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Bifidobacterium longum CCFM760 combined with lactulose alleviates loperamide-induced constipation by regulating the gut microbiota and improving short-chain fatty acid metabolism

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ABSTRACT: Constipation is a prevalent gastrointestinal disorder that severely affects people's daily life and work. *Bifidobacterium* has been proven to be effective in relieving constipation. Compared with single-strain intervention, the combined intervention of probiotics and oligosaccharides can produce more significant health effects. However, its specific efficacy and mechanism in relieving constipation have not been fully elucidated. This study aims to explore the potential of the synergistic intervention of *Bifidobacterium* and oligosaccharides in improving constipation, with a particular emphasis on investigating the regulatory effects of the combined intervention on the gut microbiota and its metabolites. In this study, based on the physiological characteristics of the strains and animal experiments, a strain of *B. longum* CCFM760 capable of relieving constipation was screened. Furthermore, nine kinds of oligosaccharides were used to replace glucose as the single carbon source respectively. According to the shortest generation time of the strain, lactulose was selected as the compounding factor to investigate how the combined intervention of *B. longum* CCFM760 and lactulose relieves constipation and reveal the involved mechanisms. It was shown that compared with the single-strain intervention, the combined intervention therapy could better relieve constipation. By promoting the intestinal colonization of *Bifidobacterium*, it could more significantly reduce the relative abundances of harmful bacteria such as *Bilophila* and *Negativibacillus*, and at the same time significantly enrich short-chain fatty acids (SCFAs)-producing bacteria such as *Bifidobacterium*, *Faecalibaculum*, and *Ileibacterium*. Therefore, it could more effectively restore the levels of SCFAs in the gut, increase the expression level of serotonin 4 receptor (5-HT4R), improve the levels of gastrointestinal active peptides (increasing the levels of gastrin (GAS) and motilin (MTL), and decreasing the level of vasoactive intestinal peptide (VIP)), as well as the expression levels of calcitonin gene-related peptide (CGRP) and brain derived neurotrophic factor (BDNF), and reduce the expressions of Aquaporin-3 (AQP3) and inducible nitric oxide synthase (iNOS) in the colon tissue, thus more effectively relieving constipation. These results lay the foundation for the development of dietary formula foods with the efficacy of relieving constipation.

Keywords: Constipation; *Bifidobacterium*; oligosaccharides; SCFAs; synergistic effect

1. Introduction

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Constipation is a prevalent gastrointestinal problem that leads to dry stools, slow intestinal transit, and decreased bowel movements[1], as well as mental health disturbances that can seriously affect patients' daily lives[2]. The pathogenesis of constipation is multifactorial and remains unclear. Although drugs have been widely used in the treatment of constipation, their prolonged use can potentially bring about side effects and pose threats to patients' health[3]. Therefore, it is vital to search for mild and long-term effective constipation relief therapies.

The gut microbiota plays a crucial role in maintaining intestinal homeostasis and can dynamically regulate intestinal motility through multiple pathways, including metabolites, neural signaling, and immune regulation, thereby improving gastrointestinal transit and fecal consistency[4]. The microbiota of constipated patients is mainly characterized by a lower proportion of beneficial bacteria (e.g., *Lactobacillus* and *Bifidobacterium*), a relative increase in potential pathogens, and a decrease in species richness[5]. This disturbance of the intestinal flora usually triggers an imbalance of metabolites in the intestinal lumen and exacerbates the process of constipation development. short-chain fatty acids (SCFAs) act as crucial signaling molecules, construct a strong link between gut microbiota and constipation. It has been reported that the levels of SCFAs such as acetic acid and propionic acid in the intestines of patients with constipation are significantly lower compared with those of normal people, the levels of SCFAs are significantly negatively correlated with the severity of constipation[6]. In addition, colon-targeted release of SCFAs can accelerate intestinal transit and achieve the effect of relieving constipation[7]. Therefore, targeting gut microbiota and metabolites may be an effective method to prevent and relieve constipation.

Bifidobacteria are widely distributed probiotics in nature, including subspecies such as *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium adolescent*. They exhibit a variety of health-promoting effects, particularly in regulating gut microbiota dysbiosis, immune responses, metabolism, and intestinal homeostasis, thereby alleviating constipation[8, 9]. It should be noted that its probiotic effects are closely related to the physiological characteristics of the strains. The prominent adherence, tolerance to intestinal fluids, and ability to utilize oligosaccharides enable them to reside and proliferate in the intestinal tract for extended periods, which is crucial for relieving constipation[10]. Recent studies have indicated that bifidobacteria with adhesive properties tend to offer superior constipation relief[11]. Supplementation with *Bifidobacterium longum* carrying the abfA gene cluster can enrich the utilization of arabinan by the gut microbiota, leading to more efficient production of SCFAs and improved constipation symptoms[12]. Functional oligosaccharides, a type of dietary fiber that are poorly absorbed in the small intestine, can selectively enhance the relative abundance of beneficial bacteria and metabolites with advantageous effects on the host, thereby exerting positive impacts on host health[13]. While previous research has indicated that the single-agent application of probiotics or oligosaccharides can bring about certain beneficial effects on host health, more and more studies have shown that the combination of bifidobacteria and oligosaccharides generates a synergistic effect. For instance, compared to single-strain

application, this combination has demonstrated remarkable superiority in multiple aspects, such as alleviating colitis[14], mitigating allergic reactions[15] and promoting infant intestinal development[16].

Oligosaccharides can serve as a carbon source, promoting its proliferation. This not only enhances the survival rate of bifidobacteria in the intestine but also ensures an increase in SCFAs, enabling bifidobacteria to gain a competitive edge in nutrient acquisition against native intestinal microorganisms[17]. A recent study has shown that synbiotics are highly effective in relieving constipation symptoms[18]. Although the combination of probiotics and oligosaccharides can exert its advantages by altering the composition and function of the gut microbiot and inhibiting the inflammatory response, its exact mechanism still remains unclear. The combination of *Bifidobacterium* and oligosaccharides may become a more effective treatment for constipation.

Considering the above factors, the main scientific questions we aim to explore in this study are as follows: (1) Does the efficacy of constipation relief correlate with the physiological properties of the strains (gastrointestinal fluid tolerance and utilization of oligosaccharides)? (2) How should the oligosaccharides for compounding be selected? And can the combination therapy of *Bifidobacterium* and oligosaccharides achieve more effective relief of constipation? What are the underlying mechanisms of action? To this end, we randomly selected eight strains of *Bifidobacterium* from the strain library and screened out effective strains for relieving constipation from in vitro to in vivo. Then, these effective strains were combined with the oligosaccharides that were selected based on the criterion of the shortest generation time for the strains' growth, and used to intervene in constipated mice, so as to further explore the potential mechanism of relieving constipation.

2. Materials and methods

2.1 Bacteria and pretreatment

Eight *Bifidobacterium* strains were obtained from Strain Bank of the Food Biotechnology Center of Jiangnan University (Table 1). These strains were cultured for 20 hours at 37℃ under anaerobic conditions in modified MRS broth supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sinopharm Chemical Reagent Co.,Ltd). All strains underwent three successive sub-culturing steps to ensure their viability and stability. Subsequently, the bacterial cultures were centrifuged at 3000×g for 10 minutes at 4℃, followed by two washes with sterile ice-cold physiological saline to remove residual culture media and other impurities. Then, the bacterial pellets were resuspended in a 30% glycerol solution and stored at -80℃ for subsequent animal experiments. The viable cell count of the bacterial suspensions was accurately determined using the colony counting method. Before animal experiments, the bacterial suspensions were washed, resuspended in sterile physiological saline, and diluted to a concentration of 10^{10} CFU/mL to meet the experimental requirements.

Table 1 Strain information

Strains	Sources

B. animalis subsp. lactis BB12	Preserved in this lab
B. breve CCFM670	Infant feces, China Center of Industrial Culture Collection
B. adolescentis CCFM744	Human, Wuxi, Jiangsu
B. adolescentis CCFM743	Human, Wuxi, Jiangsu
B longum subsp.infantis CCFM751	Human, Shanxi
B longum subsp.infantis CCFM687	Human, Wuxi, Jiangsu
B. longum subsp. longum CCFM757	Human, Wuxi, Jiangsu
B. longum subsp. longum CCFM760	Human, Wuxi, Jiangsu
B. longum subsp. longum CCFM682	Human, Wuxi, Jiangsu

2.2 Determination of physiological properties of strains

2.2.1 Determination of adhesion

The experiment referred to the methods of Kim and Adlerberth et al[19, 20] with some minor modifications. Human colorectal adenocarcinoma HT-29 cells (American Type Culture Collection, accession number HTB-38) were inoculated in six-well plates and cultured in modified RPMI-1640 medium (Gibco Inc) supplemented with 5% (v/v) fetal bovine serum, and incubated at 37℃ in 5% carbon dioxide and 95% air (cell concentration of 2×10^5 cells/mL). The adherent cells were washed twice with sterile phosphate-buffered saline (PBS, pH 7.8). Subsequently, 2 mL of *Bifidobacterium*-RPMI-1640 medium suspension (with a concentration of 10^8 CFU/mL and a volume ratio of 1:1) was added to each well. Two hours later, the cells were washed twice again with sterile phosphate-buffered saline, fixed in methanol, subjected to Gram staining, observed under a microscope, and the average number of bacteria adhering to a single intestinal epithelial cell was counted.

2.2.2 Determination of tolerance to simulated gastrointestinal fluids

The gastrointestinal fluid tolerance of *Bifidobacterium* was experimentally determined by the method of Wang et al[21] with some minor modifications. Pepsin 1:15000 and trypsin 1:250 were both purchased from Sinopharm Chemical Reagent Co., Ltd., and used for the preparation of simulated gastric juice and small intestinal juice. Specifically, the experimental strains were inoculated into modified MRS medium at an inoculation amount of 2%, incubated at 37℃ for 20 h, and then subjected to plate counting. For the determination of the gastric fluid tolerance of *Bifidobacterium*, the bacterial suspension was washed twice with sterile physiological saline and then resuspended in 5 mL of artificial simulated gastric fluid, so that the concentration of the bacterial suspension reached 10^9 CFU/mL. After culturing under anaerobic conditions at 37℃ for 3 h, samples were taken and serially diluted and transferred into 9 mL of sterile physiological saline. The suspension of 0.1 mL was incubated anaerobically on cMRS agar plates at 37℃ for 48 h, and the plates counts were carried out to evaluate the gastric fluid tolerance of the strains respectively. Similarly, for the determination of the intestinal juice tolerance of *Bifidobacterium*, the washed bacterial suspension after cultivation was resuspended in 5 mL of artificial simulated intestinal fluid. Then, after the strains were cultured for 2 h, 4 h, and 8 h successively at 37℃ in an anaerobic environment, plate counting was carried out respectively to evaluate their tolerance to intestinal fluid. The calculation formula for the survival rate is as follows:

$$\text{Survival rate}(\%) = \frac{\lg N_1}{\lg N_0} \times 100\%$$

N_0 is the number of viable bacteria in the initial bacterial solution; N_1 is the number of viable bacteria after artificial simulation of gastric or intestinal fluid treatment.

2.2.3 Utilization of oligosaccharides and growth generation time

The qualitative analysis of the utilization of oligosaccharides was carried out referring to the method of Zhang et al[22], and the effects of different oligosaccharides (Shandong Qilu Biotech, Liaocheng, China). on the generation time of the strain were determined according to the following method. A liquid culture medium was prepared by using 2% (m/v) of each oligosaccharide respectively, including galactooligosaccharides (Gos), fructooligosaccharides (Fos), xylooligosaccharides (Xos), isomaltooligosaccharides (Imo), inulin (In), soybean oligosaccharides (Sos), mannose oligosaccharides (Mos), stachyose (Sta), and lactulose (Lac), to substitute for glucose in the mMRS medium. The mMRS media with and without glucose were used as positive and negative controls, respectively. Then, the activated *Bifidobacterium* suspension was inoculated into the corresponding culture medium at a dosage of 2%, and the OD_{600} was measured every 30 minutes, continuously until entering the stable phase. Select the time points for entering the logarithmic phase and stable phase from the measured growth curve of *Bifidobacterium*, and calculate the generation time of the logarithmic phase of *Bifidobacterium* growth using the following formula：

$$G = \frac{t_2 - t_1}{(\lg OD_2 - \lg OD_1)/\lg 2}$$

G is the generation time of the logarithmic growth period (min); t_1 and t_2 are the selected time points (min); and OD_1 and OD_2 are the corresponding OD_{600} values.

2.3 Animal experimentation

2.3.1 Animals and experimental design

Experiment 1: The experimental design for screening strains with the efficacy of relieving constipation is as follows (Fig.1): Forty-eight male BALB/c mice (seven-week-old, purchased from Shanghai SLAC Laboratory Animal Co., Ltd.) were randomly divided into 6 groups. Then mice were housed in an individually ventilated cage (IVC) rodent housing system for two weeks. Then, the mice entered a two-week intervention phase I, a two-week washout period, and a two-week intervention phase II. The purpose of intervention phase I and the two-week washout period was to detect the colonization ability of the tested strains in constipated mice. Constipation in mice was induced by gavaging 0.2 mL of loperamide suspension at a concentration of 10 mg/kg body weight (Xi'an Yangsen Pharmaceutical Co., Ltd.). One hour later, the phenolphthalein group, 0.1 mL of phenolphthalein (XiAn Hengtai Biological Science and Technology Ltd) in a concentration of 70 mg/kg body weight was received. Four intervention groups were orally administered the corresponding *Bifidobacterium* bacterial suspension (1×10^9 CFU, 0.2 mL) respectively. In order to determine the colonization time of *Bifidobacterium*, the mice were only fed a basal diet during the washout

period. On the last day of the intervention phase II, the time to the first black stool was measured, and feces were collected to determine the water content of the feces. All experimental protocols used in this study have been approved by the Ethics Committee of Jiangnan University (No.: JN.No20170704 - 20170825 [86]).

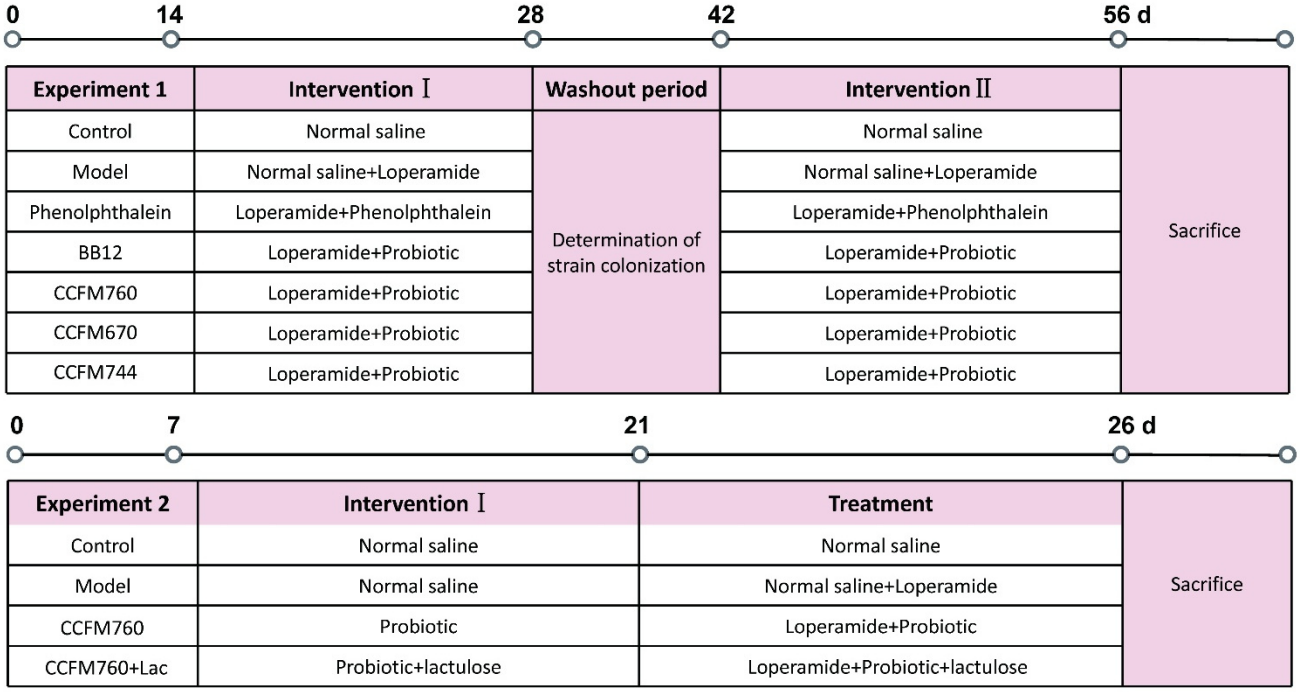


Fig. 1. Animal experiment flowchart.

Experiment 2: The experimental design for the intervention of probiotics combined with oligosaccharides is as follows: A total of twenty-four C57BL/6 mice (six-week-old, purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd.) were randomly divided into 4 groups. After one week of acclimatization, except for the control group and the model group, the probiotic intervention group gavaged with CCFM760, and the combined intervention group gavaged with a mixture of CCFM760 and Lac(Shandong Qilu Biotech, Liaocheng, China). During the first three weeks, preventive intervention was carried out: Every day, the control group and the model group were gavaged with normal saline (0.2 mL); the probiotic intervention group was gavaged with *Bifidobacterium* bacterial suspension (1×10^9 CFU, 0.2 mL), and the combined intervention group was gavaged with the 0.2 mL bacterial solution containing 6% Lac oligosaccharides(w/v). In the fourth week, except for the control group, the model group and the probiotic intervention group were gavaged with 0.2 mL of loperamide at a dose of 10 mg/kg body weight (BW) to establish the model. One hour later, the control group and the model group were gavaged with normal saline (0.2 mL). and the probiotic intervention group was gavaged with bacterial solution (0.2 mL, while the combined intervention group was administered 0.2 mL bacterial solution containing 6% lactulose oligosaccharides (w/v). One day before the end of the experiment, the time of the first black stool was measured, and feces were collected to determine the water content of the feces, SCFAs, and the composition of the intestinal microbiota. After the experiment was finished, the mice were sacrificed, and the small intestine propulsion rate was measured, and the required materials were collected. All experimental

protocols used in this study have been approved by the Ethics Committee of Jiangnan University (Approval No.: JN.No20230515c0900715[226]).

2.3.2 Determination of Gut transit time

After the mice had been fasted for 12 hours, all groups, other than the control group, received loperamide hydrochloride solution via gavage. One hour later, all mice were gavaged with 0.2 mL of ink prepared from 5% activated carbon and 10% gum arabic (Sinopharm Chemical Reagent Co., Ltd.)[23]. The duration between the mice's ingestion of the ink and the expulsion of black feces was recorded.

2.3.3 Determination of Faecal water content

The mice's fresh feces were gathered and placed into sterile small tubes, and then the wet weight of the feces of each mouse was recorded. Then feces were subjected to freeze-drying treatment, and the dry weight of the feces was recorded. The water content of the feces was calculated according to the following formula:

$$\text{Faecal water content}(\%) = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100$$

2.3.4 Determination of small intestinal propulsion rate

After the mice were fasted for 12 hours, on the next day, gavage was carried out again following the same procedures as in (2.2.2). The difference was that, 30 minutes after gavage of the ink, the mice were sacrificed by cervical dislocation under anesthesia. After opening the abdominal cavity of the mice, the intestinal tract from the pylorus to the cecum was excised, and this intestinal tract was stretched into a straight line. The length from the pylorus to the leading edge of the ink was measured as the "advancing length of the front end of the activated carbon solution", and the following formula was used to calculate the small intestine propulsion rate[21].

$$\text{Small intestine propulsion rate}(\%) = \frac{\text{length of activated carbon}}{\text{Total length of the small intestine}} \times 100$$

2.4 Quantification of bifidobacteria in feces by real-time fluorescence quantitative polymerase chain reaction (RT-qPCR)

Weigh 100 mg of the feces of mice collected during the washout period. The genomic DNA of six *Bifidobacterium* strains was obtained through extraction with the Bacterial Genomic DNA Extraction Kit (Tiangen Biotech (Beijing) Co., Ltd.). After the concentration had been measured, it was transformed into the copy number. Then, RT-PCR was performed with the primers presented in Table 2, and each sample was analyzed three times. After performing gradient dilution and absolute quantification on the samples of single *Bifidobacterium* DNA, take the logarithm of the copy number as the abscissa and the cycle number (Ct value) when the sample reaches the fluorescence threshold as the ordinate to draw a standard curve, and calculate the DNA concentration of the corresponding *Bifidobacterium* in the fecal samples. Taking the concentration of *Bifidobacterium* in the feces of mice before gavage as the reference, evaluate the colonization ability of different experimental strains in the intestines of mice by comparing the changes in

the concentration of *Bifidobacterium* in the feces of mice during the washout period. The primer information of various strains involved in this experiment and the information of the reaction system for real-time fluorescence quantitative PCR (RT-qPCR) are shown in Supplementary Table S1. The colonization ability of *Bifidobacterium* in the mouse intestine is evaluated by calculating the ratio of the number of mice with the content of *Bifidobacterium* in the fecal samples higher than the initial value to the total number of mice.

2.5 Histopathological analysis

Colon tissues were immersed in a 4% neutral paraformaldehyde solution for fixation, rinsed with tap water overnight, and then subjected to gradient ethanol dehydration. Hematoxylin and eosin (H&E) staining were performed according to standard methods[24].

2.6 Biochemical measurements

The mouse colon tissues were mixed with pre-cooled physiological saline at a ratio of 1:9. Subsequently, the mixture was homogenized and then centrifuged at 12,000g for 15 minutes at 4℃ to obtain the tissue supernatant. The concentrations of substance P (SP), motilin (MTL), gastrin (Gas), vasoactive intestinal peptide (VIP), somatostatin (SS), and calcitonin gene-related peptide (CGRP) in the colon were measured with ELISA kits (Nanjing Sembega Bio-technology Co., Ltd.) according to the manufacturer's protocols. Additionally, the concentrations of protein in the tissues were determined with the relevant assay kits (NanJing JianCheng Bioengineering Institute).

2.7 Expression of constipation-related genes in the colon

Colon tissues were placed in enzyme-inactivated zirconia beads and enzyme-free centrifuge tubes, and 1 mL of TRIzol reagent was added to extract total RNA, which was reverse-transcribed to cDNA using a reverse-transcription kit (Vizzan Biotech, Nanjing, China), and the PCR system was prepared according to the instructions of the Quantitative Polymerase Chain Reaction (qPCR) Premix (Bó Lè Associates, Hercules, CA, USA). The PCR system was prepared according to the instructions of quantitative polymerase chain reaction (qPCR) premix solution (Bó Lè Associates, Hercules, CA, USA), and the PCR system was amplified using a Bó Lè CFX384 fluorescence quantitative gene amplifier (Bó Lè Associates, USA). Real-time fluorescence quantitative PCR analysis was performed to detect the expression of β-actin, brain-derived neurotrophic factor (BDNF), aquaporin 3 (AQP3), and inducible nitric oxide synthase (iNOS) in mouse colon, and the forward and reverse primer sequences are shown in Table 2.

Table 2. RT-PCR amplified primers

Gene	Primer Fw 5'-3'	Primer Rv 5'-3'
Gapdh	TGTGGATGGCCCCTCTGGAA	TGACCTTGCCCACAGCCTTG
AQP3	GGGCCTTTGCCAACAATGAG	CGGCTGTGCCTATGAACTGA
BDNF	GCCTTTGGAGCCTCCTCTAC	CGCCGAACCCTCATAGACAT
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC

2.8 Determination of SCFAs

Referring to the study of Mao et al.[25], the collected mouse feces were lyophilized and extracted. The ether extracts were determined by gas chromatography-mass spectrometry (Thermo/Trace-1300 ISQ-Mass, Thermo Fisher, USA) equipped with a Rtx Wax column (30 m, 0.25 μm). The carrier gas, flow rate, injection volume, split ratio, injection temperature and gas chromatographic warming program were kept in line with the literature, while the mass spectral scanning was performed in selected ion monitoring mode (mass-to-charge ratios of 60, 74, 88, 73, 43, 87, 28). The concentration of SCFAs was determined by the external standard method. The reference standards for SCFAs were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.9 16S ribosomal DNA (rDNA) sequencing and bioinformatics analysis

We conducted intestinal bacterial sequencing using fresh mouse fecal samples collected one day before the experimental termination. Genomic DNA was extracted from the feces with the Fecal FastDNA Spin Kit (MP Biomedical, Cat. No. 6570200), following the manufacturer's standardized protocol. The fecal microbial bacterial genome was used as a template for polymerase chain reaction (PCR) amplification of the V4 region of the 16S rRNA gene according to the methodology previously described in the literature[26] (forward primer: 5'-AYTGGGYDTAAAGNG-3'; reverse primer: 5 '-TACNVGGGGTATCTAATCC-3'). The PCR products were purified using the Tiangen Gel Recovery Kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China) and quantified using the Quant-iT PicoGreen Double Stranded DNA Quantification Kit (a product of Life Technologies, a division of Thermo Fisher Scientific, Carlsbad, CA, USA). These samples were mixed at equal mass concentrations to construct libraries, which were subsequently sequenced in 500+7 cycles on the Illumina MiSeq sequencing platform using the MiSeq Reagent Kit (Illumina, USA, 500 cycles-double-ended sequencing, product number MS-102-2003).

2.10 Analysis of genes related to Bifidobacterium longum subspecies CCFM760

B. longum subsp. longum CCFM760 was subjected to genome sketch sequencing, and the sequencing results were automatically recognized by Linux system, and genome splicing and assembly were performed by SOAPdenovo software. Sequence annotation and functional classification of the predicted genes were performed through nr database, Swiss-Prot protein database, COG database, String database and MG-RAST web server. In addition, the whole genome of Bifidobacterium animalis milk subspecies BB12 was downloaded from NCBI and analyzed for comparison.

2.11 Statistical analysis

All the data were analyzed by one-way analysis of variance (ANOVA) using the agricolae package in R. Tukey's test was used to assess significant differences between the groups. The analysis results were shown as the mean ± standard deviation, and bar graphs were created using GraphPad Prism 10.1.2 software. The difference analysis at the genus level of the microbiota was carried out using the Stamp software, and the analysis and plotting of the microbiota for LEfSe were performed using the R packages

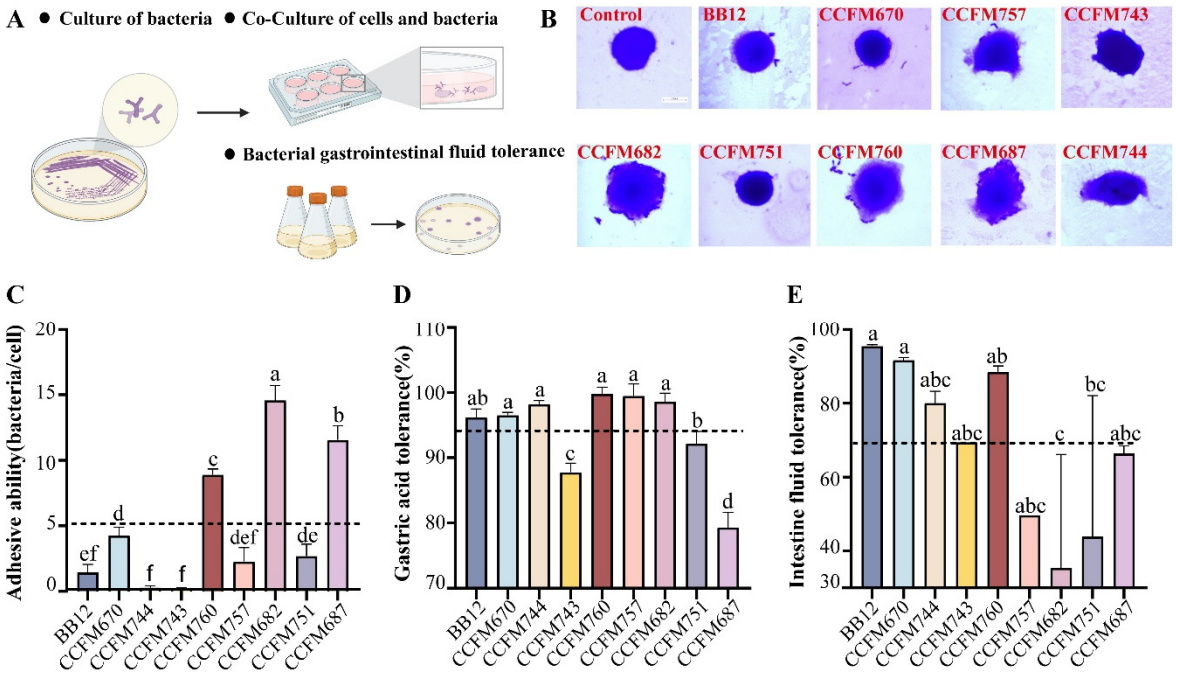
microeco, ggplot2, and magrittr. For all analyses, $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) were considered statistically significant differences.

3. Results

3.1 Three Bifidobacterium strains show excellent physiological characteristics in gastrointestinal fluid tolerance

The basic physiological characteristics of probiotic strains, including gastrointestinal transit tolerance and adhesion ability, are important factors for their probiotic function, directly related to their capacity for gastrointestinal colonization and effectiveness in alleviating constipation. It is reported that *Bifidobacterium animalis* BB12 can effectively colonize the intestine and exerts a favorable effect in relieving constipation[27]. Based on this, this study took BB12 as a positive control strain to systematically characterize the physiological properties of eight *Bifidobacterium* strains, aiming to identify potential strains that may have the effect of relieving constipation.

As depicted in Fig. 2, in terms of adhesion capacity, among the nine *Bifidobacterium* strains, *B. longum subsp. longum* CCFM682 exhibited the strongest adhesion ability, followed by CCFM687 and CCFM760, while *B. adolescentis* CCFM743 and CCFM744 were basically non-adhesive. In terms of gastrointestinal fluid tolerance, the positive control strain BB12, along with CCFM744, CCFM760, and CCFM670, maintained survival rates above the average across various time intervals in gastrointestinal fluids, indicating favorable tolerance to these harsh conditions. Principal component analysis (PCA) ultimately screened out three *Bifidobacterium* strains, CCFM760, CCFM670, and CCFM744. Their physiological characteristics were similar to BB12, particularly with respect to gastrointestinal fluid tolerance, indicating that these strains possess physiological properties comparable to those of the control strain. Therefore, they are considered potential strains for relieving constipation.



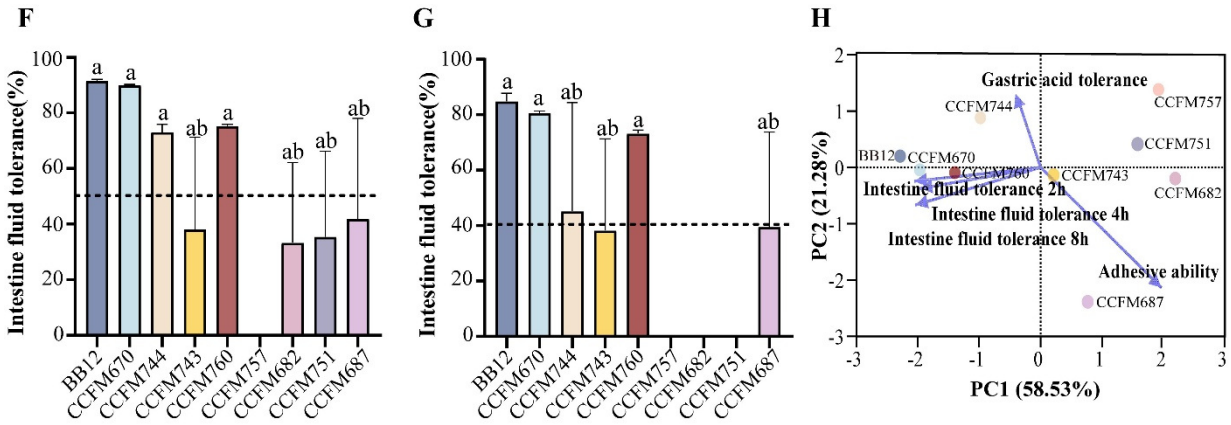


Fig. 2. Evaluation of the physiological characteristics of various *Bifidobacterium* strains. (A) Physiological characterization of strains(Created with BioRender.com). (B-C) Strain adhesion assay(n=3). (D) 3h gastric fluid tolerance assay (dashed line in the figure indicates the mean value)(n=3). (E-G) Measurement of intestinal fluid tolerance at 2h, 4h, and 8h (mean values are shown as dashed lines)(n=3). (H) PCA analysis of physiological characteristics.

3.2 Bifidobacterium longum CCFM760 relieves loperamide hydrochloride-induced constipation

Gavage administration of loperamide hydrochloride is one of the most common methods for establishing constipation models. It inhibits the transport of water and electrolytes by binding to opioid receptors, resulting in prolonged gut transit time, dry feces, and defecation difficulties. To investigate the relationship between the physiological characteristics of the strains, intestinal colonization, and constipation relief, we used the three *Bifidobacterium* strains screened in vitro to intervene in the loperamide hydrochloride-induced constipated mice respectively. During the washout period, quantitative determination of the administered strains was conducted to explore their colonization ability in vivo. Throughout the intervention period,, phenolphthalein and BB12 served as the positive control, and the capacity of the strains to relieve constipation was evaluated through the measurement of the apparent indicators of constipation.

The results showed (Fig. 3-E) that after the washout period, *Bifidobacterium* CCFM760 and CCFM670 could persist in the mouse intestine for approximately one week, and were second only to BB12, indicating their appreciable colonization capabilities. At the end of the intervention period, the spleen index in all experimental groups did not exhibit any significant alterations ($P > 0.05$). In contrast to the control group, the intestinal transit time in the model group was significantly extended($P < 0.05$), while the small intestine propulsion rate and fecal water content were significantly decreased ($P < 0.05$), indicating the successful model establishment.

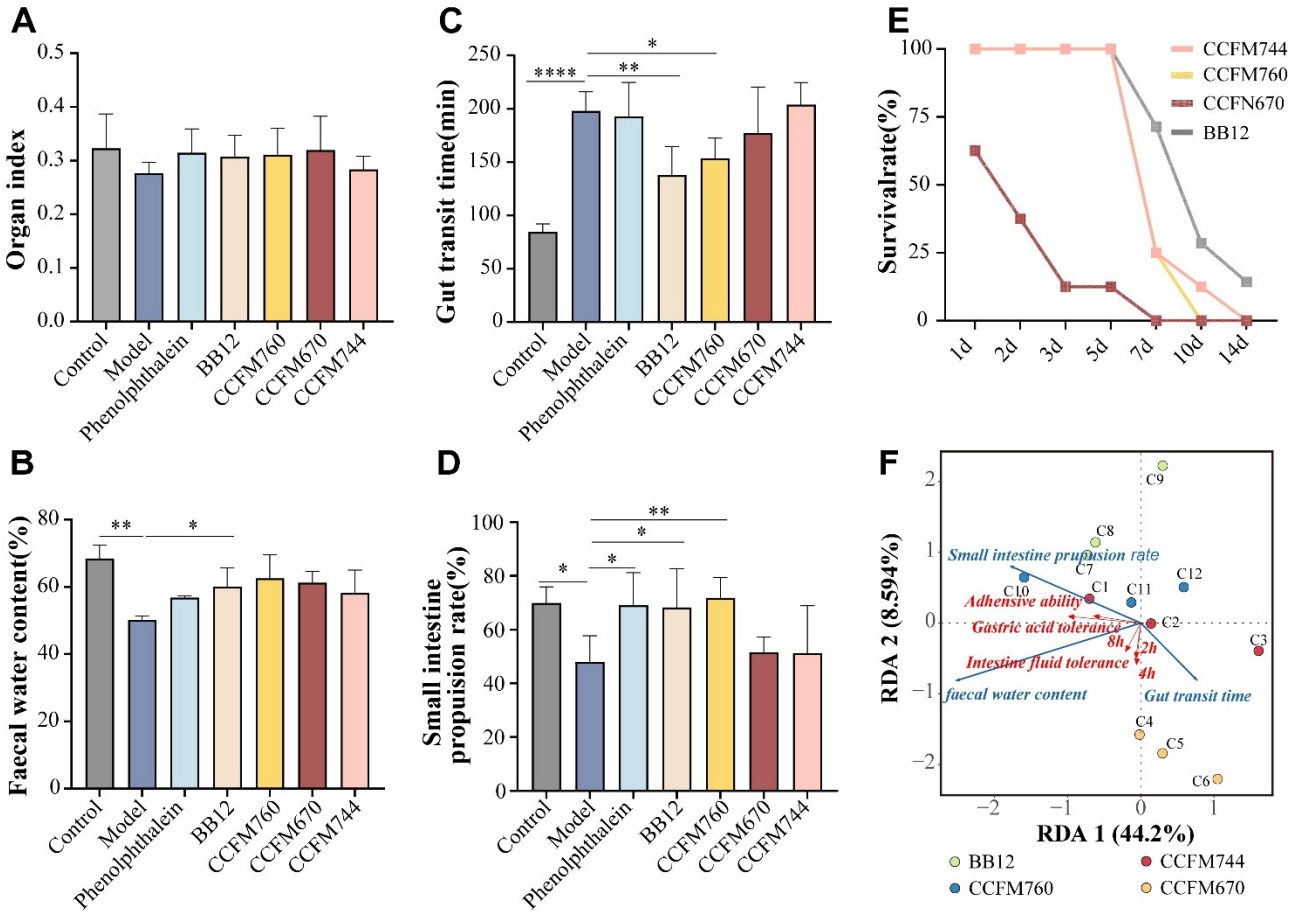


Fig. 3. Screening and Efficacy Evaluation of *Bifidobacterium* for Relieving Constipation. (A) Organ index(n=7-8). (B) Fecal water content(n=3). (C) Intestinal transit time(n=5-7). (D) Small intestinal propulsion rate(n=5-7).. (E) Intestinal colonization of bifidobacteria. (F) RDA analysis of physiological indices of strains and constipation relief indices.

In the intervention group, only *Bifidobacterium longum* CCFM760 and the positive control strain BB12 were able to relieve constipation (significantly reducing intestinal transit time, elevating fecal water content and small intestinal propulsion rate($P < 0.05$)). Further, redundancy analysis (RDA) revealed a certain correlation between the strains' tolerance to gastrointestinal fluids, adhesion ability, and constipation relief. The excellent physiological properties and intestinal colonization ability of *Bifidobacterium* CCFM760 were identified as crucial factors contributing to its efficacy in alleviating constipation. In addition, by further analysis of the strain's genome (Supplementary Fig. S1), we found that the CCFM760 genome contained 14.9% of genes encoding carbohydrate metabolism-related functions, slightly higher than that of BB12 (14.6%). This suggests that CCFM760 may also possess excellent oligosaccharides utilization capabilities.

Functional oligosaccharides can serve as a carbon source in the intestine, being utilized by Bifidobacterium and promoting its proliferation. So is it possible that CCFM760 paired with matching oligosaccharides could achieve a synergistic effect? Next, we first replaced the carbon source in the MRS medium with different types of oligosaccharides respectively and then detected the generation time of strain CCFM760 in each modified medium. The oligosaccharide with the shortest generation time was selected as the basis for formulating a combination with strain CCFM760. Subsequently, animal experiments were carried out to determine whether the combined treatment could more effectively relieve constipation

compared to the single application of CCFM760. Furthermore, the underlying mechanisms of this synergistic effect were elucidated from the perspectives of regulating the gut microbiota and its internal environment, as well as modulating gastrointestinal neurotransmitters related to constipation.

3.3 CCFM760+Lactofructose enhances the relief of constipation

In this study, we selected nine common types of oligosaccharides, each of which was used to replace glucose as the carbon source, to explore their effects on the growth of CCFM760 in vitro. Results show that, CCFM760 has a relatively low utilization ability of In and Sta, while it can well utilize the other seven kinds of oligosaccharides (Fig. 4 - Fig. S1). Moreover, in the modified MRS medium with Lac as the sole carbon source, the generation time of strain CCFM760 is the shortest. Based on this, we speculate that the combination of Lac and CCFM760 may contribute to the proliferation of this strain in the intestine and further relieve the symptoms of constipation. Therefore, in subsequent animal experiments, a combination of Lac and CCFM760 was chosen to treat constipated mice, while the group receiving only CCFM760 served as the control group.

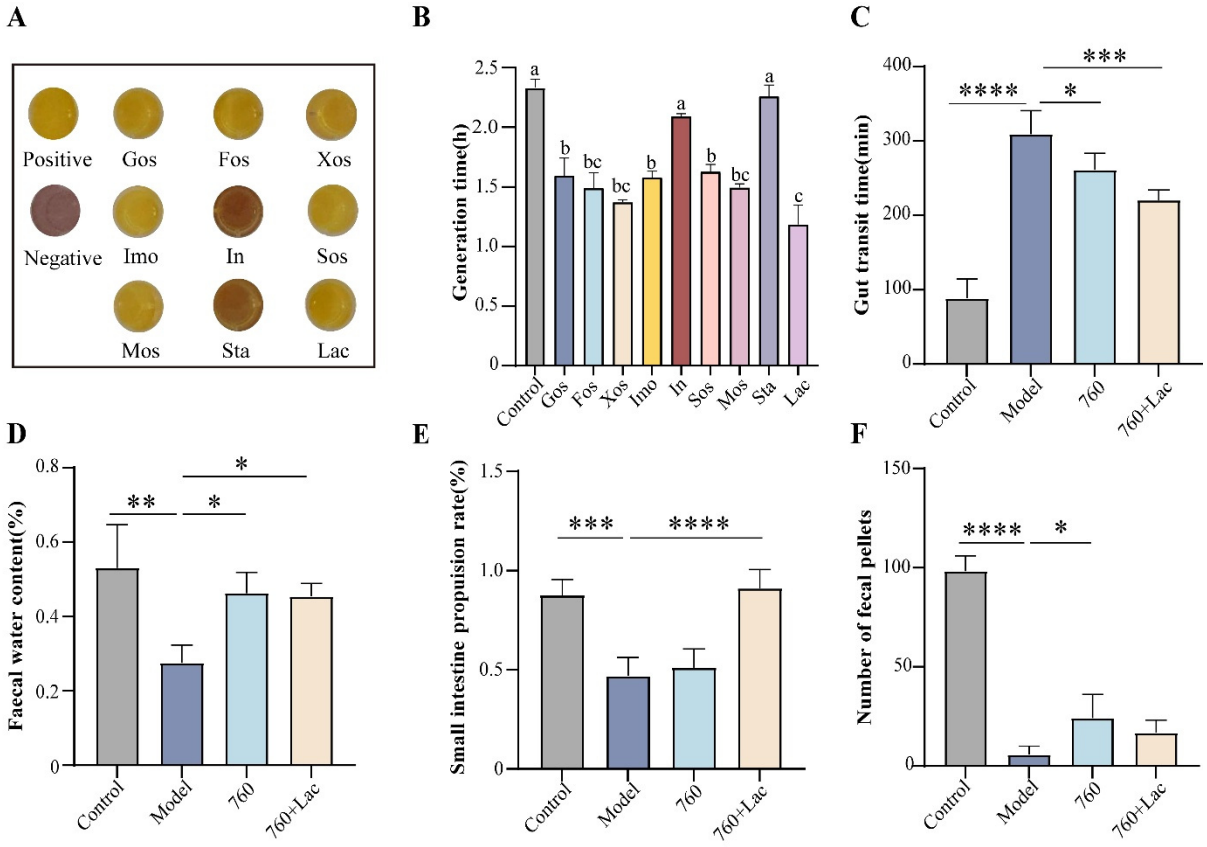


Fig. 4. Screening of oligosaccharides promoting CCFM760 growth and evaluation of efficacy in relieving constipation. (A)Utilization of oligosaccharides(n=3).. (B) CCFM760 growth generation time in the presence of different oligosaccharides(n=3). (C) Intestinal transit time(n=4-5). (D) Fecal water content(n=3). (E) Small intestinal propulsion rate(n=4-5). (F) Number of fecal pellets(n=4-5).

Results demonstrated that the combination of Lac and CCFM760 was significantly more effective than the single application of CCFM760 in relieving constipation (more significant decrease in the gut transit time and increase in the small intestine propulsion rate).

3.4 CCFM760+Lac ameliorates constipation-induced changes in intestinal flora composition

As shown in Fig.5A-C, from the perspective of microbiota diversity, neither loperamide-induced constipation nor *Bifidobacterium* intervention had a significant impact on the α-diversity of the microbiota. However, in terms of β diversity (Fig.5D-E), the gut microbiota of mice in the normal group and the model group showed deviations.

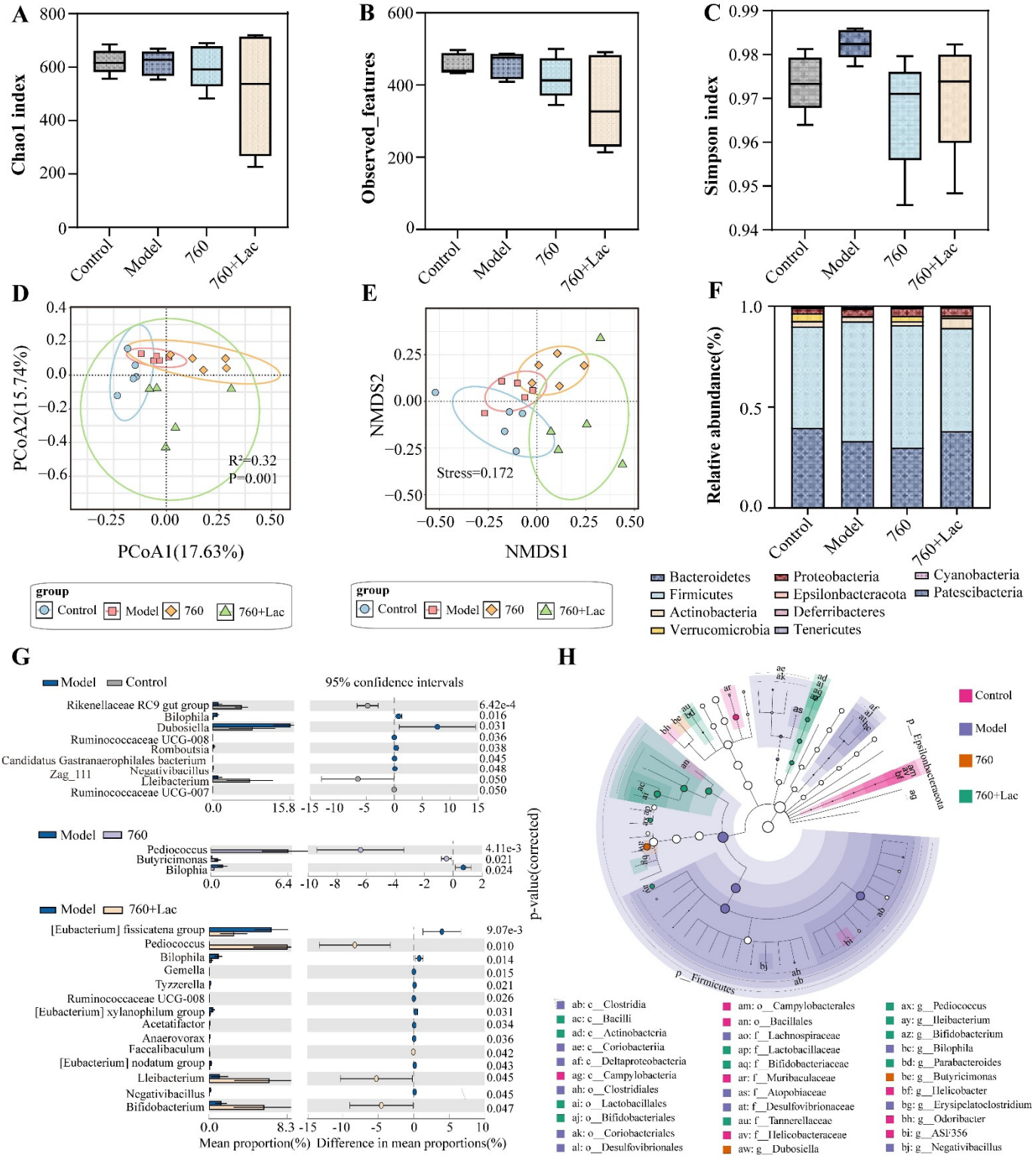


Fig. 5. Effect of CCFM760+Lac intervention on the flora of constipated mice(n=5). (A-C) α-diversity. (D-E) β diversity. (F) Colony phylum level. (G) STAMP analysis at the genus level. (H) Lefse analysis.

Compared to the CCFM760 treatment group, the confidence ellipse of the CCFM760 and Lac combination group was closer to that of the control group, indicating that the combined intervention can better rebuild the gut microbiota structure. At the phylum level (Figure 5F), Constipation induced by loperamide caused the relative abundance of Firmicutes to rise while that of Bacteroidetes declined. The combination of Lac and CCFM760 demonstrated a more pronounced alleviation effect on constipation compared to the group treated with CCFM760 alone. This combination was capable of reversing the changes in gut microbiota at the phylum level caused by constipation.

As shown in Fig.5G, at the genus level, the relative abundances of *Ileibacterium*, *Ruminococcaceae UCG-007*, and *Rikenellaceae RC9 gut group* were significantly reduced in the feces of constipated mice ($P < 0.05$), while the relative abundances of the genera *Bilophila*, *Dubosiella*, and *Ruminococcaceae UCG-008* were significantly increased ($P < 0.05$). In comparison to the group treated with CCFM760 alone, the combination group of CCFM760 and Lac could significantly increase the relative abundances of *Ileibacterium* and *Bifidobacterium* i and decrease the abundance of *Ruminococcaceae UCG-008* in constipated mice. Additionally, it also increased the relative abundances of genera including *Faecalibaculum*, *Pediococcus* and significantly decreased the relative abundance of genera such as *Bilophila* ($P < 0.05$).

Furthermore, Lefse analysis indicated the characteristic genera in the control group were *Ileibacterium*, *Rikenellaceae RC9 gut group*, *uncultured Bacteroidales bacterium*, *Helicobacter* and other genera, while the model group was typified by the *Eubacterium_ruminantium group*, *Bilophila*, and *Negativibacillus*. In the intervention groups, the characteristic genera of the CCFM760 intervention group were *Dubosiella* and *Butyricimonas*, whereas in the group intervened with the combination of CCFM760 and Lac, the characteristic genera included beneficial bacteria such as *Pediococcus*, *Parabacteroides*, *Rodentibacter*, and *Bifidobacterium*. It is particularly noteworthy that the combined intervention significantly increased the relative abundance of *Bifidobacterium*, and *Bifidobacterium* was one of the characteristic flora, which was consistent with the result of the in vitro experiment that lactulose was beneficial to the proliferation of CCFM760. In conclusion, compared with the single strain intervention, the combination of Lac and CCFM760 demonstrates greater efficacy in rectifying the gut microbiota dysbiosis caused by constipation.

3.5 CCFM760+Lac restores SCFAs levels in constipated mice

Alterations in the composition and structure of the gut microbiota usually give rise to alterations in the types and quantities of gut metabolites, such as SCFAs. Based on this, we further conducted a targeted metabolomic analysis of SCFAs in the above-mentioned intervention groups. As shown in Fig.6, loperamide hydrochloride modeling caused a notable decrease in the levels of acetic acid, propionic acid, and total SCFAs in the feces of mice with constipation($P < 0.05$), indicating a potential association between constipation and the abnormal SCFA levels. The intervention effect of the CCFM760+Lac group was superior to that of the CCFM760 treatment group. In addition to significantly raise the concentrations of acetic acid and total SCFAs in the feces, it also significantly increased the level of propionic acid ($P < 0.05$), enabling better regulation of the levels of gut SCFAs. Moreover, SCFAs can participate in the activation of the 5-HT4R receptor to

promote intestinal peristalsis. Through the determination of the gene expression of 5-HT4R in the colon tissue, it was found that compared with the control group, the expression of 5-HT4R in the model group significantly decreased ($P < 0.05$). The combined intervention of 760+Lac significantly increased the expression level of 5-HT4R compared with the single intervention of 760 ($P < 0.05$). This indicates that the combined application of Lac and CCFM760 can better restore the abnormal levels of SCFAs and further mediate 5-HT4R to relieve constipation.

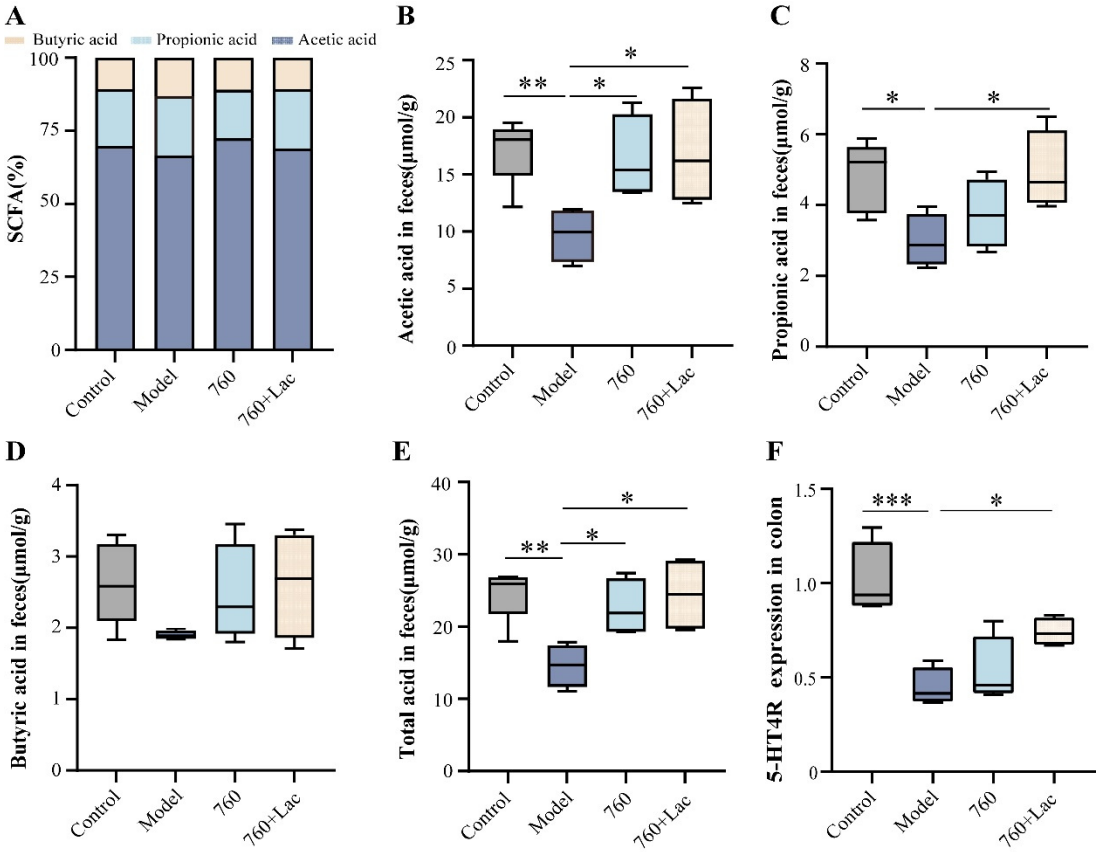


Fig. 6. Effect of CCFM760+Lac intervention on SCFAs in the feces of constipated mice(n=4-5). (A) Proportion of SCFAs. (B-E) Concentration of SCFAs in feces. (F) Colonic 5-HT4R expression.

3.6 CCFM760+Lac modulates constipation-induced intestinal mechanical and chemical barrier damage

Gut microbiota dysbiosis along with metabolite abnormalities can negatively influence the mucosal barrier of the intestinal lumen, colonic water-fluid transport, and the chemical barrier. Based on this, we further determined the relevant indicators. According to Fig.6A, in contrast to the control group, the colon mucosa of mice in the model group was damaged, and there was a certain degree of immune cell infiltration, which were significantly alleviated in the intervention groups. Gastrointestinal regulatory transmitters are important indicators for evaluating gastrointestinal peristalsis symptoms. Among them, MTL, GAS, and SP are excitatory gastrointestinal regulatory transmitters, while SS, VIP, and NO are inhibitory gastrointestinal regulatory transmitters. Among these, NO is synthesized with the participation of iNOS, which activates a series of downstream signals, leading to excessive relaxation of intestinal smooth muscles and a slowdown of intestinal peristalsis. In addition, CGRP is a neuropeptide substance that exerts a crucial function in the regulation of gastrointestinal activities.

As demonstrated in Fig.7, compared with the control group, the levels of excitatory gastrointestinal regulatory peptides (SP and MTL) in the colon of constipated mice were significantly decreased ($P < 0.05$), and the gene expression of iNOS, as well as the levels of CGRP and inhibitory gastrointestinal regulatory peptides (SS and VIP), were significantly increased ($P < 0.05$). The group treated with the combination of CCFM760 and Lac increased the levels of excitatory neurotransmitters MTL and SP in constipated mice compared with the group treated with CCFM760 ($P < 0.05$). These results show that the combined intervention of Lac and CCFM760 can better alleviate the abnormal indicators of gastrointestinal regulatory peptides and neuropeptides caused by constipation.

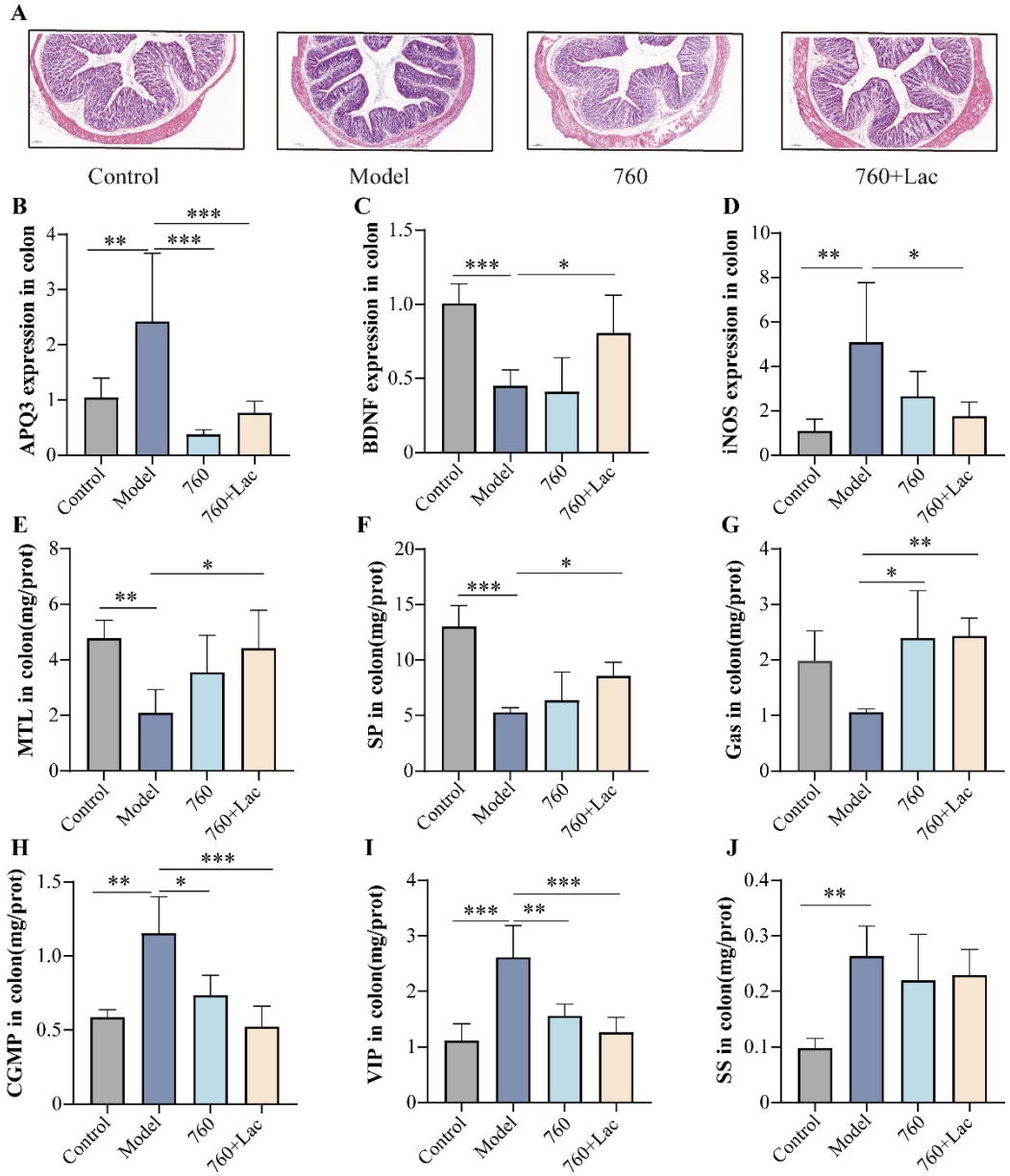


Fig. 7. Effect of CCFM760+Lac intervention on chemical indices in constipated mice(n=3-5). (A) Colonic H&E staining. (B) Colonic AQP3 gene expression level. (C) Colonic BDNF gene expression level. (D) Colonic iNOS gene expression level. (E) Protein levels of colonic CGMP. (F) Protein levels of colonic VIP. (G) Protein levels of colonic SS. (H) Protein levels of colonic CGMP. (I) Protein levels of colonic VIP. (J) Protein levels of colonic SS.

In addition, the results of qPCR showed that the expression level of AQP3 in the colon of constipated mice were significantly increased, while the expression of BDNF were significantly decreased ($P < 0.05$).

Both intervention groups significantly down-regulate the expression of AQP3 ($P < 0.05$). In addition, the expression of BDNF in the group treated with the combination of CCFM760 and Lac was significantly increased ($P < 0.05$) compared with the group treated with CCFM760 alone. Therefore, the combined use of CCFM760 and Lac can more effectively exert a synergistic impact on relieving constipation through regulating the chemical barrier of the intestinal lumen, maintaining the homeostasis of water and electrolytes, and enhancing the function of the mucosal barrier.

3.7 Relief of constipation by CCFM760+Lac is associated with modulation of SCFAs and microbiota

We performed Spearman correlation analysis to associate the above-mentioned differentially genera (31 genera in total) with SCFAs (Fig.8A-C). The results showed that the key metabolite acetic acid was significantly positively correlated with *Bifidobacterium*, while propionic acid was significantly positively correlated with *Ileibacterium*, *Bifidobacterium*, and *Faecalibaculum*. Moreover, both acetic acid and propionic acid were significantly negatively correlated with genera such as *Bilophila* and *Erysipelatoclostridium*and other genera.

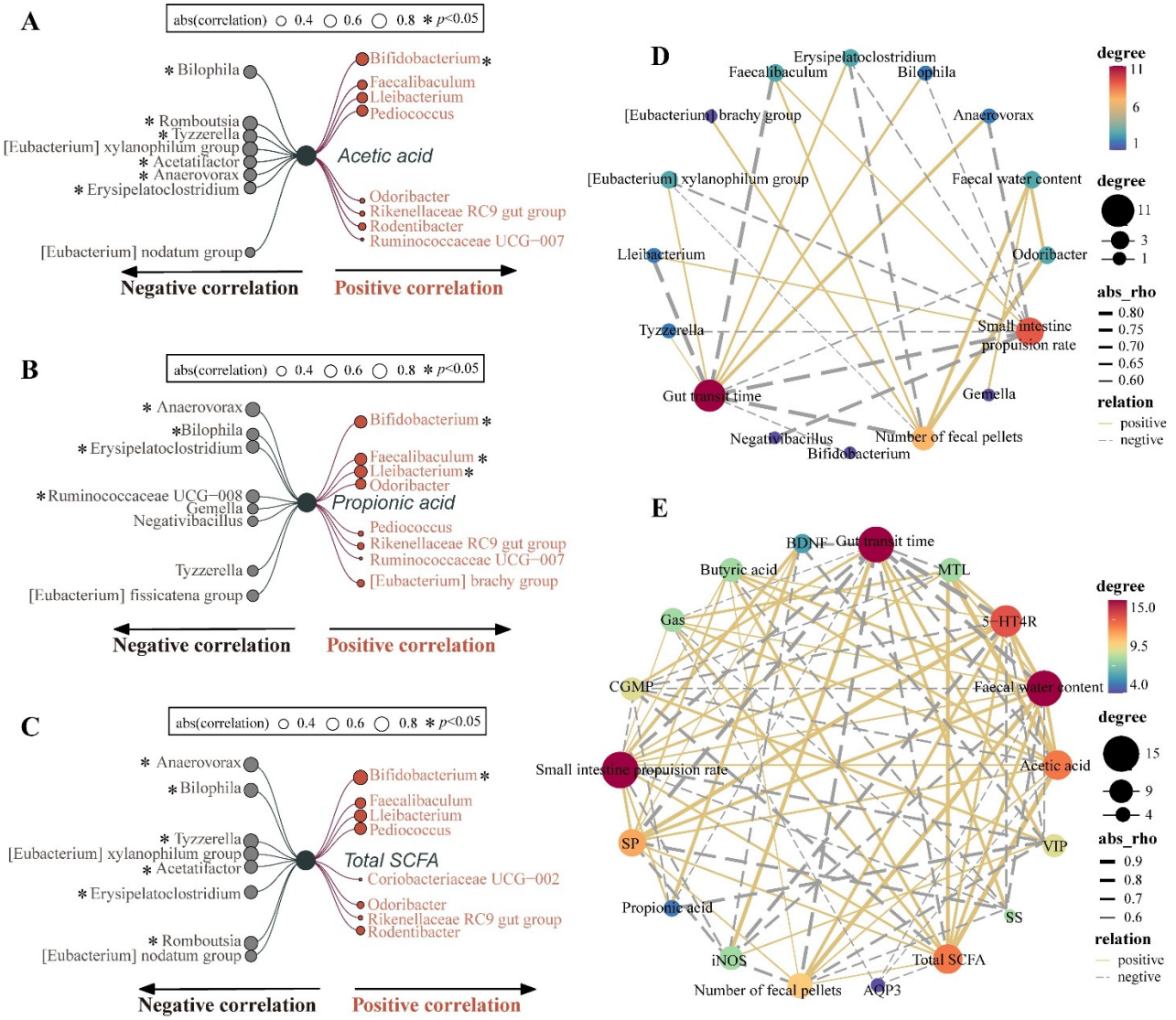


Fig. 8. correlation analysis. (A-C) Correlation analysis of SCFAs with intestinal flora. (D) Correlation analysis of bacterial genera with apparent indicators of constipation. (E) Correlation analysis of SCFAs with epigenetic and chemical indicators of constipation.

To clarify the relationship between these potential SCFAs-producing bacteria and the constipation phenotype, further correlation analysis results(Fig.8D) indicated that *Odoribacter*, a potential producer of acetic acid that was enriched in the control group, was significantly positively correlated with the constipation indicators of fecal water content and the number of fecal pellets, and was significantly negatively correlated with intestinal transit time. *Erysipelatoclostridium* and *[Eubacterium] xylanophilum group*, which were enriched in the model group, were negatively correlated with the small intestine propulsion rate and the number of fecal pellets, and positively correlated with intestinal transit time. Notably, *Faecalibaculum* and *Ileibacterium*, the potential propionic acid-producing bacteria that were significantly upregulated after regulation by CCFM760+Lac, were positively correlated with the small intestine propulsion rate and significantly negatively correlated with intestinal transit time. In contrast, the genera such as *Bilophila*, *Anaerovorax*, and *Tyzzerella*, which were significantly downregulated after the intervention, showed significantly opposite correlations. In addition, *Bifidobacterium*, which was significantly enriched in the CCFM760+Lac group, was significantly negatively correlated with intestinal transit time.

In addition, we conducted correlation analysis to clarify the relationship between the two differentially abundant metabolites, acetic acid and propionic acid, mediated by CCFM760+Lac and the relevant indicators (Fig.8E). The results evidenced that acetic acid was significantly positively correlated with gastrointestinal active peptides (MTL, GAS, SP), 5-HT4R, and significantly negatively correlated with CGMP and AQP3. Propionic acid was significantly positively correlated with BDNF, and significantly negatively correlated with CGMP and VIP.

Overall, we speculate that the intervention of CCFM760+Lac may downregulate the abundances of *Bilophila*, *Anaerovorax*, and *Tyzzerella*, and up-regulate the abundances of *Bifidobacterium*, *Faecalibaculum*, and *Ileibacterium*. In turn, it can increase the levels of acetic acid and propionic acid, repair the damaged host intestinal barrier, regulate the levels of gastrointestinal regulatory peptides and neuropeptides, and reduce the level of AQP3. Consequently, these effects may promote intestinal peristalsis, increase fecal water content, and alleviate constipation symptoms.

4. Discussion

Constipation seriously affects people's work and daily life. Current research indicates that the balance of the intestinal microecology is indeed closely related to constipation. Supplementation with *Bifidobacterium* can effectively regulate the intestinal environment (gut microbiota and metabolites), presenting itself as a potential approach to relieve constipation. Although numerous reports have demonstrated that the single intervention with specific *Bifidobacterium* strain is effective in alleviating constipation, the combined therapy involving probiotics and oligosaccharides is likely to yield more pronounced prebiotic effects[22]. The objective of this research was to explore the potential of the synergistic application of oligosaccharides and *Bifidobacterium* in enhancing the constipation relief, with a focus on investigating the regulatory effects of the combined intervention on the gut microbiota and its metabolites. Here, we screened *Bifidobacterium* strains

with the efficacy of relieving constipation and carried out interventions in combination with oligosaccharides to explore the impact of the combined formulation on the constipation relief and analyze the underlying mechanisms involved.

Bifidobacterium is a commonly used probiotic, and its efficacy is closely related to its physiological characteristics, especially the ability of the strain to tolerate gastrointestinal transit processes and its adhesion ability determines its colonization in the intestine[28], which in turn affects its probiotic effects. The ability of strains to tolerate gastrointestinal fluids, utilize oligosaccharides, and their adhesiveness have been proven to be closely associated with the alleviation of constipation[22]. Based on this, using BB12 as the positive control, we screened out three *Bifidobacterium* strains with excellent physiological characteristics through in vitro experiments: *Bifidobacterium longum* CCFM760, *Bifidobacterium breve* CCFM670, and *Bifidobacterium adolescentis* CCFM744. Furthermore, through animal experiments, we verified that one strain, *Bifidobacterium longum* CCFM760, has the efficacy of relieving constipation. Similar to BB12, *Bifidobacterium longum* CCFM760 exhibits outstanding tolerance to gastrointestinal fluids and adhesion ability in vitro, and can colonize in the intestine for a long period of time and alleviate constipation symptoms induced by loperamide. Correlation analysis reveals that the relief of constipation has a positive correlation trend with the in vitro physiological characteristics, which is consistent with previous reports[22]. Furthermore, genomic annotation analysis revealed that CCFM760 possesses remarkable carbohydrate utilization capabilities, which is higher than that of BB12 and other *Bifidobacterium* strains reported in other studies[29]. This indicates that its potent ability to utilize oligosaccharides, and it may be better for constipation relief in combination with oligosaccharides.

Functional oligosaccharides can serve as carbon sources to promote the proliferation of *Bifidobacterium*, and different probiotic strains have different preferences for oligosaccharides[30]. Through in vitro experiments, we found that the CCFM760 strain can metabolize nine common oligosaccharides. When Lac is used as the sole carbon source, it can most significantly reduce its growth generation time, which is beneficial to the growth of the strain. Furthermore, the animal experiments revealed that, compared with the intervention using the single CCFM760 strain, the combined intervention of CCFM760 and Lac could more significantly reduce the time to excrete the first black stool and improve the small intestine propulsion rate, and more effectively relieve the constipation symptoms induced by loperamide. This finding is consistent with the reports of several previous studies, which have demonstrated that the combined therapy of oligosaccharides and probiotics has better efficacy[18, 31].

The imbalance of the gut microbiota can lead to abnormal intestinal peristalsis and defecation difficulties in mice. In this study, the constipation group was enriched with harmful bacteria such as *Bilophila* and *Negativibacillus*. *Bilophila* is a harmful intestinal bacterium capable of metabolizing and producing the potentially toxic metabolite sulphide[32]. *Negativibacillus* may be a potential pathogen causing gut microbiota dysbiosis and is associated with inflammatory bowel disease[33]. After the intervention with CCFM760+Lac, the relative abundance of these harmful bacteria were significantly reduced. It should be noted that while

CCFM760 showed a certain colonization ability in the intestine in the animal experiment for strain screening, in the combined intervention experiment, the increase in the relative abundance of *Bifidobacterium* in the single-strain CCFM760 intervention group was not significant despite an upward trend (Fig. S3). This may result from the differences in the measurement methods of absolute abundance and relative abundance, as well as the interference from endogenous intestinal *Bifidobacterium*. Nonetheless, it is more worth noting that the combined intervention significantly enriched *Bifidobacterium*, which may be attributed to the fact that the combination of oligosaccharides and probiotics can mediate better colonization of *Bifidobacterium* in the intestine[34]. In addition, the intervention of CCFM760+Lac also significantly increased the relative abundances of beneficial bacterial genera such as *Pediococcus*, *Faecalibaculum*, and *Ileibacterium*. *Ileibacterium* has been reported to be a beneficial bacterium in the intestine and is related to the improvement of obesity-related metabolic disorders[35]. *Faecalibaculum* is strongly associated with constipation relief, and it has been shown that *Bifidobacterium bifidum* can positively affect constipation by enhancing the abundance of *Faecalibaculum*, which repairs the enteric nervous system[24]. Spearman correlation analysis further confirmed that these beneficial bacterial genera enriched by the intervention of CCFM760+Lac were positively correlated with the alleviation of constipation indicators. Compared with the intervention using a single strain, the intervention with CCFM760+Lac was able to better regulate the microbiota dysbiosis in constipated mice.

SCFAs are an important type of luminal metabolites for relieving constipation. It has been reported that *Bifidobacterium* is capable of generating SCFAs[36], while specific oligosaccharides can promote the growth of *Bifidobacterium*, further facilitating the enhanced accumulation of SCFAs, highlighting their complementary functions[37]. In this study, we conducted targeted determination of SCFA metabolites in the feces of mice and performed correlation analysis with the gut microbiota. The results showed that compared with the intervention of CCFM760, CCFM760+Lac could better increase the levels of SCFAs in the intestinal lumen. Previous reports have shown that *Bifidobacterium* metabolizes Lac, generating a high concentration of acetate and leading to the acidification of the intestinal lumen in both humans and mice[38].This aligns with our findings, where the CCFM760+Lac intervention led to an enrichment of *Bifidobacterium*, and a significant positive correlation was observed between *Bifidobacterium* and SCFA levels. In addition to promoting its own colonization for SCFAs production, on the other hand, CCFM760+Lac may also increase the relative abundance of SCFAs-producing bacteria in the intestine (*Faecalibaculum*, etc.) to regulate level of SCFAs. SCFAs can activate the 5-HT4R receptor to promote the release of 5-hydroxytryptamine, thereby enhancing intestinal peristalsis[39]. The correlation results showed that the intervention of CCFM760+Lac increased the expression of 5-HT4R in the colon, and there was a positive correlation between the levels of SCFAs and the expression of 5-HT4R. Therefore, we speculate that the combined intervention of CCFM760+Lac can better enhance its own colonization and enrich SCFAs-producing bacteria, further regulate the levels of SCFAs to activate the 5-HT4R receptor pathway to achieve the relief of constipation.

Gastrointestinal endocrine chemicals in the colon exert a crucial influence on intestinal peristalsis. Among them, excitatory gastrointestinal active peptides, including SP, MTL and Gas, can promote pyloric relaxation and enhance gastrointestinal peristalsis; while inhibitory gastrointestinal active peptides, including SS, VIP and CGRP, can inhibit intestinal peristalsis[4]. iNOS is involved in the synthesis of NO and participates in the regulation of intestinal peristalsis. However, the NO produced by its overexpression will lead to excessive relaxation of intestinal smooth muscles, slowing down intestinal peristalsis[40]. In our study, the intervention of CCFM760+Lac alleviated the abnormal expression of gastrointestinal regulatory peptides and iNOS caused by constipation, and was superior to the intervention of CCFM760 alone. BDNF, as a common neurotrophic factor, affects the proliferation and survival of intestinal neurons and plays a crucial role in intestinal peristalsis[41]. AQP3 in the colon is involved in the absorption of water in the intestinal lumen and is closely related to fecal characteristics[42]. Here, the intervention of CCFM760+Lac was able to reduce the expression of AQP3 and elevate BDNF expression to better relieve constipation.

Although our study demonstrated that the combination of lactulose and CCFM760 is more effective in alleviating constipation, there are still some limitations. Several previous studies have shown that lactulose has a relieving effect on constipation[43, 44], and antibiotic treatment experiments have indicated that the effect of lactulose on constipation relief depends on the modulation of the gut microbiota[45]. Due to the lack of a lactulose-only treatment group, it was unable to clarify whether the efficacy of the combined formula stems from the direct laxative effect of lactulose or its regulatory effect on the microbiota. In future studies, we will further clarify its independent effect by setting up a group treated with lactulose alone and explore its mechanism in depth by combining more experimental methods.

5. Conclusion

In conclusion, the combination of *Bifidobacterium longum* CCFM760 and Lac demonstrates a remarkable potential to significantly alleviate constipation through its potent probiotic functions. Through in vitro physiological characteristic assays and in vivo animal verification, we obtained a strain of *B. longum* CCFM760 with excellent colonization and oligosaccharide utilization capacity, and explored the effects and underlying mechanisms of its combination with the Lac in relieving constipation. In terms of physiological characteristics, *B. longum* CCFM760 possesses good gastrointestinal fluid tolerance, adhesiveness, and oligosaccharide utilization capacity, which enables it to proliferate and metabolize oligosaccharides efficiently in the intestinal tract. The combined intervention of *B. longum* CCFM760 and Lac can effectively relieve constipation symptoms, which was accomplished by altering the composition and structure of the gut microbiota, thus increasing the relative abundance of SCFAs-producing bacteria and restoring SCFA levels. Consequently, it improves the disorders of gastrointestinal regulatory peptides, repairs the damage to the intestinal mechanical and chemical barriers, accelerates colonic transit, increases fecal water content and the small intestine propulsion rate, thereby alleviating constipation.

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Declaration of competing interest

The authors declared they have no competing financial conflicts of interest.

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