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CLA-producing *Bifidobacterium breve* alleviates LPS-induced intestinal inflammation through immune modulation and gut microbiota remodeling

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ABSTRACT: *Bifidobacterium breve* is recognized as an important member of the dominant gut microbiota early in life, playing a crucial role in intestinal immunity. Although *B. breve* can bioconvert linoleic acid (LA) into conjugated linoleic acid (CLA) to exert immunomodulatory effects, its region-specific immune functions and precise roles in shaping gut microbial composition remain elusive. Our study aimed to evaluate the immunomodulatory effects and microbiota-modulating properties of CLA-producing *B. breve* strains in lipopolysaccharide (LPS)-induced inflammation model using rat pups. LPS stimulation significantly alters the relative transcriptional levels of inflammatory cytokines in intestinal tissues. Interventions with different *B. breve* strains demonstrated efficacy in alleviating LPS-induced intestinal inflammation, with *B. breve* CCFM683 exhibiting particularly broad-spectrum immunomodulatory capacity and comprehensive therapeutic effects. *B. breve* CCFM683 modulates gut immunity in rat pups via increasing interleukin-10 (IL-10) level and reducing interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interferon-gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α) mRNA expression. Furthermore, *B. breve* CCFM683 administration significantly altered the CLA level in the cecum and colon. Moreover, strain CCFM683 treatment can help restore gut homeostasis and promote beneficial alterations in gut microbiota composition by increasing the abundance of *Ligilactobacillus*. These results indicated that CLA-producing *B. breve* can alleviate LPS-induced inflammation, with *B. breve* CCFM683 had great functionality in regulating the intestinal immune responses.

Keywords: *Bifidobacterium breve*; long-chain polyunsaturated fatty acids; inflammatory cytokines; intestinal mucosal barrier; intestinal microbes

1. Introduction

The gut microbiota and its metabolites play pivotal roles in regulating intestinal immune system and maintaining immunological homeostasis [1-3]. These microorganisms are intricately linked to host physiology

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and health since they perform several essential functions with consequences not only for the gastrointestinal environment but also for remote organs of the body. *Bifidobacterium* is crucial for maintaining the intestinal micro-ecological balance necessary for immunological modulation and play an important role in the maturation of the human immune system from gestation to childhood [4, 5]. *Bifidobacterium breve* is a commensal gastrointestinal tract inhabitant that is recognized as a significant member of the infant gut microbiota [6]. Further, *B. breve* has a profound influence on both early and long-term health and development [7]. Therefore, it becomes crucial to understand how *B. breve* modifies the gut microbiota and controls immune responses.

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA) with conjugated double bonds [8]. CLA has attracted great interest in recent decades due to its biological and physiological benefits such as anti-carcinogenesis, anti-obesity, anti-inflammation, anti-diabetic and bone formation-promoting properties [9–11]. Furthermore, CLA exerts immunomodulatory effects through dual peroxisome proliferator-activated receptor (PPAR)- α/γ activation pathways: it modulates inflammatory responses via PPAR- α agonism while regulating macrophage polarization toward anti-inflammatory M2 phenotypes in a PPAR- γ -dependent manner [12]. Indeed, numerous bacterial species have been documented to biotransform free LA to CLA, though catalytic efficiency and enzymatic mechanisms vary significantly across species [13]. *B. breve* represents a recently identified species capable of single-step LA-to-CLA bioconversion, with substantial *in vitro* and *in vivo* studies reported [14–18]. Our previous studies demonstrated that among common human-associated bifidobacteria, *B. breve* generally exhibits superior CLA production capacity (conversion rate > 50.0%) [19]. However, research on how CLA-producing *B. breve* influences host immune responses remains limited, hindering a comprehensive mechanistic understanding of host-microbe interactions.

To investigate the impact of CLA-producing *B. breve* on intestinal immunity in rat pups, lipopolysaccharide (LPS) was selected for establishing intestinal inflammation models. LPS acts as a stimulant capable of leading to intestinal inflammation and immune dysregulation, causing systemic and local injury and disrupting the homeostasis of the intestinal environment, making it a valuable tool for testing interventions aimed at restoring immune and intestinal homeostasis. This study employed *B. breve* as the research subject, utilizing an LPS-induced inflammation model in rat pups to investigate the immunomodulatory effects of *B. breve* with different CLA-producing abilities, aiming to provide insights into the mechanisms by which *B. breve* regulates intestinal immunity and exerts gut microbiota-modulating effects in LPS-induced immunocompromised rats.

2. Materials and methods

2.1 Animals

2.1.1 Induction of intestinal inflammation by lipopolysaccharide

Wistar wild-type rats were purchased from SiPeiFu. All rats were kept under SPF conditions at the Jiangnan University Laboratory Animal Center. The barrier environment was controlled at a temperature of

20–26°C, a humidity of 40–70%, noise below 60 dB, and a 12 h light/dark cycle [20]. One-to-three-week-old rats were used for experiments. On postnatal day 3, pups that were either overweight or underweight were eliminated, and the sex ratio was adjusted to 1:1. Each maternal rat was fed with 10 pups. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jiangnan University. Breeding and use of samples of sacrificed mice for further experiments were approved by the Animal Ethics Committee of Jiangnan University, Jiangsu, China (approval no: JN.No20250630W0241230[388]). The scheme for the LPS-induced inflammatory was shown in **Table 1**. Body weights of the rat pups were measured and recorded at the initiation and conclusion of the experiment. Animals were euthanized at 2, 4, 6, and 8 hours post-intraperitoneal injection of LPS. Following euthanasia, the diarrhea symptoms of the rat pups were observed and recorded by photography. Then, intestinal tissues were dissected to collect duodenum, jejunum, ileum, cecum and colon sections from the rat pups. LPS was purchased from Sigma-Aldrich (L2880-10MG, USA).

Table 1 Animal experimental scheme for LPS-induced inflammatory

Group	Day 1 to 20	Day 21
Control	no treatment	PBS (intraperitoneally)
LPS-2 h	no treatment	LPS (intraperitoneally, 2.0 mg/kg body weight), sampling at 2-hours post-treatment
LPS-4 h	no treatment	LPS (intraperitoneally, 2.0 mg/kg body weight), sampling at 4-hours post-treatment
LPS-6 h	no treatment	LPS (intraperitoneally, 2.0 mg/kg body weight), sampling at 6-hours post-treatment
LPS-8 h	no treatment	LPS (intraperitoneally, 2.0 mg/kg body weight), sampling at 8-hours post-treatment

2.1.2 *Bifidobacterium breve* administration

One-week-old Wistar rats were treated with phosphate buffered saline (PBS) by gavage for 14 days followed by intraperitoneal injection with PBS (Control, n = 10), PBS by gavage for 14 days followed by intraperitoneal injection with 2.0 mg kg⁻¹ body weight LPS (LPS, n = 10), and different strains of *B. breve* by gavage for 14 days followed by intraperitoneal injection with 2.0 mg kg⁻¹ body weight LPS (n = 10, respectively) (**Fig. 1**). The serum samples and intestinal tissue samples were collected. The scheme for the feeding trial was shown in **Table 2**. Following euthanasia, intestinal tissues were dissected to collect duodenal, jejunal, ileal, cecal, and colonic sections from the rat pups. All animal experiments were strictly conducted following the instructions approved by the Animal Ethics Committee of Jiangnan University, Jiangsu, China (Approval no: JN.No20250630W0241230[388]).

Table 2 Animal experimental scheme for *Bifidobacterium breve* effect on LPS-induced inflammatory

Group	Day 1 to 6	Day 7 to 20	Day 21
Control	no treatment	PBS	PBS (intraperitoneally)
LPS	no treatment	PBS	LPS (intraperitoneally)
CCFM683+LPS	no treatment	10 ⁹ CFU/d bacterial suspension	LPS (intraperitoneally)
CCFM1198+LPS	no treatment	10 ⁹ CFU/d bacterial suspension	LPS (intraperitoneally)
CCFM1300+LPS	no treatment	10 ⁹ CFU/d bacterial suspension	LPS (intraperitoneally)
CCFM1025+LPS	no treatment	10 ⁹ CFU/d bacterial suspension	LPS (intraperitoneally)

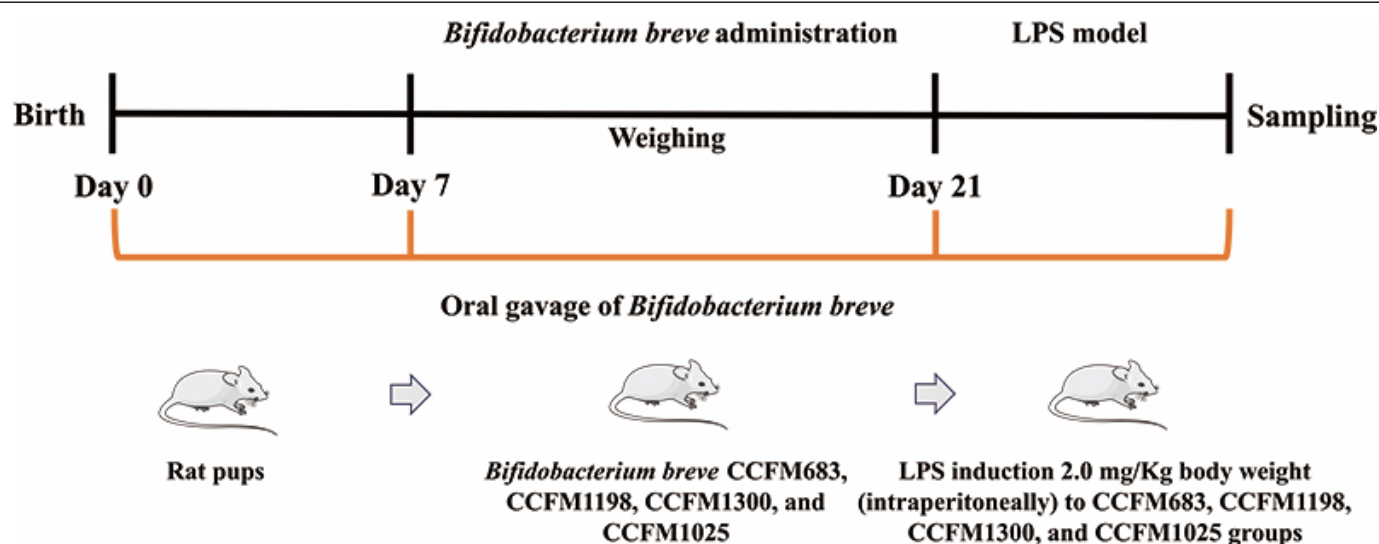


Fig. 1 The schematic diagram of the experimental design. A total of six groups were reared, with 10 rat pups per group. LPS: lipopolysaccharides.

2.2 Bacterial culture

Bacteria were preserved in the Food Biotechnology Research Center of Jiangnan University. The information of *B. breve* was shown in **Table 3**. Anaerobic bacteria were grown in de Man, Rogosa, and Sharpe (MRS) medium (10.0 g/L tryptone, 10.0 g/L beef extract, 20.0 g/L glucose, 2.0 g/L $\text{C}_2\text{H}_3\text{NaO}_2$, 5.0 g/L yeast extract, 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 2.0 g/L $\text{C}_6\text{H}_{14}\text{N}_2\text{O}_7$, 2.6 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and 1.0 g/L Tween 80) supplemented with 0.5 g/L L-cysteine. The bacteria were then cultured under anaerobic conditions (80% N_2 , 10% H_2 and 10% CO_2) at 37°C in an anaerobic chamber.

Table 3 *Bifidobacterium breve* used in this study ^[19]

Strain number	Original number	CLA bioconversion rate/%	Habitat
CCFM683	C11	90.1 ± 6.91	neonatal feces
CCFM1198	FBJCP1M6	85.4 ± 9.12	infant feces
CCFM1300	FHLJDQ3M5	55.3 ± 3.76	infant feces
CCFM1025	M2CF22M7	0.0	adult feces

2.3 GC-MS-based quantification of fatty acids

The pre-weighed intestinal content samples were extracted using a previously published method ^[21]. In brief, samples were mixed with 400 μL methanol. The mixtures were briefly vortexed and ground at 65 Hz for 30 seconds and repeat 4 to 5 times. Then, 1.0 mL of methyl tert-butyl ether (MTBE) containing an internal standard (0.02 mL $\text{C}_{15:0}$ fatty acid) was added to the samples and they were vortexed for 3 min at 452 rcf. After the addition of 400 μL of water, samples were vortexed for 1 min and centrifuged for 10 min (16, 260 rcf at 4°C). The organic upper phase was transferred to a new tube. The lower phase was extracted with 400 μL of methanol, 1.0 mL of MTBE and 400 μL of water. Then, samples were vortexed and centrifuged for 10 min (16, 260 rcf at 4°C). The upper phases were combined and dried under a gentle stream of nitrogen gas. The samples were resuspended in 1.0 mL of 2% methanol sulfate and bathed in a thermostatic oscillator for 3 h at 70°C. After cooling to room temperature, samples were mixed with 1.0 mL of hexane, and 800 μL of the upper phase of hexane fraction was transferred to a new tube for analysis.

Lipid extracts from intestinal contents were analysed using a GC2010 plus chromatograph (Shimadzu, Japan) coupled with a Rtx-5MS column ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$, Agilent Technologies) and an GCMS-QP2010 Ultra System. The following oven programmed temperature was used: 180°C (3 min), $180\text{--}190^{\circ}\text{C}$ ($10^{\circ}\text{C min}^{-1}$), 190°C (3 min), $190\text{--}220^{\circ}\text{C}$ ($5^{\circ}\text{C min}^{-1}$), 220°C (1 min), $220\text{--}230^{\circ}\text{C}$ ($2^{\circ}\text{C min}^{-1}$) and 230°C (18 min). The column flow rate was set to 1.0 mL min^{-1} and the transfer line temperature was maintained at 240°C with helium carrier gas. The following MS conditions were used: ionization mode, electron impact; electron energy, 70 eV; source temperature, 230°C ; and mass range, 50–550 Da. Analytical methods have been previously described by Lu *et al.* [22]. Different fatty acids were identified by matching their retention times and quantified based on internal standard (2.0 mg/mL C15:0).

2.4 LC-MS-based quantification of CLA

The pre-weighed intestinal content samples were extracted using the above method. Samples were mixed with 400 μL methanol. The mixtures were briefly vortexed and ground at 65 Hz for 30 seconds and repeat 5 times. Then, 1.0 mL of MTBE was added to the samples and they were vortexed for 3 min at 452 rcf. After the addition of 400 μL of water, samples were vortexed for 1 min and centrifuged for 12 min (16,260 rcf at 4°C). The organic upper phase was transferred to a new tube. The lower phase was extracted with 400 μL of methanol, 1.0 mL of MTBE and 400 μL of water. Then, samples were vortexed and centrifuged for 12 min (16,260 rcf at 4°C). The upper phases were combined and dried under a gentle stream of nitrogen gas. The samples were resuspended in 1.0 mL of methanol and centrifuged for 10 min (706 rcf at 4°C). Then, 800 μL of lipid-containing supernatants was transferred to a new tube for analysis.

Lipid extracts were analysed using an UPLC-TQD system (Waters, USA) coupled with an ACQUITY UPLC BEH C18 column ($1.7\text{ }\mu\text{m}$, $2.1 \times 50\text{ mm}$). The column flow rate was set to 0.3 mL min^{-1} and the column temperature was maintained at 40°C . Two mobile phases were used: 2-propanol and acetonitrile (70:30, v/v) (solvent A) and water with 0.1% formic acid (solvent B). The following elution gradient was used: 0–1 min, 70% A, 30% B; 1–3 min, 70% A, 30% B; 3–14 min, 70% A, 30% B; 14–15 min, 100% A; 15–15.5 min, 70% A, 30% B. The following MS conditions were used: capillary, 3.0 Kv (ESI[−]); source temperature, 120°C ; desolvation temperature, 400°C ; and desolvation gas flow, 800 L h^{-1} . CLA was identified by matching their exact masses and retention times based on authentic standards. MassLynx software (v.4.1) was used for peak identification and area integration and for generation of a calibration curve for all synthetic standards. The level of CLA was quantified by measuring the peak areas from the extracted ion chromatograms (EICs) from the MS data.

2.5 RNA isolation and real-time quantitative PCR

Total RNA was extracted from indicated rat tissues using FreeZol Reagent kit (R711-01, Vazyme) according to the manufacturer's instructions. RNA samples were quantified and reverse-transcribed into cDNA using a HiScript IV All-in-One Ultra RT SuperMix for qPCR kit (R433-01, Vazyme). The cDNA samples were amplified by real-time quantitative PCR (qPCR) with a $2 \times$ Taq Pro Universal SYBR qPCR Master Mix kit (Q712-02, Vazyme). qPCR was performed on a CFX Opus 384 instrument (Bio-Rad). The

primers for real-time qPCR are listed in **Table 4**. Expressions of the indicated genes were normalized to the expression of housekeeping gene *GAPDH* using the $2^{-\Delta\Delta C_t}$ method.

Table 4 The primers used for RT-qPCR

Gene	Forward (5'-3')	Reverse (5'-3')
<i>GAPDH</i>	AAGAAGGTGGTGAAGCAGGCATC	CGGCATCGAAGGTGGAAGAGTG
<i>IL-1β</i>	CCTCTGTGACTCGTGGGATG	CACTTGTGGCTTATGTTCTGT
<i>IL-6</i>	TTGCCTTCTTGGGACTGATG	ACTGGTCTGTTGTGGGTGGT
<i>IFN-γ</i>	TACACGCCGCGTCTTGGTTT	TTTGCCAGTTCCTCCAGATA
<i>TNF-α</i>	ATCCGAGATGTGGAAGTGGC	CACGAGCAGGAATGAGAAGAGG
<i>IL-10</i>	CTGCTATGTTGCCTGCTCTT	CCCAAGTAACCCTTAAAGTCC

2.6 ELISA measurement of cytokine responses

Rat pups were gavaged with *B. breve* and as described above. Whole blood was collected in a serum separation tube and allowed to clot for 2 h at room temperature. The serum was then collected by centrifugation at 1,383 rcf for 15 min. Serum interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interferon-gamma (IFN- γ), and interleukin-10 (IL-10) were analyzed via ELISA (EK301B/EK306/EK380/EK310, MultiSciences) according to the instructions.

2.7 Determination of serum biochemical indicators, intestinal permeability and oxidative stress-related biomarkers

Serum biochemical indicators were determined using the EXC400 automatic biochemical analyzer (zybio, China). Serum D-lactic acid (D-LA), diamine oxidase (DAO) and LPS were detected using biochemical kits (E-BC-K002-M, Elabscience) and rat ELISA kits (ELK10380/ELK8720, ELK Biotechnology) respectively. The specific operation steps were carried out in accordance with the product instructions. Serum oxidative stress levels were evaluated by quantifying malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), using the Kit-48/96 assay kit (E-EL-0060/E-BC-K020-M/E-BC-K096-M) acquired from Elabscience (Wuhan, China) following the manufacturer's protocol.

2.8 Histomorphology analysis

Duodenum, jejunum, ileum, cecum and colon tissues were fixed in 4% paraformaldehyde overnight. Hematoxylin and Eosin (H&E), Alcian Blue-Periodic Acid-Schiff (AB-PAS), and mucin2 (MUC2) staining were conducted by Wuhan Servicebio Technology Co., Ltd. These sections were scanned in the Panoramic MIDI digital section scanner, photos were taken with the Panoramic Scanner software, and the images were analyzed with the CaseViewer 2.4.0 software.

2.9 16S rRNA profiling of the gut microbiota

2.9.1 DNA extraction and PCR amplification

Microbial DNA was extracted from rat pup samples using the YH-Soil FastPure Soil DNA Isolation Kit (MJYH, Shanghai, China) according to manufacturer's protocols. The final DNA concentration and purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific,

Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR reactions were conducted using the following program: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C, and a final extension at 72°C for 10 min. PCR reactions were performed in triplicate 20 µL mixture containing 10 µL of 2 × Pro Taq, 0.8 µL of each primer (5 µM), 10 ng of template DNA and supplement ddH₂O to 20 µL. The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol.

2.9.2 Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.9.3 Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window. (ii) Primers were exactly matched allowing 2 nucleotide mismatching, and reads containing ambiguous bases were removed. (iii) Sequences whose overlap longer than 10 bp were merged according to their overlap sequence.

Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (<http://rdp.cme.msu.edu/>) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%. The data were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com).

2.10 Statistical analysis

Results are shown as the mean ± SD. The multiple comparisons were assessed by one-way ANOVA with Tukey's multiple comparisons test among three groups. A two-tail unpaired *t*-test was performed between the two groups. *P* values of < 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 8.0 and Excel 2019.

3. Results

3.1 LPS-induced intestinal inflammation in rat pups

3.1.1 LPS induced diarrhea and body weight loss in rat pups

On postnatal day 21, rat pups were intraperitoneally injected with 2.0 mg kg⁻¹ body weight LPS. Samples were collected at 2, 4, 6 and 8-hour intervals post-injection. As depicted in **Fig. 2**, compared with

the control (PBS), the body weight of rat pups in the LPS gradually decreased. Notably, their body weight significantly decreased to (53.8 ± 0.9) g by 8 hours post-injection ($P < 0.05$). Initial clinical manifestations observed at 2 hours included fecal softening, reduced motor activity and painful behavior. Subsequent observations at the 8-hour timepoint revealed that the rat pups were sluggish in response, curled and shivering, had no voluntary activities, had moist fur, red and swollen anus, and unformed feces. The results show that intraperitoneal injection of LPS can lead to weight loss and diarrhea in rat pups, thus proving the successful setting up of the LPS-induced rat model for this study.

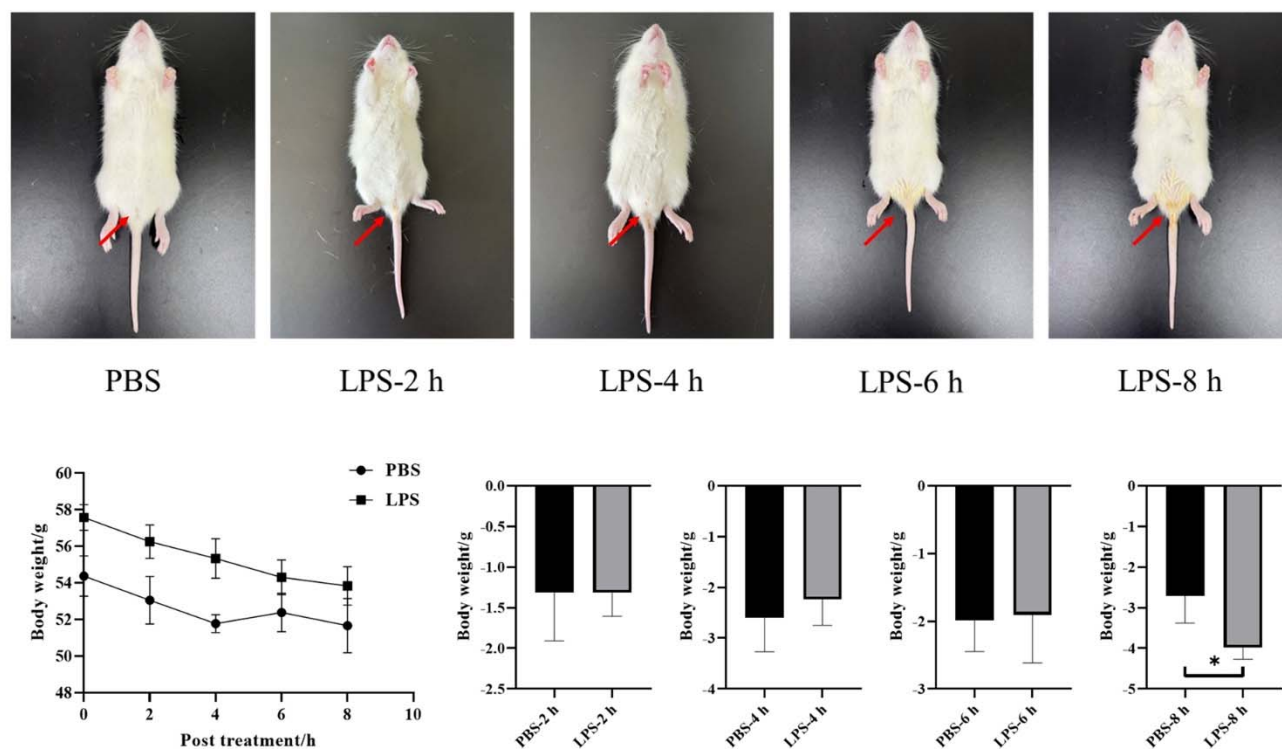


Fig. 2 Effect of LPS on diarrhea and body weight in rat pups (N = 6).

3.1.2 LPS induced inflammatory responses in the duodenum

The effect of LPS on the mRNA expression levels of inflammatory cytokines in the duodenum of rat pups is shown in **Fig. 3A**. Two hours post-LPS stimulation, the mRNA expression levels of *IL-1 β* , *IL-6*, *IFN- γ* , *TNF- α* , and *IL-10* in the duodenum were significantly elevated ($P < 0.001$). By six hours, *IL-6* and *IL-10* expression remained markedly higher than in controls ($P < 0.001$). Notably, *TNF- α* and *IL-10* expression was significantly upregulated from 2 to 6 hours post-treatment ($P < 0.05$), indicating sustained LPS-induced transcriptional activation. These findings demonstrate that LPS triggers an acute inflammatory response in the duodenum and induces prolonged *TNF- α* and *IL-10* expression in rat pups.

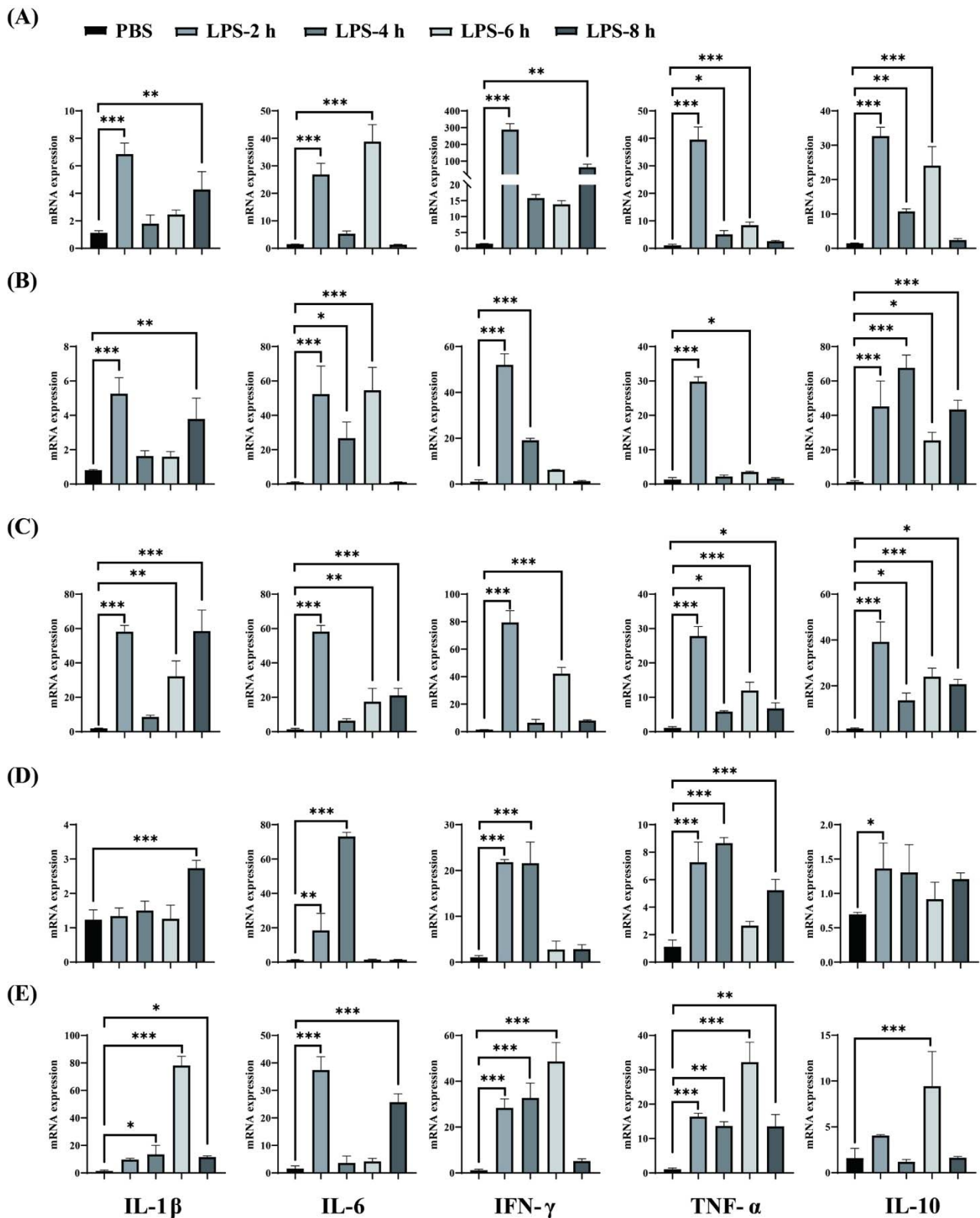


Fig. 3 Effect of LPS on the mRNA expression levels of inflammatory cytokines in the duodenum (A), jejunum (B), ileum (C), cecum (D) and colon (E). mRNA expression indicates the difference of the target gene in the treatment group compared with that in the PBS group. Significance analysis is to compare the mean of each group with the mean of the PBS group.

Data were represented as mean $\pm$ SD (N = 3). * P < 0.05, ** P < 0.01, *** P < 0.001.

3.1.3 LPS induced inflammatory responses in the jejunum

The effect of LPS on the mRNA expression levels of inflammatory cytokines in the jejunum of rat pups is shown in **Fig. 3B**. At 2 hours post-LPS administration, the mRNA expression levels of *IL-1 β* , *IL-6*, *IFN- γ* ,

TNF- α , and *IL-10* in the jejunum were markedly elevated ($P < 0.001$). By 4 hours, *IL-10* expression showed further significant upregulation ($P < 0.001$). A significant increase in *IL-6* expression was observed at 2, 4, and 6 hours ($P < 0.05$), while *IL-10* remained elevated at 8 hours ($P < 0.05$). These results indicate that LPS induces a time-dependent inflammatory response in the jejunum of rat pups, with peak effects occurring at 2 hours post-treatment.

3.1.4 LPS induced inflammatory responses in the ileum

The effect of LPS on the mRNA expression levels of inflammatory cytokines in the ileum of rat pups is shown in **Fig. 3C**. Compared with the PBS group, *IL-1 β* and *IL-6* expression exhibited significant upregulation at 2, 6, and 8 hours post-LPS administration ($P < 0.01$). Similarly, the mRNA expression level of *IFN- γ* was markedly elevated at 2 and 6 hours ($P < 0.001$). Notably, *TNF- α* and *IL-10* expression demonstrated significant elevation from 2 to 8 hours following LPS challenge ($P < 0.05$). These results demonstrate that LPS triggers a robust inflammatory response in the ileum, characterized by persistent *TNF- α* and *IL-10* induction throughout the experimental period.

3.1.5 LPS induced inflammatory responses in the cecum

The effect of LPS on the mRNA expression levels of inflammatory cytokines in the cecum of rat pups is shown in **Fig. 3D**. Cecal *IL-6*, *IFN- γ* , *TNF- α* , and *IL-10* expression showed significant upregulation at 2 hours post-LPS administration ($P < 0.05$). This response intensified by 4 hours, with *IL-6*, *IFN- γ* , and *TNF- α* all demonstrating marked elevation ($P < 0.001$). While *TNF- α* remained significantly elevated at 8 hours ($P < 0.001$), neither *IL-1 β* nor *IL-10* exhibited significant changes throughout the 2-6 hours and 4-8 hours respectively ($P > 0.05$). These findings reveal a selective inflammatory response in the cecum, characterized by sustained *TNF- α* induction and slight altered *IL-1 β* and *IL-10* expression.

3.1.6 LPS induced inflammatory responses in the colon

The effect of LPS on the mRNA expression levels of inflammatory cytokines in the colon of rat pups is shown in **Fig. 3E**. At 2 hours post-LPS administration, colonic *IL-6*, *IFN- γ* , and *TNF- α* mRNA levels showed marked elevation ($P < 0.001$). This response persisted at 6 hours, with significant upregulation of *IFN- γ* and *TNF- α* ($P < 0.001$). By 6 hours, *IL-1 β* and *IL-10* expression exhibited robust increases ($P < 0.001$). Notably, *TNF- α* expression remained consistently elevated throughout the 2-8 hours observation period ($P < 0.01$). These findings demonstrate that LPS induces a sustained inflammatory response in the colon, with particularly persistent upregulation of *TNF- α* expression.

3.2 Effects of *Bifidobacterium breve* on body weight and organ indices in rat pups

As depicted in **Fig. 4A**, the *B. breve*-treated groups showed no obvious increase in body weight and was almost in line with the PBS group. The organ indices for the liver, spleen, kidneys and thymus are shown in **Fig. 4B-E**. The organ indices for the liver, spleen, and thymus in the LPS exhibited a decrease relative to the PBS group, while the kidney index showed an increase compared to the PBS group. Compared to LPS group, treatment with *B. breve* CCFM1300 and CCFM1025 increased the spleen indices,

and *B. breve* CCFM683 and *B. breve* CCFM1198 resulted in increased kidney indices. Furthermore, treatment with *B. breve* CCFM683 increased the thymus indices. However, all statistical analysis revealed no significant differences among the groups.

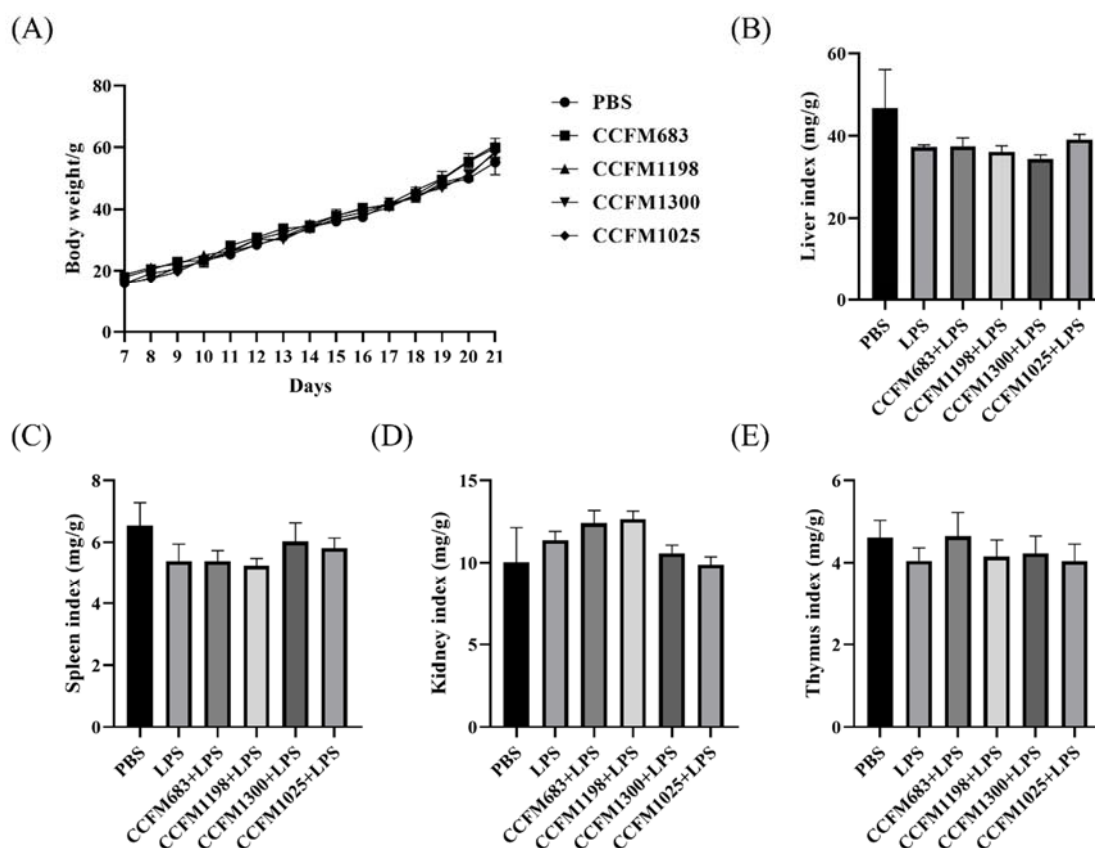


Fig. 4 *Bifidobacterium breve* effect on the body weight and organ indices. (A) body weight; (B) liver indices; (C) spleen indices; (D) kidney indices; (E) thymus indices. Data were represented as mean $\pm$ SD from four animals of each group. Organ indices were measured using the formula = organ weight (mg)/body weight (g).

3.3 *Bifidobacterium breve* maintains the integrity of intestinal mucosal morphology

Histological evaluation of intestinal segments in rat pups following *B. breve* intervention was performed using H&E staining. Compared with the PBS group, LPS challenge induced villous disappearance or damage in the small intestine of rat pups, while *B. breve* CCFM683 effectively attenuated LPS-induced intestinal injury (**Fig. 5**). Furthermore, LPS administration significantly reduced villus height and crypt depth in both duodenum and jejunum ($P < 0.001$, **Fig. 6A and B**). All strains significantly increased duodenal villus height and crypt depth compared to LPS group ($P < 0.001$, **Fig. 6A**). They also markedly enhanced jejunal crypt depth ($P < 0.001$) but reduced ileal villus length ($P < 0.05$) (**Fig. 6B and C**). These findings demonstrate that *B. breve* interventions exerted consistent protective effects against LPS-induced duodenal damage, while showing strain-dependent variations in jejunal and ileal injury mitigation. Histological examination of colon tissue revealed that *B. breve* CCFM683 and CCFM1198 provided protection against LPS-induced colonic damage. In contrast, strains CCFM1300 and CCFM1025 showed negligible ameliorative effects on colonic injury.

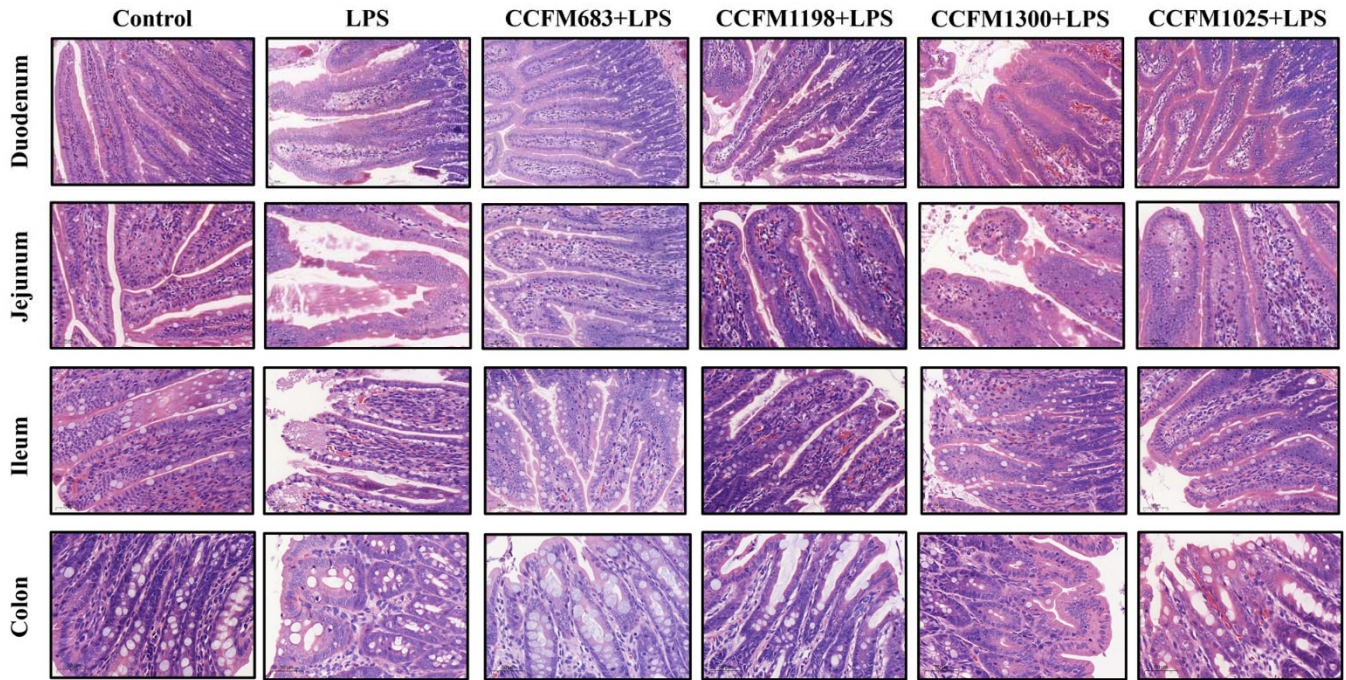
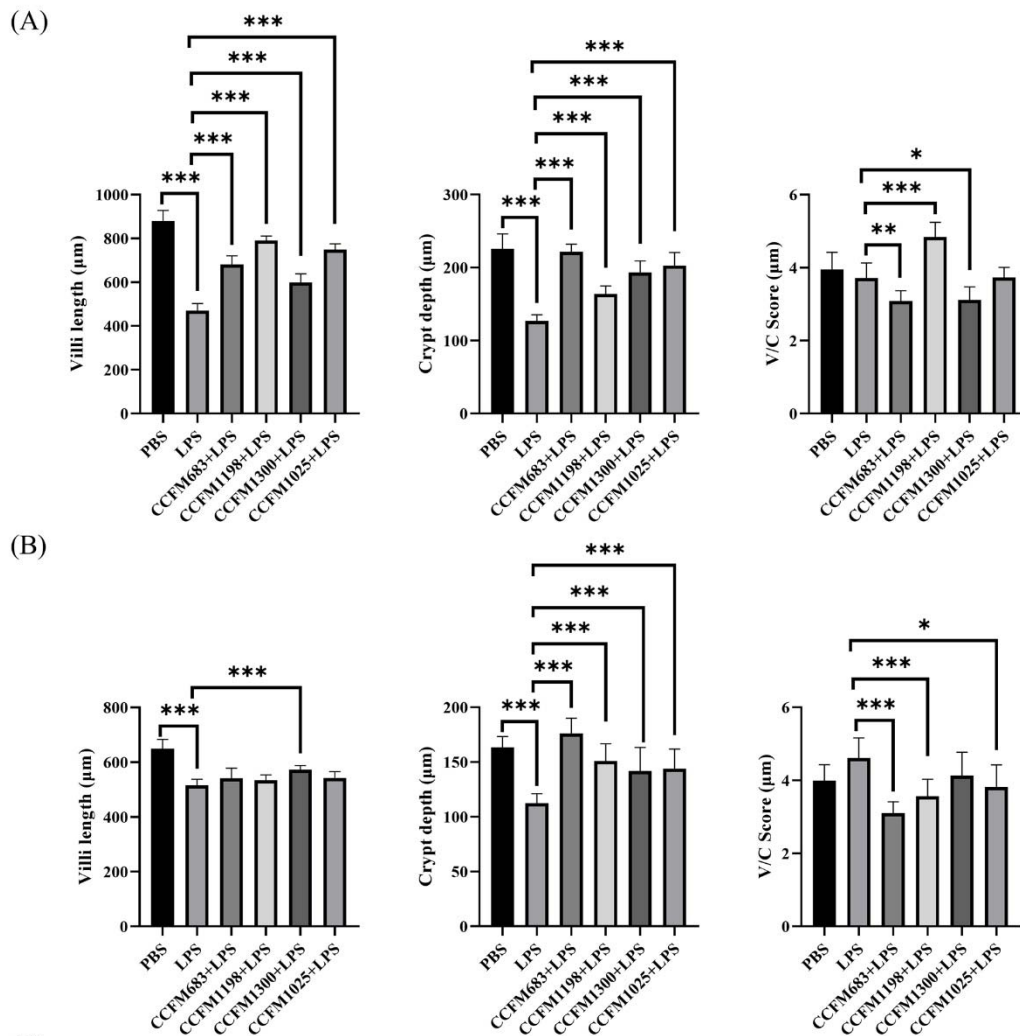


Fig. 5 Intestinal tissues stained with H&E for histological analysis. Representative images of the duodenum, jejunum, ileum and colon are shown, with a scale of 50 μm.



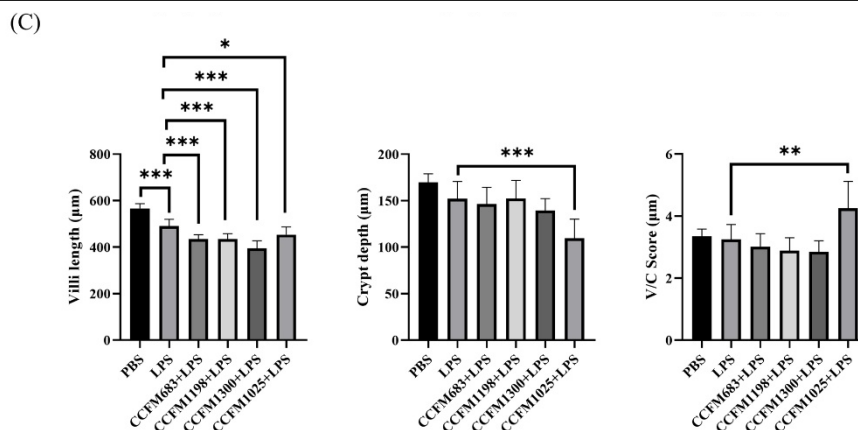


Fig. 6 Histological indexes of duodenum (A), jejunum (B), and ileum (C). Significance analysis is to compare the mean of each group with the mean of the LPS group. Data were represented as mean $\pm$ SD (N = 8). * P < 0.05, ** P < 0.01, *** P < 0.001.

3.4 *Bifidobacterium breve* alleviates LPS-induced intestinal inflammatory responses

To investigate the role of CLA-producing *B. breve* in regulating intestinal health and preventing LPS-induced inflammation, rat pups were supplemented with strains of *B. breve* with different CLA-producing abilities (Table 3). As shown in Fig. 7, *B. breve* CCFM683 and CCFM1198 markedly downregulated the mRNA expression of *IL-1 β* , *IL-6*, and *IFN- γ* in the duodenum (P < 0.001, Fig. 7 A). Furthermore, *B. breve* CCFM683 demonstrated a marked downregulation of *TNF- α* mRNA expression in the duodenum (P < 0.001), and *B. breve* CCFM1198 exhibited significant upregulation of *IL-10* mRNA expression in the duodenum (P < 0.001). *B. breve* CCFM1300 and CCFM1025 demonstrated marked upregulation of multiple pro-inflammatory cytokines at transcriptional levels, with a concomitant increase in *IL-10* expression (Fig. 7A). This comparative analysis demonstrates the superior immunomodulatory efficacy of *B. breve* CCFM683 and CCFM1198 in the duodenum compared with CCFM1300 and CCFM1025 strains. *B. breve* CCFM683 and CCFM1198 significantly downregulated the mRNA expression levels of *IL-6*, *IFN- γ* , and *TNF- α* in the jejunum while enhancing *IL-10* mRNA expression (Fig. 7B). Furthermore, *B. breve* CCFM1198 suppressed the transcriptional level of *IL-1 β* in the jejunum. In contrast, *B. breve* CCFM1300 and CCFM1025 markedly increased jejunal *IFN- γ* mRNA expression while upregulating *IL-10* transcriptional expression (P < 0.001, Fig. 7B). These findings indicate that *B. breve* CCFM1198 demonstrates superior immunomodulatory efficacy in the jejunum, followed by *B. breve* CCFM683. *B. breve* CCFM683 and CCFM1198 downregulated the mRNA expression levels of *IL-1 β* , *IFN- γ* , and *TNF- α* in the ileum while elevating *IL-10* mRNA expression (Fig. 7C). In contrast, *B. breve* CCFM1025 upregulated the transcriptional levels of these pro-inflammatory cytokines. This indicates that *B. breve* strains with high CLA bioconversion capacity exhibit superior regulatory efficacy in the ileum.

Similarly, *B. breve* CCFM683 downregulated mRNA expression of *IL-1 β* , *IL-6*, *IFN- γ* , and *TNF- α* in both the cecum and colon, while elevating *IL-10* mRNA expression (Fig. 7D and E). Conversely, *B. breve* CCFM1300 and CCFM1025 upregulated transcriptional levels of specific pro-inflammatory cytokines in the cecum and colon. Moreover, *B. breve* CCFM683 not only modulated inflammatory cytokine transcriptional

levels in intestinal tissues, but also reduced serum concentrations of IL-1 β , IL-6, and IFN- γ , while significantly increasing serum IL-10 levels ($P < 0.01$, **Fig. 8**). Collectively, *B. breve* CCFM683 exhibits comprehensive immunomodulatory effects with more pronounced efficacy observed in the cecum and colon. And, *B. breve* CCFM683 is superior to *B. breve* CCFM1198, CCFM1300 and CCFM1025 in ameliorating symptoms of LPS-induced inflammation.

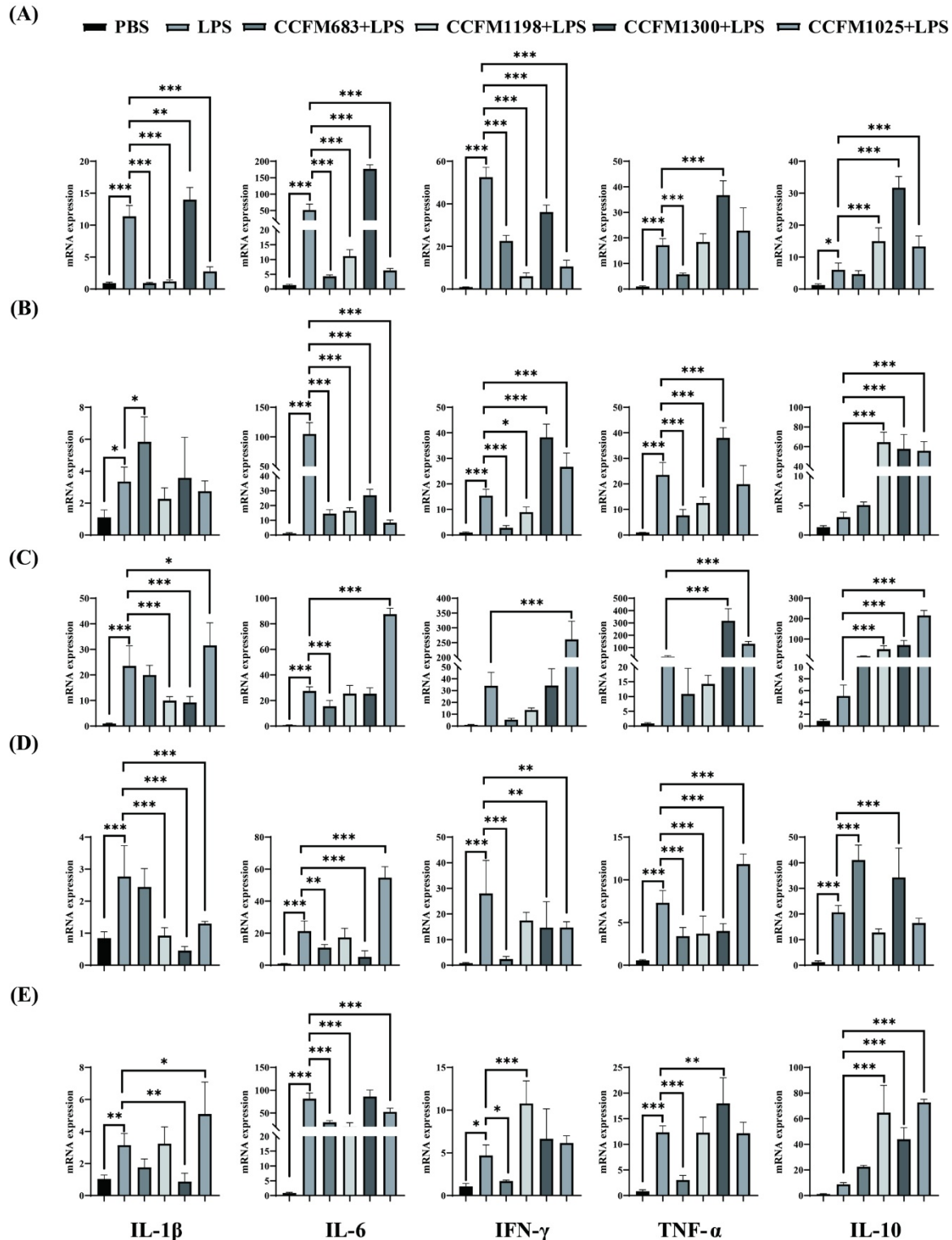


Fig. 7 *Bifidobacterium breve* effect on LPS-induced inflammation in the rat pups. Effect of *B. breve* on the mRNA expression levels of inflammatory cytokines in the duodenum (A), jejunum (B), ileum (C), cecum (D), and colon (E). mRNA expression indicates the difference of the target gene in the treatment group compared with that in the PBS group. Significance analysis is to compare the mean of each group with the mean of the LPS group. Data were represented as mean $\pm$ SD (N = 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

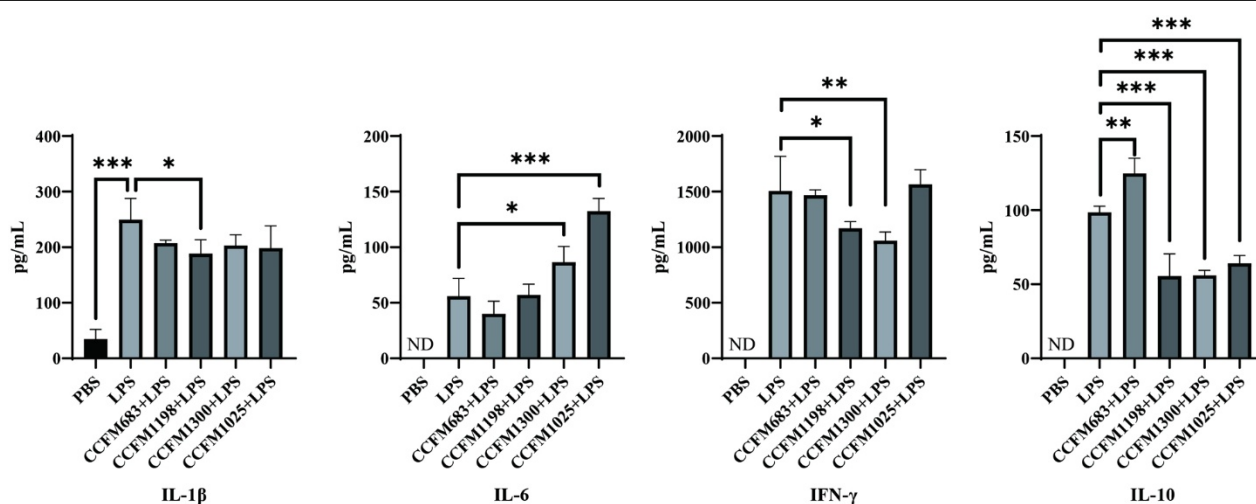


Fig. 8 The influence of *B. breve* on the levels of serum inflammatory cytokines. ND, not detected. Significance analysis is to compare the mean of each group with the mean of the LPS group. Data were represented as mean $\pm$ SD (N = 4). * P < 0.05, ** P < 0.01, *** P < 0.001.

3.5 *Bifidobacterium breve* CCFM683 protects intestinal barrier function

To evaluate the safety of *B. breve* CCFM683 and its protective mechanisms on the intestine, we assessed serum biochemical parameters, intestinal permeability, oxidative stress-related markers, and intestinal mucosal barrier. Analysis of serum biochemical parameters in rat pups revealed differences in the levels of triglycerides (TG), urea, glucose (Glu), albumin (ALB), and alanine aminotransferase (ALT) among the PBS, LPS, and CCFM683+LPS groups. Compared with the PBS group, the serum Glu level was significantly decreased in the LPS group (P < 0.05). In contrast, the CCFM683+LPS group exhibited a significant increase in ALB levels compared to the LPS group (P < 0.05) (**Table 5**). Furthermore, no significant differences were found in multiple serum biochemical parameters between the CCFM683+LPS group and the PBS group, indicating that *B. breve* CCFM683 had minimal impact on serum biochemical parameters and no adverse effects. AB-PAS staining and MUC2 immunostaining revealed that LPS stimulation led to a decrease in colonic mucin, and *B. breve* CCFM683 was able to reverse the LPS-induced reduction in MUC2 expression (**Fig. 9A and B**). The indicators of intestinal permeability are shown in **Fig. 10A**. Compared with the PBS group, serum levels of D-LA, DAO, and LPS were significantly elevated in the LPS group (P < 0.05), while CCFM683 administration significantly reduced these levels (P < 0.05), indicating that *B. breve* CCFM683 can suppress the LPS-induced increase in intestinal permeability. Moreover, measurements of serum MDA, SOD, and GSH-Px levels showed no significant differences in MDA level among the different groups (**Fig. 10B**). Compared to the LPS group, the CCFM683+LPS group significantly increased SOD (P < 0.05) and GSH-Px (P < 0.01) levels (**Fig. 10B**), suggesting that CCFM683 possesses redox regulatory capacity.

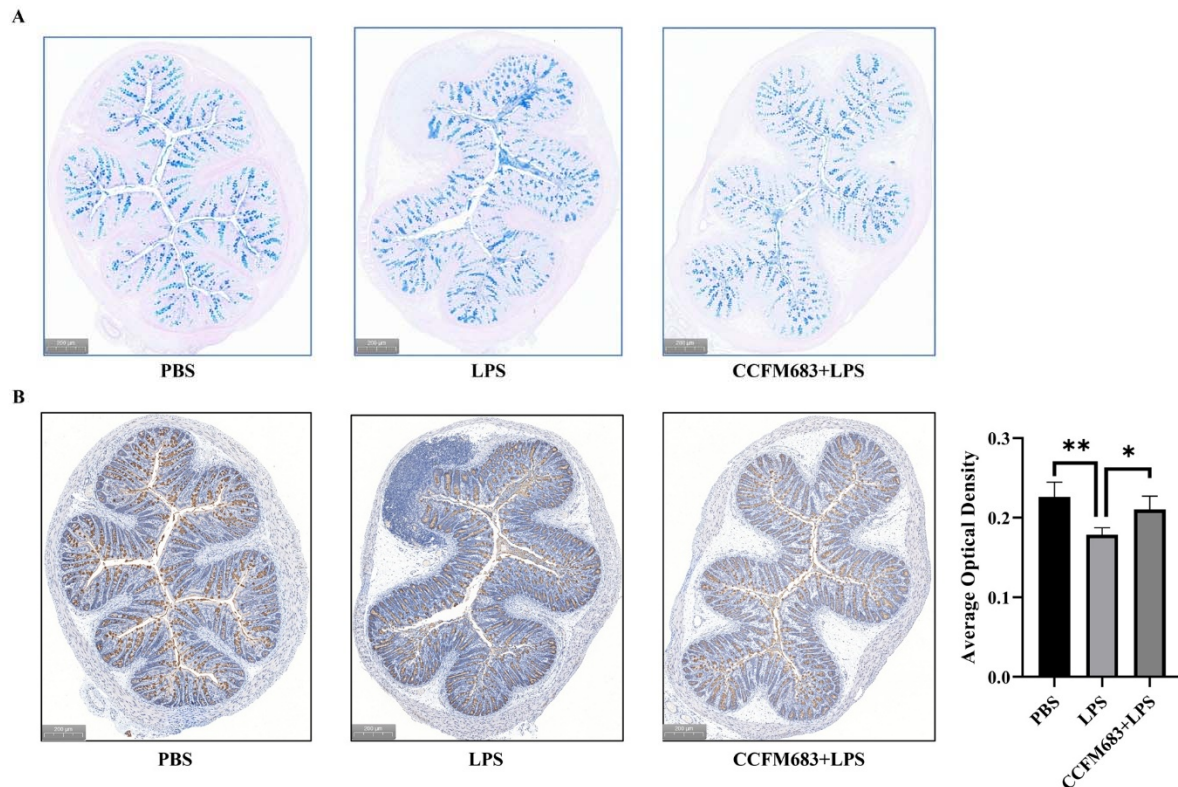


Fig. 9 *Bifidobacterium breve* CCFM683 protects the intestinal mucosal barrier. Representative images of the colon are shown, with a scale of 200 μm . (A) AB-PAS staining. (B) MUC2 immunostaining. Significance analysis is to compare the mean of each column with the mean of every other column. Data were represented as mean $\pm$ SD (N = 4). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

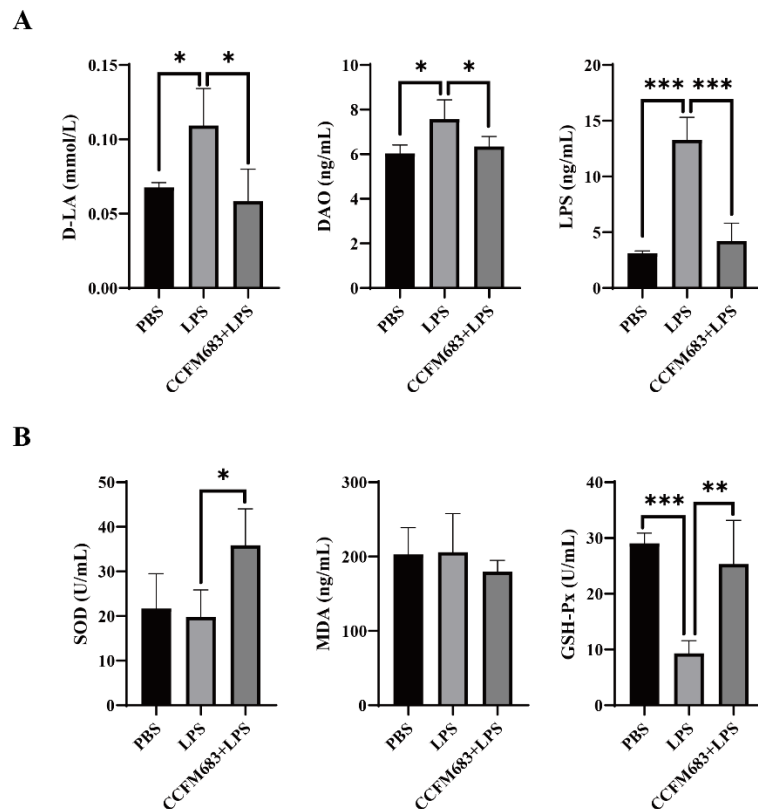


Fig. 10 *Bifidobacterium breve* CCFM683 improves intestinal permeability and oxidative stress. The indicators of intestinal permeability include D-LA, DAO, and LPS (A). Oxidative stress-related markers include SOD, MDA, and GSH-Px (B). Significance analysis is to compare the mean of each column with the mean of every other column. Data were represented as mean $\pm$ SD (N = 4), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5 Serum biochemical indicators

Biochemical indicators	PBS group	LPS group	CCFM683+LPS group
TC (mmol/L)	3.76 ± 0.46	3.34 ± 0.59	3.09 ± 0.26
TG (mmol/L)	0.52 ± 0.01 ^b	1.35 ± 0.70 ^{ab}	1.83 ± 0.50 ^a
CREA (μmol/L)	47.97 ± 2.57	47.46 ± 4.11	51.95 ± 10.09
UREA (mmol/L)	3.92 ± 0.29 ^b	5.03 ± 1.20 ^{ab}	5.84 ± 0.94 ^a
LDL-C (mmol/L)	2.17 ± 0.30	2.01 ± 0.38	1.90 ± 0.17
AST (U/L)	104.60 ± 7.26	111.69 ± 18.96	151.40 ± 44.44
HDL-C (mmol/L)	0.48 ± 0.06	0.40 ± 0.13	0.41 ± 0.02
GLU (mmol/L)	8.50 ± 1.57 ^a	5.61 ± 1.98 ^b	7.33 ± 1.16 ^{ab}
ALB (g/L)	13.50 ± 1.57 ^{ab}	12.05 ± 2.30 ^b	15.15 ± 0.99 ^a
TP (g/L)	38.80 ± 3.52	35.08 ± 7.11	43.20 ± 2.80
ALT (U/L)	17.67 ± 1.15 ^b	20.94 ± 4.02 ^{ab}	25.08 ± 2.20 ^a
IgA (g/L)	0.41 ± 0.01	0.41 ± 0.01	0.41 ± 0.01
IgM (g/L)	0.20 ± 0.00	0.20 ± 0.00	0.21 ± 0.00
IgG (g/L)	1.66 ± 0.09	1.48 ± 0.10	1.68 ± 0.17
CRP (mg/L)	3.05 ± 0.06	3.08 ± 0.05	3.08 ± 0.05

Different letters in the same row indicate significant differences, with $P < 0.05$ (N=4)

3.6 *Bifidobacterium breve* CCFM683 enhances conjugated linoleic acid level in the intestinal content

Treatment with *B. breve* CCFM683 showed no significant effects on palmitic acid (PA), stearic acid (SA), oleic acid (OA), LA, and α -linoleic acid (ALA) levels in the small intestine, cecum, and colon contents, but significantly increased the CLA level in both the cecum and colon (**Fig. 11A and B**). Compared to the PBS group, the LPS group exhibited a significant increase in CLA level in cecal contents but a decrease in colonic contents (**Fig. 11B**). In the cecum, the CLA level in the LPS group was (20.88 ± 5.58) $\mu\text{g/g}$ sample, approximately 2.6-fold higher than that in the PBS group [(8.00 ± 1.37) $\mu\text{g/g}$ sample] (**Fig. 11B**). As to the colon, the CLA level in the LPS group was (0.37 ± 0.05) mg/g sample, lower than that in the PBS group (0.47 ± 0.03) mg/g sample, indicating that LPS-induced intestinal inflammation altered CLA levels in both cecal and colonic contents. Compared to the LPS group, CCFM683 treatment significantly improved colonic CLA level ($P < 0.001$), increasing from (0.37 ± 0.05) mg/g sample to (0.62 ± 0.09) mg/g sample (**Fig. 11B**).

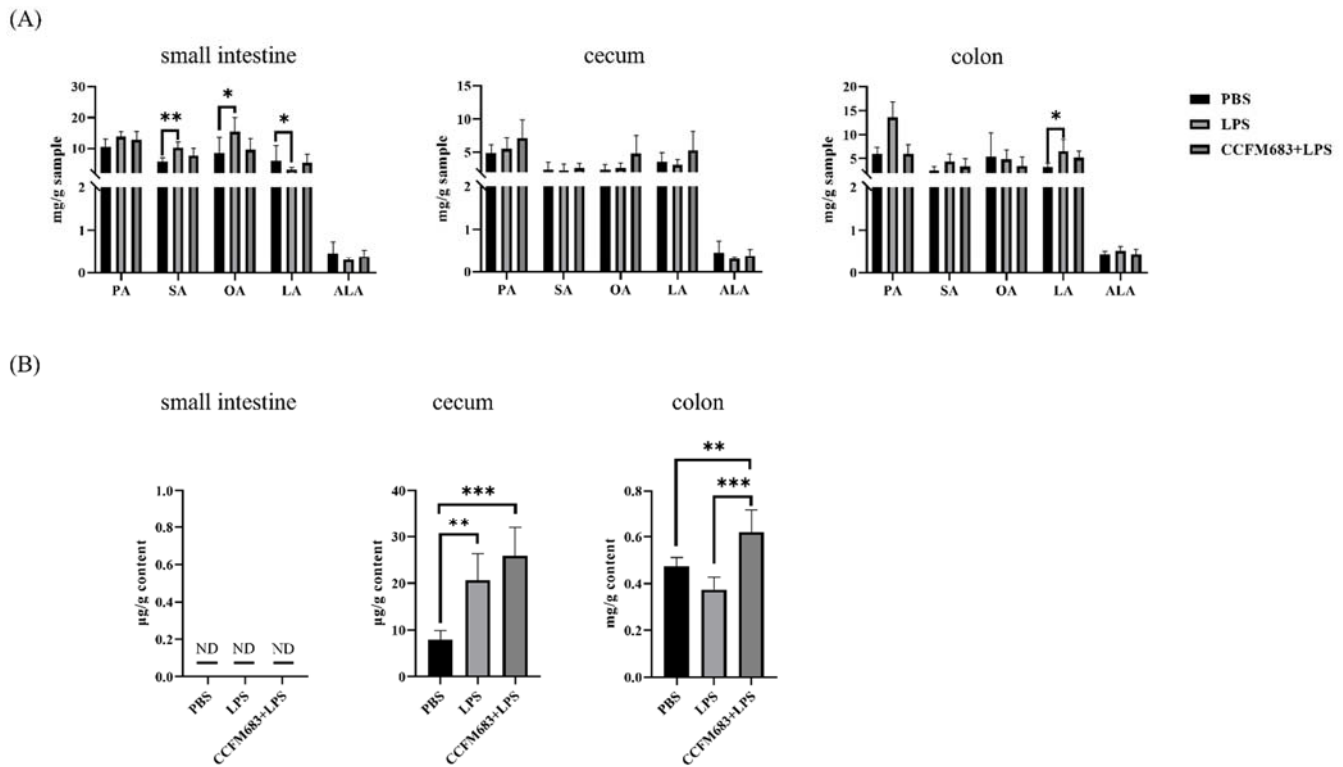


Fig. 11 *Bifidobacterium breve* CCFM683 improves conjugated linoleic acid level in the intestinal content. (A) Levels of PA, SA, OA, LA, and ALA in the content of small intestine, cecum and colon of rat pups, measured by GC-MS. (B) Detection of CLA in the content of small intestine, cecum and colon of rat pups, measured by LC-MS. ND, not detected. Data were represented as mean $\pm$ SD (N = 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.7 Probiotic function of *Bifidobacterium breve* CCFM683 in hindgut

We analyzed 16S rRNA gene data from 9 fecal samples across 3 groups (PBS, LPS, and CCFM683+LPS) to evaluate microbial diversity changes. **Fig. 12A-C** presents the Shannon, Chao, and ACE diversity indices across all groups, showing differences between the control and treatment groups. Additionally, metrics such as Shannon, Chao, and ACE indices indicated significant differences in alpha diversity between the LPS group and the PBS group ($P < 0.05$). In contrast, there are no substantial changes in alpha diversity between the PBS and CCFM683+LPS groups ($P < 0.05$), suggesting that treatment with *B. breve* CCFM683 can improve LPS-induced intestinal microbiota imbalance. For beta diversity, PCoA plot and NMDS analysis using the Bray-curtis distance matrix revealed clustering within the all groups (**Fig. 12D and E**), indicating that the PBS group and the CCFM683+LPS group had more similar species compositions. These findings suggest that CCFM683 treatment can improve the changes in intestinal microbiota diversity caused by LPS.

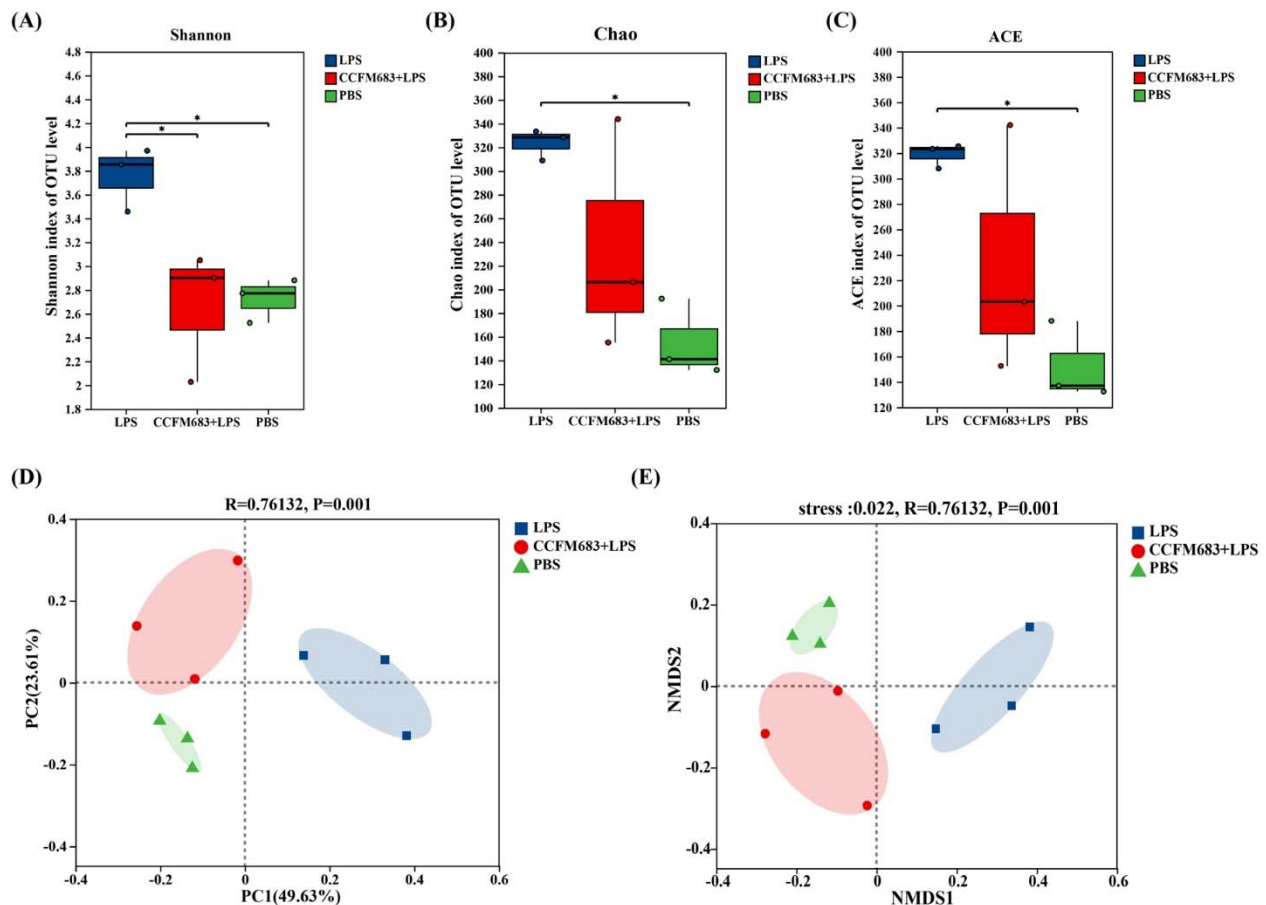


Fig. 12 Analysis of bacterial community diversity. The alpha diversity and richness of bacterial communities across the PBS, LPS and CCFM683-treated groups are evaluated using the Shannon (A), Chao (B), and ACE indices (C). The beta diversity of bacterial communities across the various groups was evaluated using the PCoA plot (D) and NMDS of Bray-curtis dissimilarity (E). Statistical significance was determined using a non-parametric Kruskal-Wallis test followed by a Tukey *post hoc* test.

To further investigate the changes in gut microbiota dynamics between the LPS-treated and CCFM683-administered groups, we analyzed the gut microbiota composition. The analysis revealed that *Bacillota* (51.11%-72.09%) and *Bacteroidota* (26.67%-45.76%) were the most common phyla in the samples, with *Bacillota* being particularly prevalent in the CCFM683+LPS groups, while *Bacteroidota* were more abundant in the PBS and LPS groups (**Fig. 13A and B**). In the PBS group, the predominant genera were *norank_f_Muribaculaceae* (19.98%) and *Limosilactobacillus* (17.62%). These abundances decreased in the LPS group. In contrast, the CCFM683-treated groups showed higher relative abundances of *Limosilactobacillus* (14.25%) compared to the LPS groups. Specifically, the abundances of *Ligilactobacillus* in the PBS and CCFM683+LPS groups were 11.36% and 16.25%, respectively. The heatmap presented in **Fig. 13C**, generated through cluster analysis, revealed that the fecal microbiota in the CCFM683-treated group and PBS group primarily clustered around the genera *Limosilactobacillus*, and *Ligilactobacillus*. Given the substantial individual variation in gut microbiota, these results represent preliminary experimental findings.

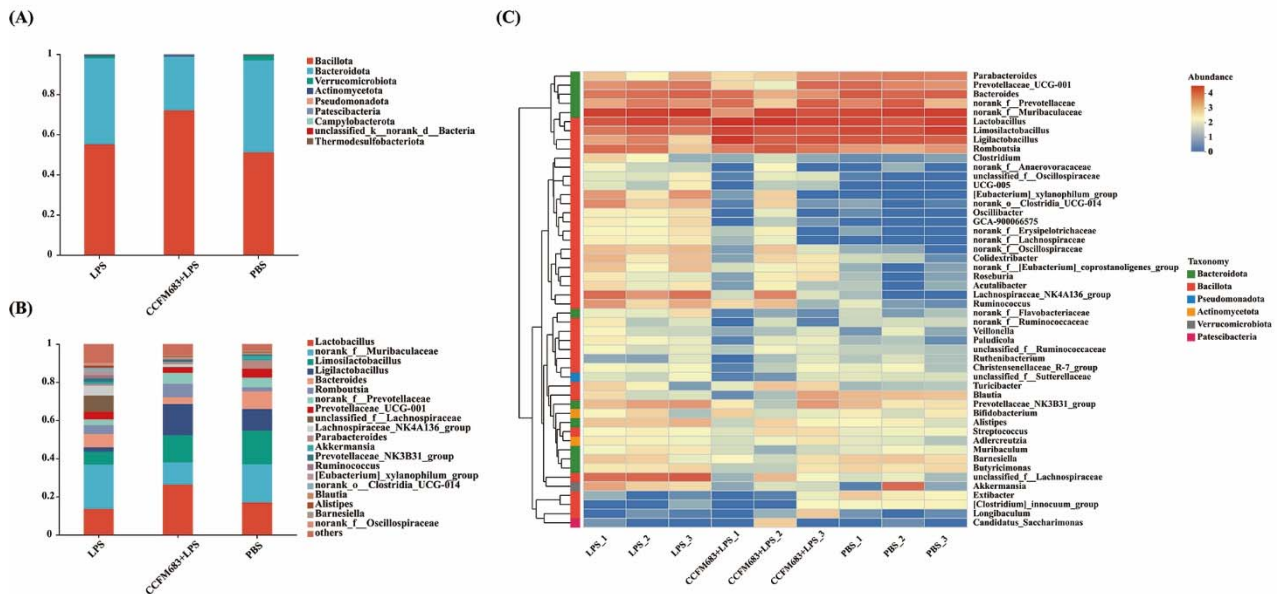


Fig. 13 Comparison of the relative abundances of gut microbiota taxa among the PBS, LPS, and CCFM683-treatment groups. The gut microbiota composition is analyzed at the phylum level (A) and the genus level (B). A heatmap highlights the most common genera with different expression levels in the gut microbiota (C).

4. Discussion

To investigate the impact of CLA-producing *B. breve* on intestinal immunity in rat pups, LPS was used to establish intestinal inflammation models. LPS can induce systemic and localized inflammatory responses and is widely used in inflammation models and immunocompromised models, which made it an important tool for testing interventions aimed at restoring immune and intestinal homeostasis [23, 24]. Following LPS challenge, significant alterations in mRNA transcript levels of multiple inflammatory cytokines (IL-1 β , IL-6, IFN- γ , TNF- α , and IL-10) were detected throughout the duodenum, jejunum, ileum, cecum, and colon. Furthermore, temporal variations in response to LPS stimulation were observed across different intestinal segments, indicating intestinal segment-specific immune responses. It should be emphasized that the dosage of intraperitoneal LPS administration is critical for model establishment. Substantial differences in LPS tolerance were observed across rodent strains and developmental stages. Xiao *et al.* [25] established a model by intraperitoneally administering 10.0 mg/kg body weight to C57BL/6 mice (male, 4 weeks old), whereas Abbas *et al.* [26] employed a dosage of 2.0 μ g/g body weight in C57BL/6 mice (male, 6 weeks old) as reported in their study on mulberry-derived postbiotics alleviating intestinal inflammation. In contrast, the present study selected an optimal dose of 2.0 mg/kg body weight for Wistar rats (3 weeks old) based on preliminary experiments. Compared to adult rats, rat pups exhibit greater sensitivity in their immune response to exogenous LPS. Under normal physiological conditions, the concentration of LPS in plasma remains extremely low and does not induce inflammatory responses [27, 28]. When exogenous LPS injection disrupts this balance, the level of LPS in the bloodstream rises sharply, provoking a potent immune response. Rat pups demonstrate lower tolerance in this context, so a lower dose can induce a severe inflammatory response.

Intestinal inflammation impacts body weight and organ indices. The change in body weight is closely correlated with alterations in the organism's nutritional status and physiological functions [29]. Within 2-8 hours after intraperitoneal LPS injection, the body weight of rat pups in both the LPS and PBS groups showed a decreasing trend. This is attributed to the fasting implemented following the LPS injection. An additional reason is that LPS induces severe diarrhea and fluid loss, resulting in a significant decrease in body weight at the 8 h compared to the PBS group. The liver, spleen, thymus, and kidneys are crucial components of the immune system and are essential for regulating immune responses [30-32]. They play a significant role in the development of immune cells, the production of immune factors, and immune regulation. Under normal physiological conditions, their values remain relatively constant. However, when organs are affected by chemical compounds, phenomena such as organ congestion, edema, hyperplasia, hypertrophy, and even tissue apoptosis may occur, thereby altering the organ indices [33]. Our findings indicate that these strains do not affect the normal growth and development of rat pups. Villus height and crypt depth are important indicators for assessing small intestinal function. CLA-producing *B. breve* strains can alleviate the LPS-induced reduction in duodenal villus height and crypt depth, and increase jejunal crypt depth, with strain CCFM683 exhibiting superior alleviative capacity, suggesting *B. breve* CCFM683 effectively protect the function of the small intestine.

During intestinal inflammation, pro-inflammatory and anti-inflammatory cytokines undergo continuous dynamic change. When their equilibrium is severely disrupted, it triggers immune system dysregulation. This imbalance manifests as pathological symptoms of varying severity, including epithelial barrier dysfunction and systemic inflammatory responses. LPS activates the immune system via the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and the MyD88-independent pathway [34, 35]. LPS induces inflammation by MyD88-dependent pathway via the toll-like receptor 4 (TLR4)-mediated signaling pathway and nuclear factor kappa-B (NF- κ B) activation, inducing the production of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α [36-39]. Dong et al. [4] confirmed that *B. longum* BL-10 significantly reduced the protein expression of TLR4 and NF- κ B p65, thereby reducing the inflammatory level in mice. The immunomodulatory mechanism of *B. breve* CCFM683 involves downregulating pro-inflammatory cytokine mRNA expression (*IL-1 β* , *IL-6*, *IFN- γ* , and *TNF- α*), upregulating anti-inflammatory cytokine transcript levels, reducing serum concentrations of pro-inflammatory cytokines, and elevating IL-10 levels in serum. The research of Qu et al. [40] also indicated that *B. breve* JSNJNM2 possessing strong IL-10-producing activity could alleviate dextran sulfate sodium-induced colitis symptoms in normal mice and exhibit potential functions in antibiotic cocktail-treated mice. Furthermore, *Bifidobacterium* can stimulate intestinal mucosal immune cells to secrete cytokines, with an important role in inducing proliferation and differentiation of immune cells and enhancing the immune response [41, 42].

B. breve CCFM683 can protect intestinal barrier function through multiple mechanisms. *B. breve* repairs LPS-induced intestinal damage by increasing MUC2 levels. MUC2 is mainly produced by goblet

cells and plays a crucial role in epithelial defense [43]. Intervention with CCFM683 elevates MUC2 expression, thereby enhancing the protective capacity of epithelial cells. This is consistent with the findings reported by Chen et al. [44], which demonstrated that at high doses (10^{10} CFU/d and 10^9 CFU/d), *B. breve* CCFM683 alleviated dextran sulfate sodium-induced colitis by upregulating MUC2 and tight junction protein concentrations. Serum D-LA, DAO, and LPS are three typical biomarkers of intestinal permeability. Xiao et al. [45] found that *Clostridium tyrobutyricum* ATCC25755 alleviated the LPS-induced increase in D-LA, DAO, and LPS levels in mice, which is consistent with our results. Oxidative stress is closely associated with inflammatory responses. SOD, MDA, and GSH-Px collectively constitute the endogenous antioxidant defense system and participate in immune regulation. SOD is an important antioxidant enzyme that can neutralize highly reactive superoxide radicals, thereby preventing oxidative stress and cell damage [46]. GSH-Px catalyzes the reaction of hydrogen peroxide with reduced glutathione to produce water and oxidized glutathione, thereby protecting the structure and function of cell membranes from peroxide-induced disruption and damage. Following intervention with *B. breve* CCFM683, the activities of SOD and GSH-Px were higher compared to the LPS group, indicating its potent antioxidant capacity. Overall, *B. breve* CCFM683 exerts anti-inflammatory effects via maintaining the homeostasis of the intestinal barrier, which is closely related to preserving colonic permeability, enhancing mucin expression, and elevating antioxidant levels.

In addition to microorganisms themselves, certain metabolites derived from both microbial and host sources can also modulate host's immunity [21, 47–49]. Previous studies have established that microbial CLA modulates innate immunity and adaptive immunity [2]. *B. breve* CCFM683 demonstrated a high CLA conversion rate (approximately 90.0%), which may contribute to its immunomodulatory efficacy. CLA exhibits multiple biological activities including anti-inflammatory, anti-carcinogenesis, anti-obesity, and anti-diabetic effects [9]. In the context of inflammation research, CLA was shown to suppress NF- κ B activation and promote IL-10 production through the extracellular regulated protein kinase pathway, and it functions as a PPAR- γ ligand to activate immune pathways and modulate macrophage polarization, thereby exerting immunomodulatory effects [12, 50]. Furthermore, dietary LA can be converted by gut microbiota into CLA, modulating CD4⁺ intraepithelial lymphocytes expressing CD8 α in the small intestine [21]. *B. breve* CCFM683 significantly increased CLA levels in colonic contents ($P < 0.001$), indicating its substantial conversion capacity within the intestinal tract, which may be the key process to exert its immunomodulatory effect. Chen et al. [44] reported that *B. breve* CCFM683 can reduce intestinal mucosal damage and prevent further deterioration of dextran sulfate sodium induced colitis. Further, Chen et al. [51] demonstrated that CLA and the CLA-producing gene were the key metabolites and genes for CCFM683 to prevent colorectal cancer. Our study investigated the effects of CLA-producing *B. breve* strains on intestinal immunity. However, the current study lacks investigation into the effect of *B. breve* concentration on LPS-induced intestinal inflammation, and we do not have a CCFM683 strain that is unable to biotransform CLA.

Therefore, future studies should explore the dose-response relationship between LPS and *B. breve* and whether CLA acts as a key metabolite mediating these immunomodulatory effects.

Intestinal inflammation can lead to gut microbiota dysbiosis, and supplementation with *Bifidobacterium* may facilitate the restoration of microbial homeostasis. Among different strains, *B. breve* CCFM683 demonstrates a more comprehensive immunomodulatory effect, which is particularly pronounced in the colon. *B. breve* CCFM683 treatment enriched beneficial gut microbiota, such as *Limosilactobacillus* and *Ligilactobacillus*, which suggests its immunoregulatory function is partially executed through microbiota remodeling. *Limosilactobacillus* and *Ligilactobacillus* are common probiotics, among which *Limosilactobacillus reuteri* and *Ligilactobacillus gasseri* are representative species. Numerous studies have investigated their effects on the control of intestinal flora, anti-inflammatory and intestinal barrier [52]. Previous studies have confirmed that *L. reuteri* could regulate immune responses of host and improve intestinal mucosal integrity [53, 54]. It has also been approved that *B. breve* has the functions of anti-infection and regulating host immune system [55], which is consistent with our research results. *B. breve* CCFM683 increased the relative abundance of *Limosilactobacillus* and *Ligilactobacillus*, at the same time, CCFM683 improved the symptoms of LPS-induced inflammation. These evidences helped us to conclude that CCFM683 altered the structure of the intestinal microbiota in rat pups, specifically increasing the abundance of *Limosilactobacillus* and *Ligilactobacillus* to regulate immune response. In fact, improving the immune response by controlling the intestinal micro-ecology is one of the important ways for *Bifidobacterium* to exert its immunomodulatory effect. Previous studies revealed that *Bifidobacterium* might indirectly modulate the immune system by interacting with gut microbiota. For example, Dong et al. [4] demonstrated that *B. longum* BL-10 increased the relative proportions of the Clostridia UCG-014 and Lachnospiraceae NK4A136 group while decreasing the proportions of Escherichia-Shigella to boost the intestinal immune. Another example, Huang et al. [54] suggested that breast-fed infants-derived *B. breve* FPHC4024 increased the abundance of *Verrucomicrobiaceae Akkermansia* and ameliorated dextran sulfate sodium-induced colitis in mice. Altogether, *B. breve* CCFM683 alleviates LPS-induced intestinal inflammation through immune modulation and gut microbiota remodeling.

5. Conclusion

The present study demonstrated the beneficial effects of CLA-producing *B. breve* on the intestine of rat pups. Administration of 10^9 CFU/d *B. breve* CCFM683 restored the integrity of intestinal mucosal morphology, and reduced the mRNA expression of pro-inflammatory cytokines (IL-1 β , IL-6, IFN- γ and TNF- α), it also increased production of the anti-inflammatory cytokine IL-10. Furthermore, *B. breve* CCFM683 improved CLA level in the cecum and colon, and it significantly promoted the proliferation of several beneficial friendly bacteria, as well as the adaptability of intestinal microbiota. Collectively, these findings provided theoretical foundation for CLA-producing *B. breve* as a protective agent for intestinal health early in life.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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