulfovibrio	0.020066	0.014579	0	0.012477	0.006124	0.039159
g_Roseburia	0.022248	0.023682	0.004398	0.012702	0.000812	0.012860
g_Treponema	0.012635	0.027199	0	0.007472	0.001399	0.017375
g_Clostridia_UCG-014	0.093741	0.108704	0.163258	0.114673	0.034540	0.112123
g_(Eubacterium)_xylanophilum_group	0.017205	0.013593	0.006758	0.009861	0.002359	0.023704
g_Alistipes	0.011792	0.010228	0.016478	0.005330	0.006724	0.039409
g_Prevotellaceae_NK3B31_group	0.003611	0.004154	0	0.005193	0.006545	0.039409
g_Bacteroides	0.007134	0.011833	0.029125	0.022835	0.030505	0.102168
g_Alloprevotella	0	2.59e−07	0	0.010590	0.000867	0.012861

Group A, *Lactobacillus rhamnosus* OF44 treatment group; group B, *Saccharomyces boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group. FDR, false discovery rate.

than that of CP rats with no intervention. This may suggest that intervention with *L. rhamnosus* OF44 and *S. boulardii* lower the rats' spatial motion ability. However, no exact conclusion can be drawn since no significant difference was observed.

Through data analysis of ELISA experiments, we found that intervention with *L. rhamnosus* OF44 and *S. boulardii* had a significant impact upon the levels of cytokines from rat
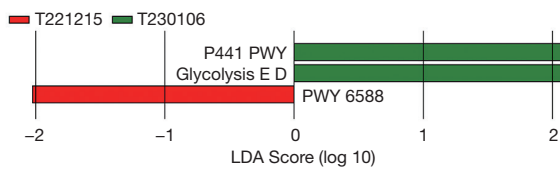
Figure 12 Differential metabolic pathways in the group A rats at different intervention times. T221215 stands for Dec. 15th, 2022, or day 7 of the experiment. T230106 stands for Jan. 6th, 2023, or day 28 of the experiment. Glycolysis ED pathway refers to Entner-Doudoroff pathway. LDA, linear discriminant analysis.
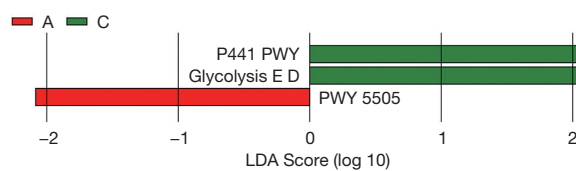
Figure 13 Differential metabolic pathways between group A and group C. Group A, *Lactobacillus rhamnosus* OF44 treatment group; group C, physiological saline treatment group. Glycolysis ED pathway refers to Entner-Doudoroff pathway. LDA, linear discriminant analysis.
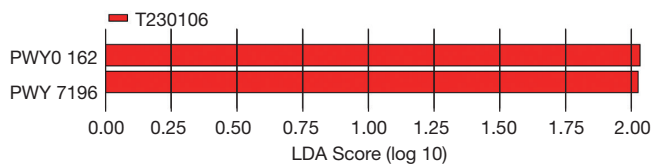
Figure 14 Differential metabolic pathway in group C rats on day 28. Group C, physiological saline treatment group. LDA, linear discriminant analysis.

serum. (I) The levels of IL-1β and IL-6 increased among rats with CP over time. (II) Interventions with both *L. rhamnosus* and *S. boulardii* had no significant effect in reducing the amount of IL-1β and IL-6 in CP rats. (III) *L. rhamnosus* intervention had some effect on slowing the increase of IL-6 levels. (IV) Intervention with *L. rhamnosus* had an effect on the IL-8 levels in rats, but a relatively long intervention time was needed. (V) Intervention of *S. boulardii* appeared to reduce the IL-8 levels in rats, but the intervention seemed to be unstable. (VI) *L. rhamnosus* OF44 intervention had some overall effect in reducing the amount of TNF-α.

Our observations regarding cytokine levels suggests that as CP progresses, cytokine levels increase in rats, supporting the notion that CP is associated with inflammation (35). In previous research, intervention with *L. rhamnosus* OF44 had a similar effect to that of *S. boulardii* in reducing cytokine levels in the serum of CP mice (19), and in our study, *L. rhamnosus* OF44 demonstrated a better effect compared to *S. boulardii*. This supports the hypothesis the intervention with *L. rhamnosus* OF44 can reduce cytokine levels in CP rats, alleviating their inflammatory response and relieving their CP symptoms. However, to obtain more definitive results, longer intervention periods are needed.

We further found that though another study suggests that interventions with dietary fiber, lactic acid-producing bacteria, and butyric acid-producing bacteria can significantly alter gut microbiota diversity for children with CP (8), intervention with *L. rhamnosus* OF44 changed the gut microbial composition of CP rats to some extent, but the overall composition and diversity of intestinal flora were not significantly changed. Analysis of 16S rRNA sequencing data revealed some differential bacterial taxa among the different groups of rats. Metabolism-related bacteria were mainly affected after both *L. rhamnosus* OF44 treatment and *S. boulardii* treatment. *Prevotella* was a notable example, as it had a mean relative abundance of 0 in the gut of healthy rats but a mean relative abundance of about 0.0117 in the gut of CP rats. This result was consistent with a previous report that *Prevotella* is a signature taxon of CP (36). *Lachnoclostridium* UCG 006 is a strain of bacteria that is usually found in the gut microbiome and is highly abundant in the human intestine. It has been shown to promote the function of the intestinal mucosal barrier, thereby helping to protect intestinal health. Moreover, it produces beneficial metabolites, such as propionic acid, which help maintain the stability of the intestinal environment (37). *Roseburia* bacteria, as a type of probiotic, is a constituent of the intestinal microbiota in the human body and is primarily found in the colon. These bacteria have the ability to produce short-chain fatty acids (SCFAs), such as acetic acid, butyric acid, and propionic acid on (38). These SCFAs contribute to maintaining gut health, regulating the immune system, and reducing inflammation (39). Research indicates that a decrease in the abundance of *Roseburia* bacteria is linked to several illnesses, including inflammatory bowel disease, enteritis, metabolic syndrome, obesity, and type 2 diabetes (40). *Desulfovibrio* bacteria are a Gram-negative sulfate-reducing bacterium primarily found inside the human digestive tract. They have the ability to use organic compounds and hydrogen gas to reduce sulfates, producing organic acids such as propionate, isobutyrate, and acetone,

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while also generating hydrogen sulfide (41). The presence of these bacteria suggests that treatment with *L. rhamnosus* OF44 and *S. boulardii* may improve constipation in rats with CP by influencing the metabolism of the gut microbiota.

Our sequencing analysis showed after 1 week of intervention, the PWY-6588 pathway was downregulated in the group A rats. This may indicate that the intervention with *L. rhamnosus* OF44 led to a downregulation of energy metabolism since PWY-6588 is the pyruvate fermentation to acetone pathway (32). After 4 weeks, the GLYCOLYSIS-E-D pathway was upregulated in the group A rats, possibly indicating that the rats gradually adapted to the intervention of *L. rhamnosus*, and their energy metabolism returned to a normal state. In comparison to the rats in the other groups, the group A rats showed a downregulation of the PWY-5505 pathway. PWY-5505 is also a pathway associated with energy metabolism, and its downregulation suggests a decrease in metabolic levels (33). This could be due to the impact of *L. rhamnosus* OF44 intervention on energy metabolism in rats with CP. Confirmation of this speculation requires further experimental validation is. In comparison to the other groups, group C showed an upregulation of both the P441-PWY pathway and the GLYCOLYSIS-E-D pathway, indicating an increase in metabolic levels (30,31). This could potentially be due to inflammation caused by CP, but additional research is needed to confirm this hypothesis.

Overall, the findings of this study support the use of treating CP with probiotics and suggest that intervention with probiotics can be an effective and feasible means to reducing the amount of cytokines, altering gut microbiota, and changing the behaviors of CP rats, especially when the intervention is long lasting. Therefore, pharmaceutical companies should consider to investing more resources and support in developing and implementing probiotics therapy in CP treatment.

However, some limitations to this study should be mentioned. First, the sample was relatively small, which limits the generalizability of results. Second, we only measured cytokines levels and conducted 16S rRNA sequencing, while only two behavioral experiments were performed; this may not capture the full range of the behaviors and symptoms of CP rats. Third, the data of the healthy control group was only measured on the first day, which might have had an impact on data analysis and comparison. Finally, the study began when the rats are 100 days old, which might have influenced the outcomes of the intervention.

To address these limitations, subsequent studies should be conducted that include larger and more representative samples, measure a more extensive array of metrics representing the health state of rats with CP, establish more complete and comprehensive control groups, and begin when the rats are younger. Moreover, future research could explore other aspects of probiotics treatment, for example, the specific mechanisms by which probiotics affect physiological activity, and examine the possibility treating CP with probiotic pharmaceuticals.

Conclusions

The CP rats in our study exhibited a higher level of inflammation than did healthy rats before intervention. After intervention, rats treated with both *L. rhamnosus* OF44 and *S. boulardii* had lower levels of cytokines in their serum. Moreover, *L. rhamnosus* OF44 appeared to be more efficient in lowering the levels of cytokines. In addition, *L. rhamnosus* OF44 and *S. boulardii* altered the gut microbial composition and metabolic rate of CP rats. Some altered bacteria taxa were the same as those in our previous studies concerning the link between CP and the gut microbiota. However, in contrast to other studies, the CP rats in our study did not exhibit depression-like behaviour. Overall, our findings indicate that *L. rhamnosus* OF44, similar to *S. boulardii*, has potential as a treatment for CP.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-23-566/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-23-566/dss

Peer Review File: Available at https://tp.amegroups.com/article/view/10.21037/tp-23-566/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.

com/article/view/10.21037/tp-23-566/coif). Y.Z. and Z.M. are from BGI Nutrition Precision Co., Ltd., which provided *L. rhamnosus* OF44 for this study. The other authors have no 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. The animal study protocol was approved by the Institutional Review Board of Department of Pediatrics of Longgang District Maternity & Child Healthcare Hospital of Shenzhen City (ethical approval number: LGFYYXLLLQ-2020-002), in compliance with the Chinese national standards GB14925 and GB14922, as well as the standard operating procedures of Shenzhen TOP Biotechnology Co., Ltd., for the care and use of animals.

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