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Synthetic microbial consortia for the treatment of *Clostridioides difficile* infection in mice model

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Abstract

Clostridioides difficile infection (CDI) as of recent has become a great concern to the impact on human health due to its high hazardous risk and rate of recurrence. Live bacterial therapeutics is a promising method to treat or prevent CDI. Here, a synthetic microbial consortia (SMC) B10 was constructed using probiotic strains with antibacterial and anti-quorum sensing activities, and the therapeutic effect of SMC B10 against *C. difficile* infection was evaluated in vitro. Compared to the model group, the treatment of SMC B10 significantly increased the survival rate. The clinical signs of mice were significantly ameliorated, especially the cecum injury, while the secretion of pro-inflammatory associated cytokines such as IL-1α, IL-6, IL-17A and TNF-α was reduced, the expression of TLR4 was inhibited, which alleviated the inflammatory response, and the expression of the tight junction protein Claudin-1 was increased, ultimately promoting the recovery of host health. The treatment of B10 restored gut microbiota dysbiosis and led to a healthy intestinal microbiota structure, significantly improved alpha diversity, suppressing potentially harmful bacteria and restoring other core bacterial species. In conclusion, SMC B10 can effectively treat CDI through modulate gut microbiota and attenuate the inflammatory response.

INTRODUCTION

Clostridioides difficile is a Gram-positive, strictly anaerobic, conditionally pathogenic bacterium that can cause serious infections within the intestinal tract (Sandhu & McBride, 2018). *C. difficile* infection (CDI) is a severe contagious colitis that can cause pseudomembranous colitis, intense diarrhoea, and even death and has a global morbidity and mortality profile, especially in the United States, where it has become the most common healthcare-associated infection, with an increasing trend in the incidence of community-associated infections (Burke & Lamont, 2014; Guh & Kutty, 2018). It is commonly believed that the occurrence of CDI is associated with the disruption of intestinal microbiota due to the misuse of antibiotics, particularly clindamycin, broad-spectrum penicillins, cephalosporins and fluoroquinolones (Abad & Safdar, 2021). After the imbalance of intestinal colonies, endogenous or exogenous *C. difficile* proliferates and replaces the ecological niche of the original microbiota where it then produces toxins (TcdA and TcdB) and spores, leading to host disease and relapse (Baloh et al., 2022; Carter et al., 2015). TcdA and TcdB act on colonic epithelium and immune cells, both of which are capable of inducing host innate immunity and pro-inflammatory responses, but TcdB may be the trigger for multi-organ dysfunction syndrome (Carter et al., 2015; Steele et al., 2013). Meanwhile, *C. difficile* can acquire antibiotic resistance through biofilm formation, transfer of plasmids, etc., thus affecting treatment (Boekhoud et al., 2020; Peng et al., 2017). The dysbiosis of the gut microbiota results in a decrease in the

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production of short-chain fatty acids, and secondary bile acids, along with an increase in the production of primary bile acids, this in turn promotes the germination of *C. difficile* and aggravates the inflammatory process (Aguirre et al., 2021; Mullish et al., 2019).

Previously, treatment of CDI relied on antibiotics such as metronidazole, vancomycin and fidaxomicin, but with increasing resistance and the recurrence of CDI, faecal-microbial transplantation (FMT) is progressively becoming a more attractive alternative therapy for recurrent CDI. FMT is designed to treat CDI by restoring the diversity of the intestinal microbiota, rebuilding the intestinal mucosal barrier and recovering the metabolism of the microbiota (Mullish et al., 2019). FMT has been approved by FDA to treat recurrent CDI. Some research indicated that FMT not only can treat colitis-related diseases but it also has the potential to be applied to treat irritable bowel syndrome (El-Salhy et al., 2020), depression (Fond et al., 2020) and even cancer (Chen, Luan, et al., 2019; Chen, Wu, et al., 2019; Pernigoni et al., 2021). Nevertheless, the potential risks of FMT should be taken into consideration, especially in immunocompromised patients who are more likely to suffer infections after receiving FMT. Previously two cases of the transfer of antibiotic-resistant microorganisms due to FMT with one resulting in the death of a patient have been reported, by the FDA in June 2019, and a similar report was published in March 2020 (Green et al., 2020). Several consensus documents and national guidelines support the screening of donors for multidrug-resistant organisms while progressively establishing procedures for SARS-CoV-2 testing (Ng et al., 2023). In parallel, FMT treatment may have some uncertain associations with certain diseases, including peripheral neuropathy, idiopathic thrombocytopenic purpura, Sjögren's syndrome and rheumatoid arthritis (Wang et al., 2019), as well as having profound ethical implications. Washed microbiota transplantation (WMT), based on a modified version of FMT, provides a precise dose of enriched microbiota and can effectively reduce the type and amount of viruses, as well as metabolites with pro-inflammatory effects, during the washing process (Zhang et al., 2020), but WMT still cannot avoid the transfer of antibiotic-resistant microorganisms to the recipient.

Synthetic microbial consortia (SMC) are a crossover field between synthetic biology and microbiome, that is, a synthetic co-culture system containing multiple species. Unlike natural microbial communities with intricate internal relationships, artificially selected microbial communities can be used for different purposes, such as studying the interaction between two groups of bacteria or for therapeutic purposes after establishing an equilibrium state containing multiple groups of bacteria (Durán et al., 2018; Stecher, 2021). Compared with FMT, the composition of the SMC is clearer, and the background of the strains is purer. As a result, SMC are in general the safer treatment option. When compared with single probiotic bacteria, they can better adjust to environmental changes, additionally the cooperation and interdependence of various divisions of labour between the species allows the whole system to accomplish more complex tasks. For example, RBX2660, which has completed Phase III, has shown therapeutic effects against CDI (Khanna et al., 2022). This study screened candidate strains from existing probiotic strains to construct synthetic microbial consortium for the treatment of CDI. Anti-bacterial, anti-quorum sensing and anti-toxin production were used as screening criteria with the aim of avoiding the potential harm caused by FMT, and the synthetic microbial consortium was employed in the treatment of *C. difficile* infection in mice model and the potential mechanisms were also illustrated.

EXPERIMENTAL PROCEDURES

Construction of SMC based on probiotics

Probiotics strains and culture conditions

Lactobacillus rhamnosus ATCC 7469, *Lactobacillus paracasei* ATCC 25302, *Bifidobacterium longum* ATCC 15707, *Bifidobacterium breve* ATCC 15700 and *Bifidobacterium bifidum* ATCC 29521 were cultured in MRS broth supplemented with 0.1% cysteine hydrochloride, and *Bifidobacterium* spp. were cultured anaerobically. *Bacteroides ovatus* JCM 5824, *Bacteroides fragilis* ATCC 25285, *Clostridium butyricum* NCTC 7423, *Akkermansia muciniphila* ATCC BAA-835 and *C. difficile* ATCC 9689 were cultured anaerobically in modified Brain Heart Infusion (BHIM) broth added with 0.5% yeast extract, 0.1% cysteine hydrochloride and 0.1% mucin. *Bacillus licheniformis* ATCC 14580 and *Escherichia coli* OP50 were cultured in Nutrient Broth (NB). Cefoxitin cycloserine fructose agar (CCFA) was used for solid media to culture *C. difficile*. All the above bacteria were cultured at 37°C. *Vibrio harveyi* BB120 as well as BB170 were incubated in Lysogeny broth (LB) at 30°C.

Determination of the effects of the selected strains on the growth of *C. difficile*

The strain to be tested was cultured in a suitable liquid medium for 24 h and then centrifuged and filtered to obtain the cell-free supernatant (CFS). 100 μL of the overnight culture of *C. difficile* was taken, added to sterilized CCFA medium (100 mL) at approximately 50°C, mixed, and poured into Petri dishes. Equal amounts of CFS as well as the corresponding freshly prepared sterile medium, approximately 100 μL per well, were injected after punching holes in the agar and cultured overnight at 37°C. The diameter of the inhibition circle was measured after culture according to the discoloration of the CCFA medium.

While in the situation of co-culture in liquid, 1 mL of CFS together with 100 μL of the overnight culture of *C. difficile* was added to 3 mL of BHIM medium and the corresponding sterile medium was used as a blank control. The OD_{600} of the cultures was measured at multiple time points taken within 12 h after mixing. All the above experiments were repeated three times.

Determination of AI-2 in co-culture of *C. difficile*

The AI-2 assay was based on the previously published protocol (Bassler et al., 1993). Autoinducer Bioassay (AB) medium contains 0.3 M NaCl, 0.05 M $MgSO_4$, 0.2% casamino acids and pH adjusted to 7.5 with KOH. Sterile 1 mL pH 7.0 K_3PO_4 (1 M, pH 7.0), 1 mL arginine (0.1 M), 0.1 mL vitamin B2 (10 μg/mL), 0.1 mL vitamin B1 (10 mg/mL), and 2 mL glycerol were added per 100 mL after sterilization and cooling. *V. harveyi* BB170 was chosen as the reporter strain, with *V. harveyi* BB120 used as a positive control for AI-2 production. *V. harveyi* BB170 was first incubated overnight in AB medium and then diluted to fresh AB medium at a ratio of 1:5000. 180 μL of the mixed AB medium and the overnight incubated CFS of *V. harveyi* BB120 were added to white 96-well plates, while sterile AB medium was used as a negative control. The luminescence production was measured every hour for 6 h, using an enzyme marker (Spark 20M; TECAN) to determine when the luminescence produced by the reporter strain peaked. The broth of the selected strains after 24 h incubation and the overnight culture of *C. difficile*, after centrifugation, the precipitate was washed twice with sterile Phosphate buffer solution (PBS) and resuspended in sterile BHIM medium, and the quantity ratio of *C. difficile* to the selected strains was adjusted to 1:10 and injected into fresh BHIM medium (total volume of 430 μL). Based on Lee and Song's study (2005), 20 μL of CFS after 8 h co-culture was mixed with 180 μL of diluted *V. harveyi* BB170 in a white 96-well plate, while sterile BHIM medium was used as a negative control, and the luminescence was measured after 5 h culturing at 30°C and 170 rpm.

Determination of *C. difficile* TcdB toxin

The supernatant was removed by centrifugation of the strains to be tested after 24 h incubation in BHIM, and the precipitate was washed with sterile PBS, mixed with *C. difficile* in an adjusted quantity ratio of 1:10 and inoculated into fresh BHIM. After co-culture for 24 h, the CFS was taken and the TcdB concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Sinobestbio).

Probiotic properties of selected bacterial strains

Haemolytic activity

The haemolytic activity test was modified according to the prior released method (Talib et al., 2019). All screened strains were inoculated in triplicate on Brain Heart Infusion (BHI) agar plates supplemented with 5% (w/v) sheep blood and incubated anaerobically at 37°C for 48 h. After finishing the culture, a check was performed to determine whether there were any signs of β-haemolysis (clear area around the colony), α-haemolysis (green area around the colony), or γ-haemolysis (no area around the colony) around the colonies.

Antibiotic sensitivity test

The assessment of antibiotic susceptibility was based on the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute guidelines. The antibiotics selected were the usual broad-spectrum antibiotics and those available for intestinal preparation (Hangzhou Microbial Reagent Co., Ltd.), including Erythromycin (15 μg), Streptomycin (10 μg), Chloramphenicol (30 μg), Neomycin (30 μg), Ampicillin (10 μg), Cefradin (30 μg) and Vancomycin (30 μg). The selected strains were inoculated individually into a liquid medium, and after 24 h culture, 100 μL was taken to mix with 100 mL sterilized agar medium at about 50°C and poured into Petri dishes. The antibiotic paper sheets were then placed on the agar surface for 24 h at 37°C and then the diameter of the inhibition circle around the paper sheets was measured. Depending on the size of the diameter, the strains were labelled as sensitive (S), intermediate (I), or resistant (R).

Human gastrointestinal tract environmental tolerance simulation

Resistance to low pH. The strains were first inoculated into the corresponding liquid medium and incubated at 37°C for 24 h. The cultures were inoculated at approximately 1×10^9 CFU, 100 μL, into a freshly prepared liquid medium adjusted to different pH values (2.0, 2.5, 3.0) with HCl, and the normal medium was used as a control group. Each group of experiments was duplicated thrice. OD_{600} was measured after 24 h incubation using an enzyme marker. The

OD_{600} of the low pH group, the control group, and the fresh medium were named A_1, A_0 and A_M (Wang et al., 2020), respectively. The survival rate was calculated as:

$$\text{Survival rate} = \frac{A_1 - A_M}{A_0 - A_M} \times 100\%.$$

Resistance to bile salt. The strains were inoculated at approximately 1×10^9 CFU, 100 μL after 24 h of incubation in liquid mediums into the corresponding medium containing bile salts at concentrations of 0.1%, 0.3%, and 0.5%, and the medium without bile salts were used as a control group. Each group of experiments was repeated three times. The OD_{600} of the bile salt group, the control group, and the fresh medium were named A_2, A_0 and A_M (Wang et al., 2020), respectively, after 24 h incubation using an enzyme marker. The survival rate was calculated as:

$$\text{Survival rate} = \frac{A_2 - A_M}{A_0 - A_M} \times 100\%.$$

In vitro free radical scavenging assay

DPPH free radical scavenging assay. Firstly, the CFS was prepared by centrifugation at 12,000 rpm for 2 min after 24 h of incubation, and the supernatant was filtered through a 0.22 μm membrane. 1.2 mL DPPH (0.2 M) solution prepared with methanol was mixed with 0.3 mL CFS, while bacteria-free liquid medium was substituted for CFS as the control group, and then centrifuged at 8000 rpm for 1 min, and the OD_{517} was measured by spectrophotometer after the light-proof reaction for 25 min. The OD_{517} of the experimental group and control group were named A_{D1} and A_{D0}, respectively, and the clearance rate was calculated as:

$$\text{Clearance rate} = \frac{A_{D0} - A_{D1}}{A_{D0}} \times 100\%.$$

Hydroxyl radical scavenging assay. 1.4 mL of freshly mixed solution containing sodium salicylate (5 mM), $FeSO_4$ (5 mM) and H_2O_2 (3 mM) was added with 100 μL of CFS, and a sterile liquid medium was used as the control group replacing CFS, and the reaction was carried out for 50 min at room temperature followed by centrifugation at 8000 rpm for 1 min, and OD_{510} was measured by spectrophotometer. The OD_{510} of the experimental group and the control group were named A_{H1} as well as A_{H0}, and the clearance rate was calculated as:

$$\text{Clearance rate} = \frac{A_{H0} - A_{H1}}{A_{H0}} \times 100\%.$$

Superoxide anion radical scavenging assay. In the cuvette, 0.25 mL of CFS, 0.25 mL of pyrogallol solution (0.05 M), and 1.5 mL of Tris-HCl (0.1 M, pH 8.0) were added in this order. After mixing, the OD_{325} was measured by UV spectrophotometer at the 30 and 300 s of the reaction and recorded as A_{30s} and A_{300s}, respectively. In addition, a sterile liquid medium was used as the control group to replace CFS, and A_{30s} and A_{300s} were also recorded. The values of the difference between A_{300s} and A_{30s} were calculated as A_{S1} and A_{S0} for the experimental and control groups, so that the clearance rate of was calculated as:

$$\text{Clearance rate} = \frac{A_{S0} - A_{S1}}{A_{S0}} \times 100\%.$$

Bacteria-bacteria interaction in SMC

Determination of the ratio of individual strain in the co-culture system

DNA of the selected strains was first extracted using a bacterial genomic DNA extraction kit (TIANamp Bacteria DNA Kit; Tiangen), and DNA concentrations were determined spectrophotometrically and then diluted to concentrations of 0.01, 0.1, 1, 10, and 100 ng/μL with TE buffer. Quantitative real-time PCR was performed using a LightCycler system (Roche Diagnostics) with the ChamQ Universal SYBR qPCR Master Mix (Vazyme) according to the method previously described (Kato et al., 2005). The strains and their corresponding primers are listed in Table S4, and all PCR products were verified by 1.5% agarose gel electrophoresis for the target strains to detect the bands unaffected by the existence of genomic DNA of other strains. Quantitative real-time PCR started with a pre-denaturation lasting 30 s at 95°C and went through 40 cycles. Each cycle consisted of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s, as well as a fluorometric assay at the end of extension. The specificity of the qPCR products was assessed by melting curve analysis, which consisted of a 15 s denaturation at 95°C, annealing at 60°C for 1 min, and a 15 s ramp-up to 95°C with continuous detection of fluorescence. Each sample was assayed three times and the Ct values were taken logarithmically to make a standard curve.

After adjusting the selected strains to approximately 1×10^7 CFU, they were mixed and cultured in BHIM medium for 24 h. This synthetic microbiota was called Bacteria 10 (B10). The bacterial genomic DNA was extracted from the medium before and after co-culture, respectively, and the concentrations of the extracted DNA were unified. Based on the Ct values, the mass was calculated by substituting the standard curve to determine the proportion of the strain in the overall co-culture system.

Quantification of biofilms

Biofilm formation was referenced to the protocol (Slater et al., 2019). Tissue culture-treated 24-well plates were anaerobically plated for 48h prior to use. Overnight cultures of *C. difficile* were diluted with BHIM medium supplemented with 0.1M glucose (BHIG) (Đapa et al., 2013) at a ratio of 1:100. B10 and CFS incubated in BHIG anaerobically for 24h were mixed separately with *C. difficile* and then incubated anaerobically for 72h and wrapped in membrane to avoid evaporation of the liquid.

The biofilms were quantified at 24 and 72h after co-culture, respectively. After co-culture, the wells of the 24-well plates were first washed twice with sterile PBS and dried in air for 10min. Then, the biofilms were stained with 1mL of aseptic 0.2% crystalline violet (CV) filtered through a 0.22μm membrane and incubated anaerobically for 30min at 37°C (Slater et al., 2019). After incubation, the CV was removed and each well was washed twice with sterile PBS. Then 1mL of methanol was added to each well and incubated at room temperature and under aerobic conditions for 30min. The methanol-extracted CV was diluted at a ratio of 1:10, and OD_{570} was measured using a spectrophotometer. Each group of tests was carried out three times.

Animal experiment

Ethics statement

In this research, the use of mice was approved by the China Pharmaceutical University Animal Care and Use Committee (20210412) and all experimental animal procedures followed the guidelines of the Institute Animal Care and Use Committee of China Pharmaceutical University.

CDI model

Male C57BL/6J mice (6weeks old, GemPharmatech) were randomly divided into three groups after 1week of acclimatization feeding under standard feeding conditions (12 light/12 dark cycle, 22–24°C with humidity set at 40%–50%) for a 14-day experiment. The CDI model was modified based on the existing study (Asai et al., 2022). The three groups of mice were treated by gavage from Day 1 to Day 5 as follows: 0.1mL PBS (standard control group, $n=6$; CDI group, $n=10$), 0.1mL synthetic microbiota (B10 group, 1×10^8CFU, $n=10$). During gavage, mice were assessed for body weight, behaviour and faecal morphology based on the clinical symptom score (CSS) criteria (Shelby et al., 2020). Before CDI, the CDI and B10 groups were treated with a 72h (Day −6 to Day −3) antibiotic cocktail (0.215mg/mL metronidazole, 0.2mg/mL neomycin and 0.045mg/mL vancomycin), and regular water intake was resumed after treatment. Clindamycin (10mg/kg) was administered intraperitoneally at 24h prior to infection. On Day 0, *C. difficile* (1×10^8CFU, 0.1mL) was orally given to the CDI group and the B10 group. Body weight and food intake of mice were monitored daily after infection.

Histologic injury score (HIS)

After the sacrifice of the mice, cecum and colon tissues were collected and fixed in a 4% paraformaldehyde solution, followed by paraffin embedding. Sections were stained with haematoxylin—eosin and images were taken by microscopy. Injuries to the cecum and colon were assessed based on the HIS criteria (Shelby et al., 2020).

Detection of inflammatory cytokines and TcdB toxin in serum

Serum collected from mice after execution is used for inflammatory cytokine detection, including interleukin 1α (IL-1α), IL-1β, IL-6, IL-17A, IL-22, tumour necrosis factor alpha (TNF-α) and TcdB toxin in serum were measured using an ELISA kit (Sinobestbio).

Quantitative real-time PCR

Total RNA from colonic tissue was extracted using TRIzol (Vazyme). Reverse transcription, as well as amplification and relative quantification of mRNA ($2^{-\Delta\Delta CT}$ method), were performed in triplicate using a Q2000A Quantitative Real-time PCR System (LongGene) and HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the internal reference gene. The primer sequences are listed in Table S5.

Microbiota analysis

The colon contents of mice after execution were used for sequencing. The bacterial DNA was extracted from faecal samples with a QIAamp Fast DNA stool Mini Kit (Qiagen, Cat# 51604), and PCR amplification was conducted with barcoded specific bacterial primers targeting the variable region 3–4 (V3–V4) of the 16S rRNA gene: forward primer 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and reverse primer 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ (Chen, Luan, et al., 2019; Chen, Wu, et al., 2019). Construction of sequencing libraries and paired-end sequencing was performed on an Illumina NovaSeq6000 platform at Biomarker

Technologies Co, Ltd. according to standard protocols. The raw sequenced sequences were subjected to quality control, including low-quality filtering, length filtering to obtain high-quality sequences and clustering/denoising of high-quality sequences to classify OTUs/ASVs. Paired-end reads were merged using FLASH v1.2.7 (Yang et al., 2020), and tags with more than six mismatches were discarded. The merged tags with an average quality score <20 in a 50 bp sliding window were determined using Trimmomatic and those shorter than 350 bps were removed (Sheng et al., 2019). Possible chimeras were further removed and the denoised sequences were clustered into operational taxonomic units (OTUs) with 97% similarity using USEARCH (version 10.0). Taxonomy was assigned to all OTUs by searching against the Silva databases (Release128) using QIIME software. Raw sequences were deposited in the Sequence Read Archive database (http://www.ncbinlm.nih.gov/sra), with the accession numbers ranging from SAMN* to SAMN*. Analysis of all microbiota-related analysis was performed using BMKCloud (http://www.biocloud.net).

RESULTS

Selection of probiotic strains for construction of the SMC

With the aim of determining the inhibitory effect of individual strains, it was first investigated whether the CFS from the selected strains could inhibit the growth of *C. difficile* on agar plates. The results with obvious inhibitory zone are listed in Table 1 and were either *Bifidobacterium* spp. or *Lactobacillus* spp. The other five strains are not listed as the shower no signs of inhibition circles. To further understand the specific effects of the selected strains at various periods of *C. difficile* growth, we divided the selected strains into three groups according to the required medium and measured OD_{600} several times before reaching the platform growth period. As similar to the situation found on agar plates, CFS from *B. breve*, *B. bifidum*, *L. rhamnosus* and *L. paracasei* all completely inhibited the growth of *C. difficile*; while CFS from *B. longum* produced significant differences from the control after 2.5 h and could markedly slow down the growth rate of *C. difficile*. Similarly, the CFS from species *B. longum*, *B. licheniformis*, *A. muciniphila* and *C. butyricum* could significantly delay the growth of *C. difficile*, while causing a decrease in the bacterial concentration at the point at which the plateau growth phase is reached. In contrast, CFS from two strains of *Bacteroides* spp. had no apparent influence on the growth of *C. difficile* (Figure 1A). These results suggested that most of the CFS from selected strains could in vitro inhibit the growth of *C. difficile*.

TABLE 1 Antibacterial activity of the selected strains against *Clostridioides difficile*.

	Inhibition zone diameter (mm)			
Selected strains	**1**	**2**	**3**	**Average**
Bifidobacterium longum	8.2	8.5	8.1	8.27
Bifidobacterium breve	12.3	13.4	12.8	12.83
Bifidobacterium bifidum	14.0	13.2	13.7	13.63
Lactobacillus rhamnosus	12.5	13.2	10.4	12.03
Lactobacillus paracasei	9.6	9.1	8.4	9.03

Due to the presence of an AI-2 type quorum sensing system in *C. difficile* and its association with biofilm formation and expression of virulence factors, it was investigated whether the selected strains would have any impact on AI-2 secretion by *C. difficile* under co-culture conditions (Chandra et al., 2022; Vuotto et al., 2018). It was first ascertained that luminescence reached a maximum of five hours after the induction of bioluminescence in wild-type *V. harveyi* BB170 (Figure 1B). Secondly, according to Lee and Song, the maximum secretion of AI-2 by *C. difficile* was reached after 8 h of culture (Lee & Song, 2005), as such the induction of bioluminescence was opted for after 8 h of co-culture. It was worth noting that the level of AI-2 production did not strictly correspond to the result of growth inhibition by CFS. *B. longum* and *A. muciniphila*, which had positive growth inhibitory effects, did not inhibit AI-2 synthesis and secretion of *C. difficile*, whereas *B. fragilis* and *B. ovatus*, which had no growth inhibitory effects, did. Meanwhile, all other six strains could significantly reduce the amount of AI-2 in the system when co-cultured (Figure 1C). To exclude the selected strain's own AI-2 production from the co-culture system, we monocultured the selected strains for 8 h and induced bioluminescence with CFS. The results showed that *A. muciniphila* could probably also inhibit AI-2 secretion by *C. difficile*; however, *B. longum* remained ineffective in the suppression of AI-2 synthesis. In the case of *B. ovatus* (Figure 1D), the AI-2 content was significantly higher in monoculture than in co-culture, as such the level of AI-2 production in co-culture was not simply superimposed, but from the perspective of the synthetic microbiota it could inhibit AI-2 synthesis and secretion of *C. difficile*.

Since TcdB is associated with fulminant infection (Riegler et al., 1995; Steele et al., 2013), we tested the concentration of TcdB in the culture system after each member of B10 was co-cultured with *C. difficile* for 24 h in the viable state. Six of the ten strains were effective in reducing TcdB secretion, while the other four strains, all *Bifidobacterium* and *L. paracasei*, did not

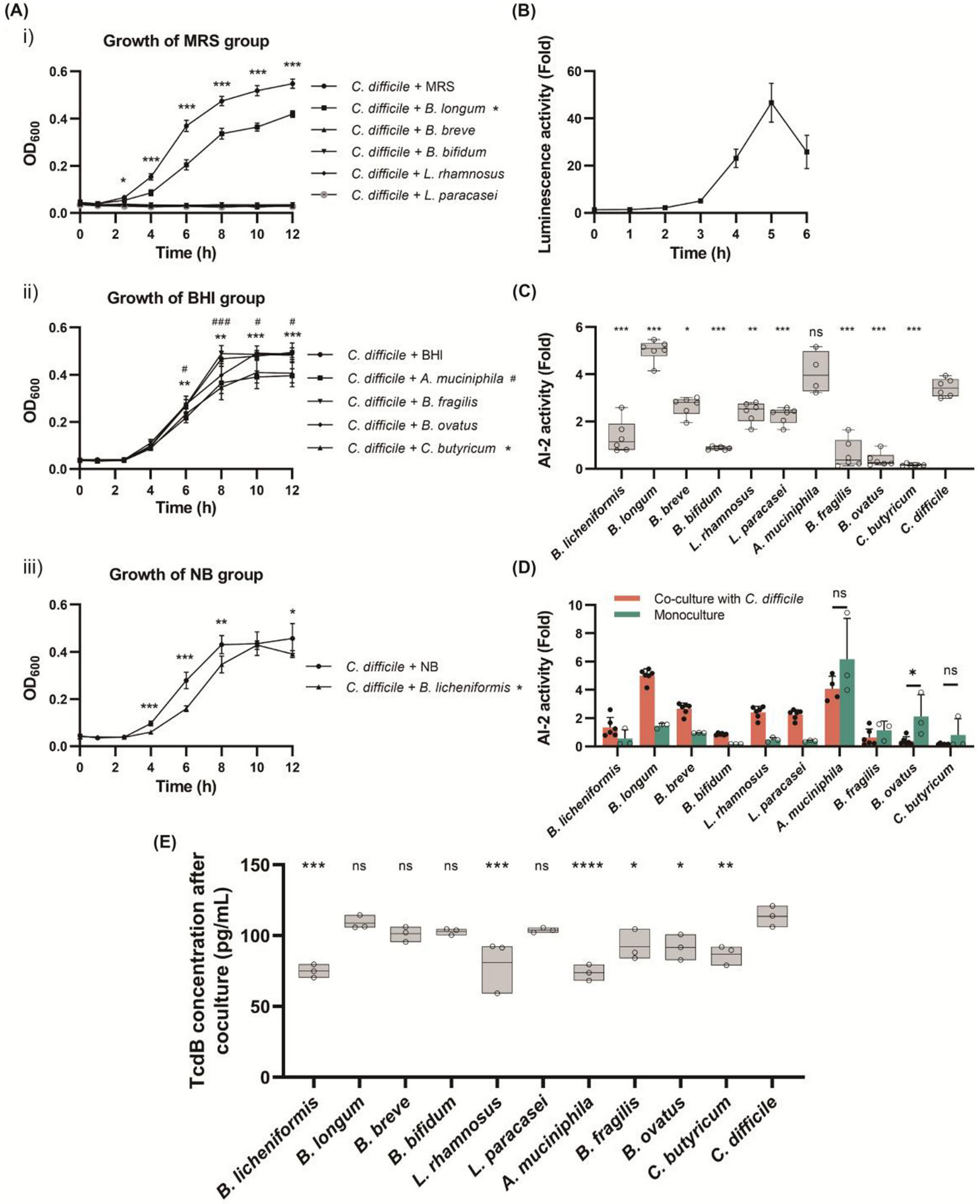

FIGURE 1 Selected strains can differentially inhibit the activity of *Clostridioides difficile*. (A) Growth curves of *C. difficile* when co-cultured with a cell-free supernatant of different strains, (i) MRS group, * represents *Bifidobacterium longum* vs. MRS, (ii) BHI group, * represents *Clostridium butyricum* vs. BHI, # represents *Akkermansia muciniphila* vs. BHI, (iii) NB group, $n=3$. (B) The intensity of fluorescence expression of the reporter strain *Vibrio harveyi* BB170 at different times. (C) The expression of AI-2 in the whole system when the live bacteria were co-cultured with *C. difficile*, $n=6$. (D) Expression of AI-2 in the system when the living bacteria were co-cultured and when they were monocultured, $n=3$. (E) Concentration of TcdB in the system when the living bacteria were co-cultured with *C. difficile*, $n=3$. Significance calculated using unpaired *t* test (A, D) and ordinary one-way ANOVA (C, E). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$.

inhibit TcdB (Figure 1E). This is a contrary result to the inhibitory effect of CFS and the inhibition of secretion of AI-2; thus, it can be speculated that *Bifidobacterium* and *Lactobacillus* directly inhibit the growth of *C. difficile* through certain substances released in the culture; however, starting cell culture at the same time will not produce the same end result. Nevertheless, 60% of the B10 members could significantly inhibit the synthesis of TcdB.

Probiotic properties of the selected strains

The incapability to cause haemolysis is necessary to screen for therapeutic strains. When grown on agar plates containing 5% (w/v) sheep blood, all strains exhibited γ-haemolysis (the lack of haemolysis around a bacterial colony) (Figure S1).

Likewise, the lack of antibiotic resistance is a prerequisite for therapeutic purposes. Under the conditions of the seven commonly used broad-spectrum antibiotics selected, all strains were sensitive to two or more of these antibiotics, which indicated that the selected strains are safety to be used to construct microbial consortia. Antibiotic susceptibility of selected antibiotics against probiotic strains is listed in Table S1.

Since gastric and bile acids constitute the first line of defence for the gastrointestinal tract in vivo, this necessitates the strain's survival within these conditions. In vitro conditions with low pH (2.0, 2.5, 3.0) or the presence of bile salts (0.1%, 0.3%, 0.5%, w/v) need to be established to simulate those conditions within the gastrointestinal tract and to investigate the strains survival when introduced via gavage. Under conditions pH 2.0, the lowest survival rate for *B. fragilis* was 1.74%, whereas under conditions with 0.3% bile salt concentration, the lowest survival rate for *B. licheniformis* was 1.16%. The mean values of the survival rates (Figure 2A,B) show that the selected strains possess gastric acid tolerance and bile salt tolerance. In addition, the results and measurements can be used as a reference, in order to adjust the concentration of gavage applied.

Free radicals in organisms cause oxidative damage to the body when in excess, including oxygen and nitrogen radicals, so the selected strains need to play a role in scavenging free radicals. It was found that all strains except *B. licheniformis* had more than 50% scavenging effect on DPPH radicals and superoxide anion radicals, while they only had less than 40% scavenging ability on hydroxyl radicals. Strangely, the scavenging rate of *B. licheniformis* for DPPH radicals and hydroxyl radicals was negative (Figure 2C). Overall, the constructed SMC B10 showed positive antioxidant activities.

B10 can stably exist in vitro and exert the desired effects against *C. difficile*

A prerequisite for the synthetic microbiota to function in vivo is that the stability of a single group of strains can be maintained while cultured in vitro and that the ecological niche is not completely replaced. Therefore, we used qPCR to ascertain the respective proportions of the different members of B10 across the system before and after 24 h co-culture. Compared to before co-culture, only *B. breve*, *L. rhamnosus*, *L. paracasei*, and *C. butyricum* showed an increasing trend in their proportions. *B. breve* occupied almost all the resources of the *Bifidobacterium* while the massive expansion of *L. paracasei* and *C. butyricum* led to a significant shrinkage of other strains, most notably *A. muciniphila* declined by 96.69% (12.09% to 0.40%) (Figure 3A,B). It was postulated that the motility of the bacteria themselves and the slow metabolism with more demanding nutrient requirements may account for these observations. The results of the in vitro simulations are not a good representation of the actual conditions when applied in vivo, but they can provide a reference for adjusting the actual gavage concentrations; the results also indicate that members of B10 can stably survive.

Biofilm is a major contributing factor to drug resistance and virulence in pathogenic bacteria, thereby inducing cell death, delaying wound healing, promoting inflammatory responses and adding difficulty to treatment (Peng et al., 2017). This study explored the effect of B10 and its CFS on biofilm formation of *C. difficile* in vitro successively. CV method was used to quantify biofilms as described in method and material. B10 itself formed biofilm, but after co-culture, the biofilm formation in the mixed group (co-culture of B10 and *C. difficile*) was significantly decreased (Figure 3C). Meanwhile, compared with the *C. difficile* group, both live bacteria co-culture, and CFS co-culture, B10 could significantly repress biofilm formation, and the extent of CFS reduction was more obvious when compared with the other groups (Figure 3D). Therefore, the constructed microbiota could effectively inhibit the biofilm formation of *C. difficile*.

Treatment with B10 ameliorated clinical signs caused by CDI in mice

As shown in the Figure 4A, the CDI models were constructed by oral gavage of 1×10^7 CFU *C. difficile* on day 0, and 100 μL B10 or PBS was orally gavaged daily on days 1–5, where the concentration of B10 was 1×10^8 CFU/mL, and the animals were executed at 48 h after the last gavage. Mouse body weight and food intake were monitored on each day after CDI. Weight change is an important indicator of the severity of CDI and one can visually determine the course of the disease from it; however in our study, there were no

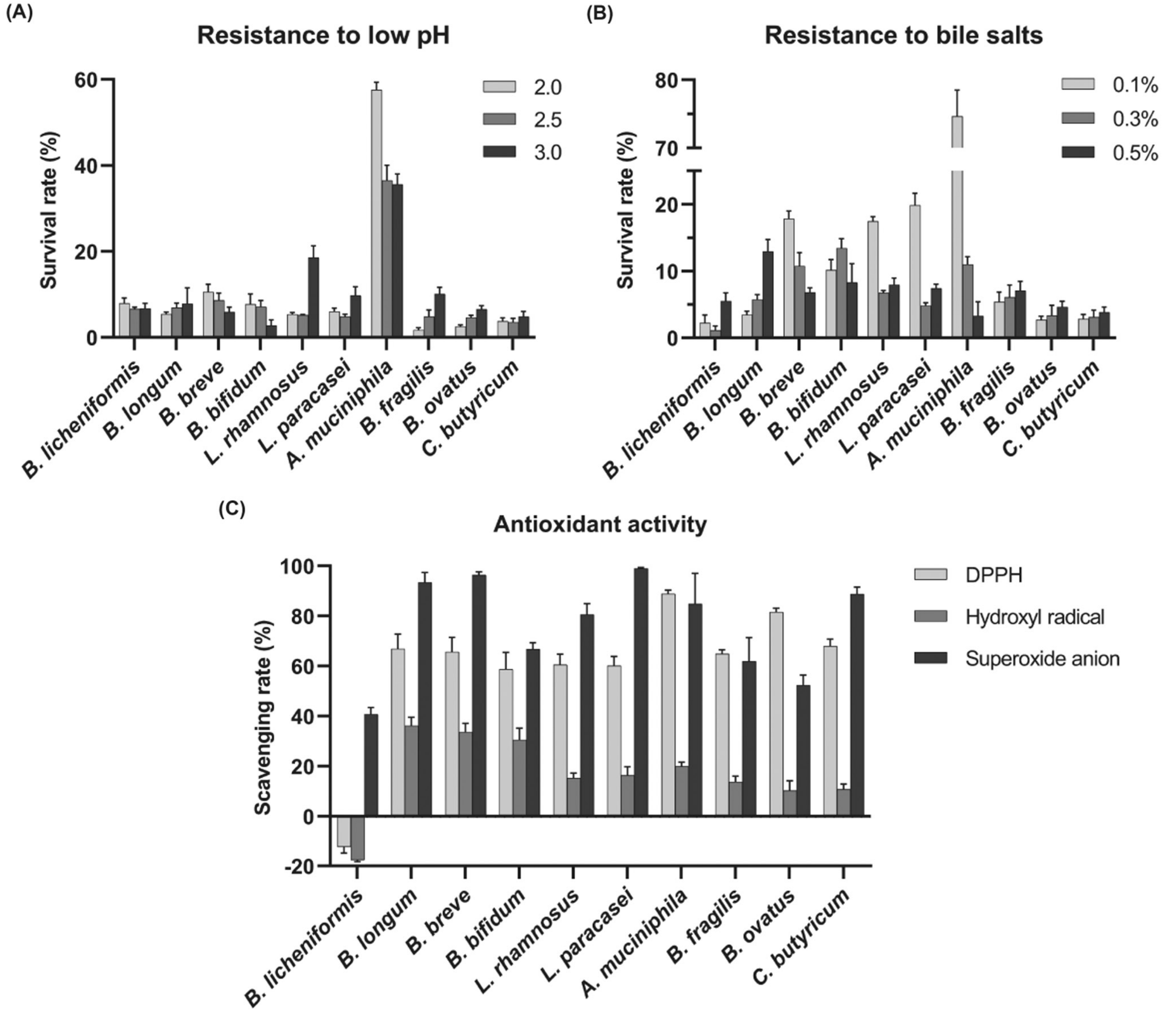

FIGURE 2 Probiotic properties of B10 members, $n = 3$. (A) Survival in gastric acid environment. (B) Survival in bile salt environment. (C) DPPH radical scavenging ability, hydroxyl radical scavenging ability and superoxide anion scavenging ability.

significant differences between the CDI group and the B10 group, and the two groups maintained a decreasing trend until the end of the experiment (Figure 4B). Another indicator, survival, showed the therapeutic effect of B10 on the mice. The control group and the B10 group had no deaths after infection; on the other hand, the CDI group had a total of four mice succumb to the infection on the second day, fifth day and seventh day (Figure 4C). CSS was used to index in order to determine the severity of the infection, with a total score ranging from normal (0–2) to mild (3–5), moderate (6–8), or severe (9–12), depending on the mouse stool, behaviours, and weight loss. In the five-day CSS study, there was a significant decrease in the B10 group when compared to the CDI group, that is, from moderate to mild, but did not return to the level of the control group by the end of the experiment (Figure 4D). We also measured the colorectal lengths of the mice, and similarly to CSS, B10 significantly alleviated the intestinal shortening symptom caused by *C. difficile* (Figure 4E,F). Thus, B10 greatly diminished the clinical symptoms caused by CDI.

B10 attenuates the inflammatory response and upregulates Claudin-1 expression

H&E staining of the cecum and colon of mice after paraffin embedding restored the cecum and colon of the B10 group to a level close to that of the control group that is: a more intact mucosal barrier, an intact cecum wall, and a clearly visible crypt of the cecum and colon (Figure 5A). Conversely, in the CDI group, the cecum was severely damaged, with a completely damaged mucus layer, the presence of obvious broken tissue and a fairly significant neutrophil infiltration, whilst the

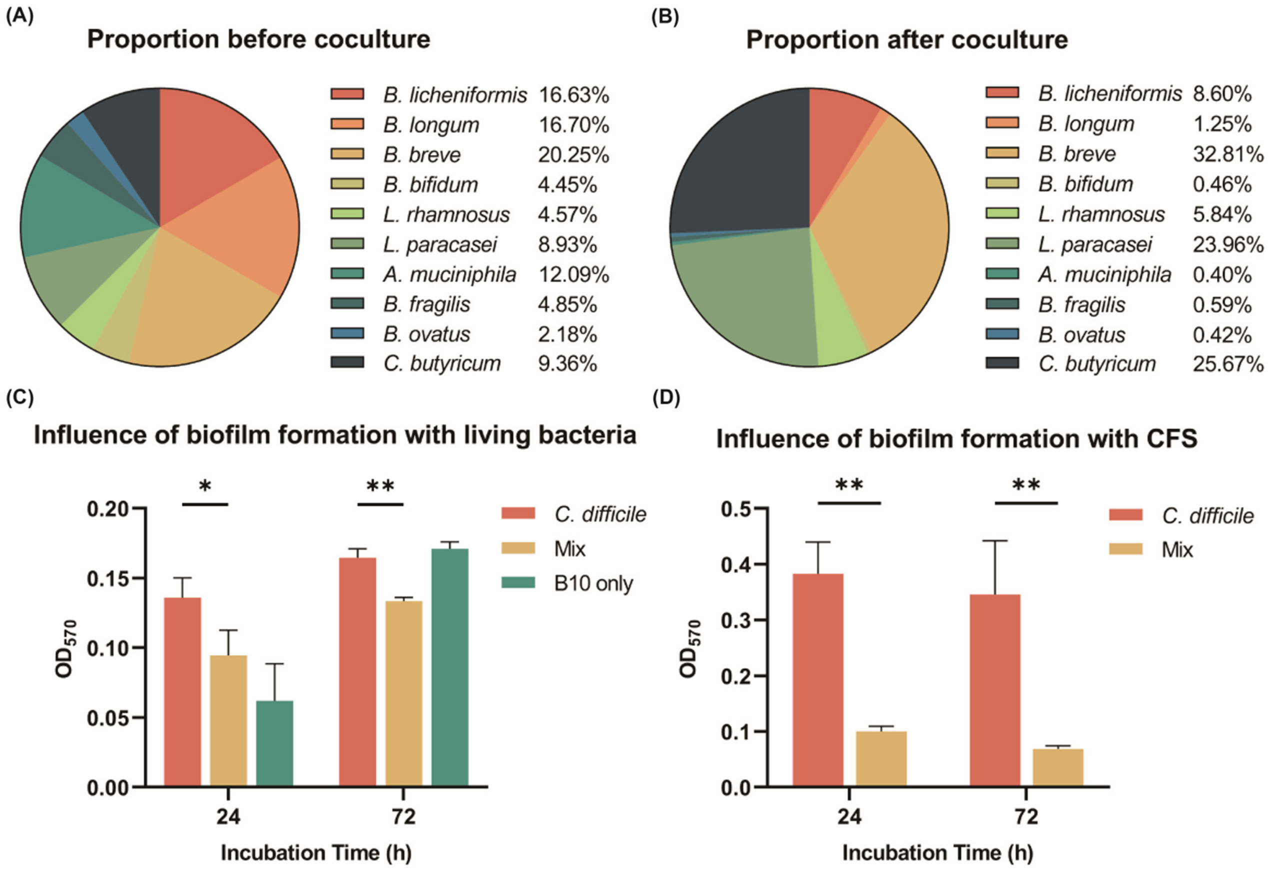

FIGURE 3 B10 can exist stably in vitro environment and achieve the designed effects, $n=3$. (A) Proportion of each member of B10 before co-culture. (B) Proportion of each member of B10 after co-culture. (C) The amount of biofilm formed by *Clostridioides difficile* monoculture, B10 co-culture with *C. difficile* and B10 monoculture. (D) The amount of biofilm formed by *C. difficile* monoculture versus CFS of B10 co-cultured with *C. difficile*. Significance calculated using unpaired *t* test (C, D). $^{*}p<0.05$ and $^{**}p<0.01$.

colon showed epithelial cell loss, mucus layer damage, and local neutrophil infiltration. Although the intestine in the B10 group had a slight submucosal oedema as in the CDI group, it may still be assumed that B10 treatment can have an important and noticeable effect on intestinal recovery in mice.

Furthermore, we evaluated the actual effect of B10 treatment on infected mice based on the established scoring criterion with pathological sections and extended application to the cecum (Figure 5B). Similar to the results presented from the H&E staining findings, the B10 group scored significantly lower than the CDI group in the cecum HIS. And within the six samples submitted for inspection in each group, only two samples (33%) in the B10 group scored greater than or equal to 3, that is, there was severe impairment, while four samples (67%) in the CDI group, and even two samples scored 8 (the upper limit was 9). Also, although there was no significant difference in the scores of the colon between the two groups, all samples (100%) in the CDI group had scores greater than or equal to 3, while only four samples (67%) in the B10 group did.

Six inflammation-associated cytokines including interleukin 1α (IL-1α), IL-1β, IL-6, IL-17A, IL-22, tumour necrosis factor-alpha (TNF-α) in the serum were measured. All the remaining cytokines except IL-1β and IL-22 showed a significant decrease after treatment when compared to the CDI group (Figure 5C). The decreases in IL-1α and IL-6 revealed a lower activation and expression of the NF-κB pathway (Zhou et al., 2001), and the decrease in IL-17A implied a recovery in the metabolism of secondary metabolites such as short-chain fatty acids and secondary bile acids in the intestine (Bhaskaran et al., 2018; Hang et al., 2019; Luu et al., 2019), the same for TNF-α indicated a decline in overall inflammation levels, and the lack of significant changes in IL-22 may suggest that B10 treatment did not influence the immunity associated with the intestinal crypts and thus contributed to the recovery of the host's health (Chiang et al., 2022).

The expression levels of mRNA in colon tissue were measured according to three aspects: mucin, tight junction proteins and NF-κB pathway (Figure 5D). When compared to the control group, the mRNA expression levels of Myd88 and TLR4 were dramatically increased in the CDI group, while the mRNA expression levels of CD14 and ZO-1 were significantly decreased. In comparison with the CDI group, treatment with B10 resulted

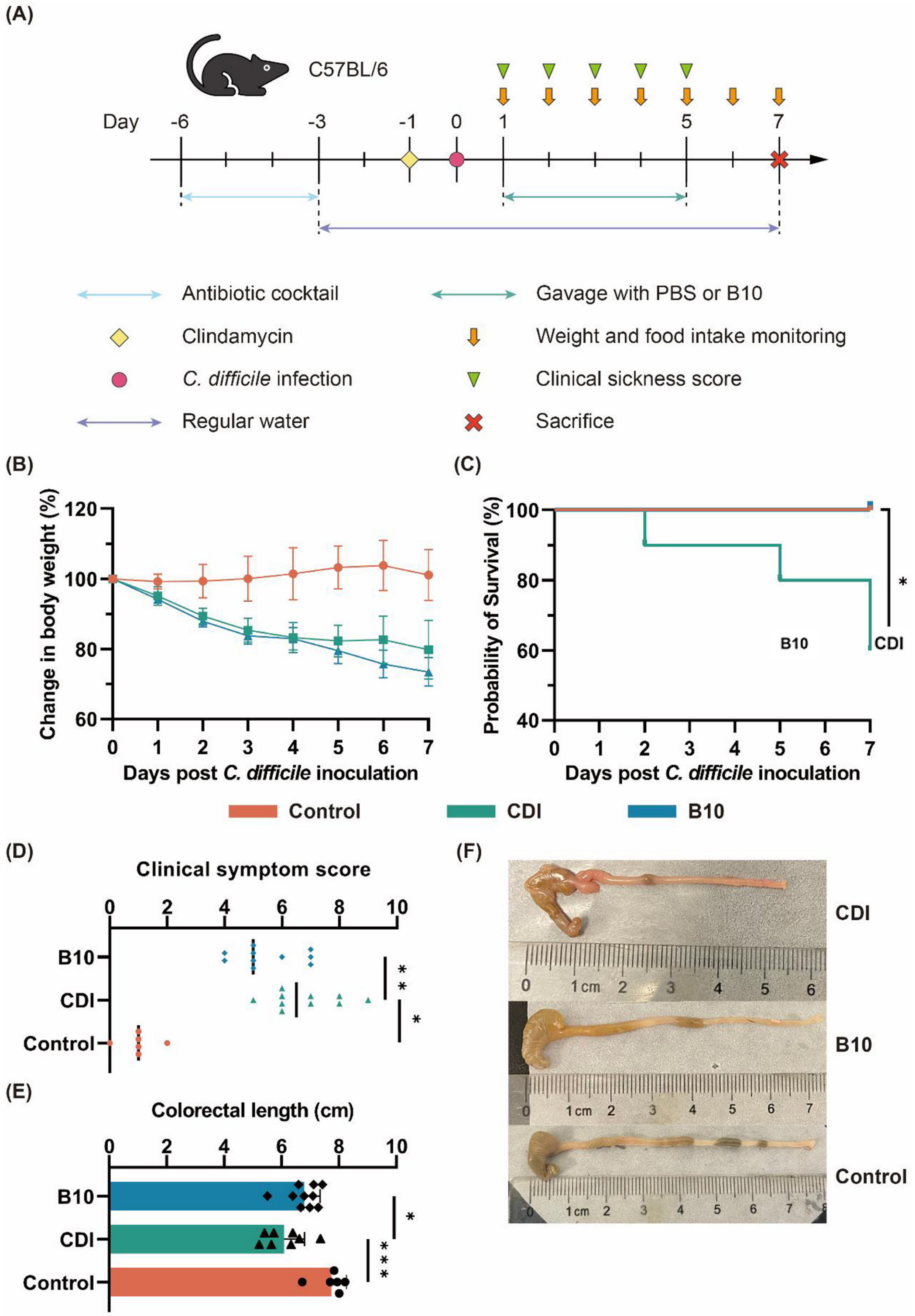

FIGURE 4 B10 ameliorates clinical signs caused by *Clostridioides difficile* infection (CDI). (A) Timeline of animal experiments. (B) Relative weight change after infection, animals were culled the next day after death. (C) Change in mortality after infection. (D) Highest Clinical Sickness Score for each animal within five days after infection. (E) Comparison of colorectal lengths of the three groups. (F) Representative colorectal photographs of the infected group versus the treated group. The number of independent samples used for each group was: Control group, $n = 6$; CDI group, $n = 10$; and B10 group, $n = 10$. Significance calculated using Mantel-Cox test (C), Wilcoxon test (D) and ordinary one-way ANOVA (F). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

in a positive increase in the mRNA expression of Claudin-1 and a remarkable decline in the TLR4 while the Muc2 and ZO-1 showed an upwards increasing trend, as well as a tendency to downregulate Myd88. TLR4 can bind to lipopolysaccharide (LPS), and it has been reported that in vivo levels of Proteobacteria increase

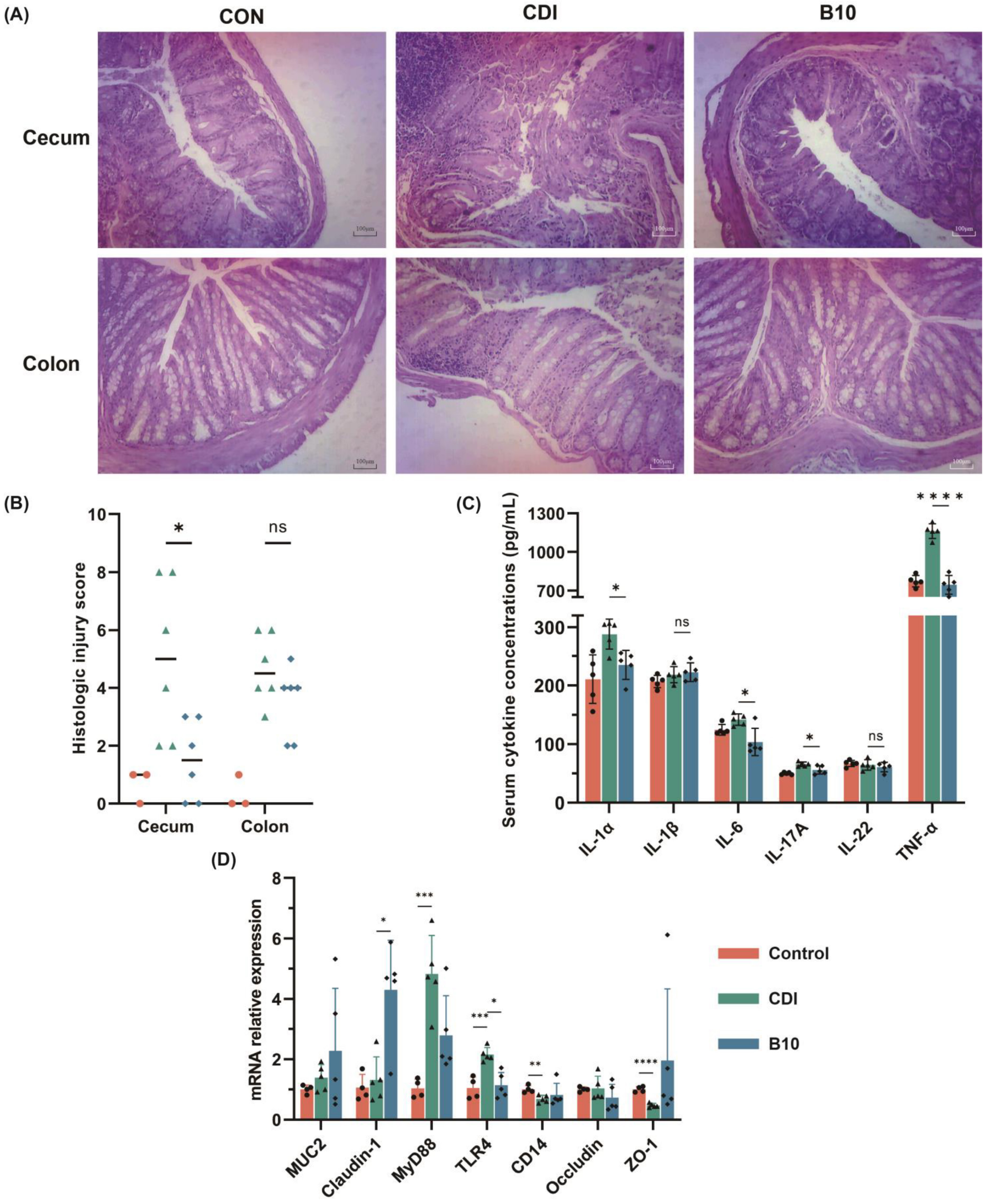

FIGURE 5 Treatment with B10 attenuated the inflammatory response and increased the expression of tight junction protein. (A) Representative H&E-stained images of three groups of cecum and colon. (B) Histologic injury scores (HIS) of cecum and colon based on H&E staining results ($n=6$ per group). (C) Cytokine concentrations in serum ($n=5$ per group). (D) Relative expression levels of mRNA in colon tissue ($n=5$ per group). Significance calculated using unpaired *t* test (B–E). *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

after CDI, which leads to an increase in LPS levels and thus upregulation of Tlr4 expression (VanInsberghe et al., 2020). B10, on the other hand, can reduce the inflammatory response by restoring the colony balance, diminish the share of Proteobacteria, and decrease TLR4-mediated NF-κB expression. It also increases the expression of mucins and tight junction proteins, even if only certain tendencies, and recovers the host's intestinal health.

B10 revives the gut microbiome at multiple levels

A total of 1,115,503 pairs of Reads were sequenced from 15 samples (Control = 5, CDI = 5, B10 = 5), and 1,112,453 Clean Reads were generated after double-end Reads QC and splicing, with at least 42,344 Clean Reads per sample and an average of 74,164 Clean Reads (Table S2). Analysis based on OTU level, according to the ACE and Chao1 indices, the CDI and B10 groups had statistically lower species richness when compared to the control group, which was a result of the use of an antibiotic cocktail during the modelling period (Figure 6A,B). In addition, the species richness was significantly higher after treatment with B10 than in the CDI group that did not receive therapy. The PD whole tree calculated based on the phylogenetic tree equally

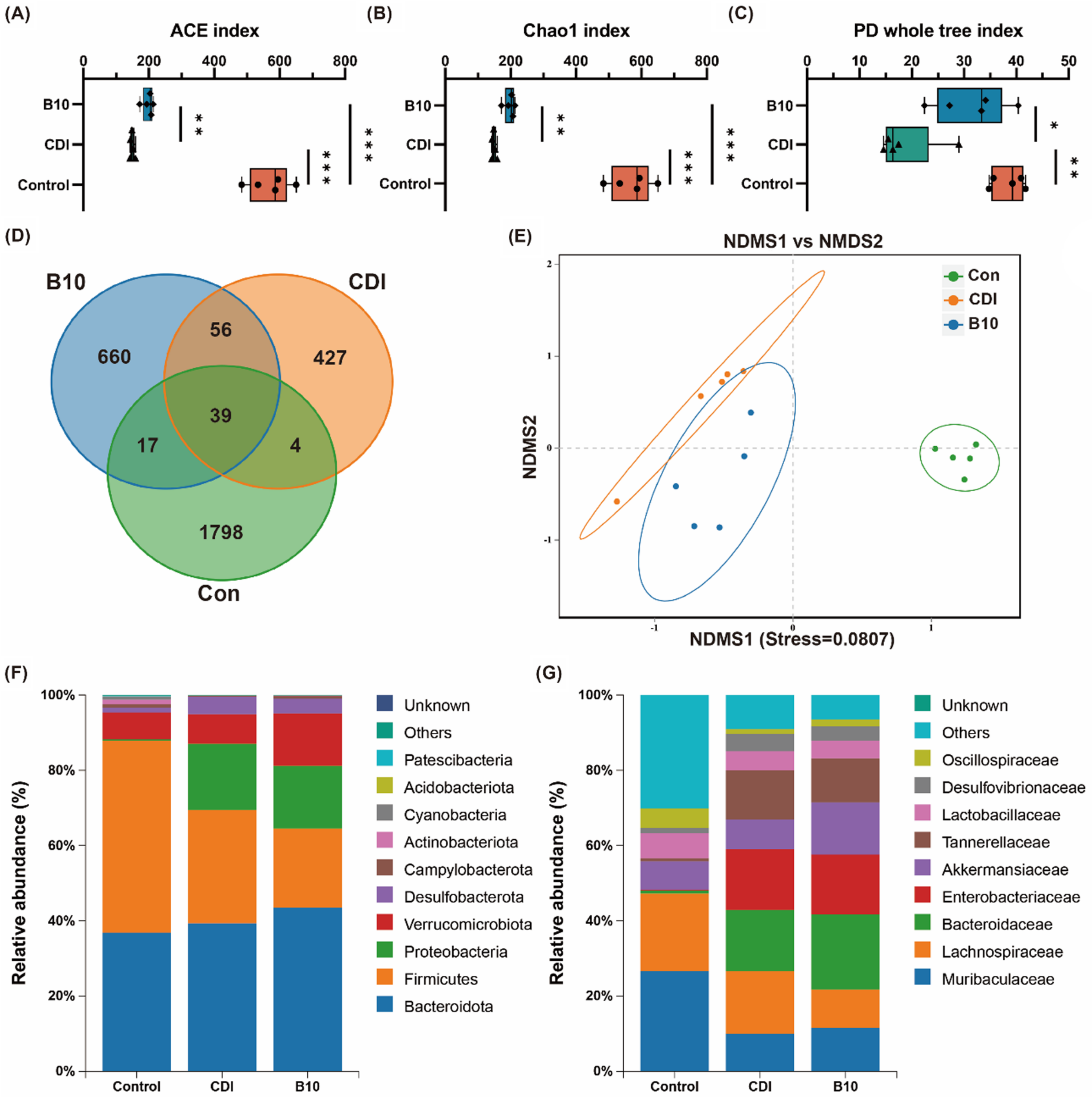

FIGURE 6 B10 restores the alpha diversity of the intestinal microbiota while affecting the microbiota constitution. (A) ACE index. (B) Chao1 index. (C) PD whole tree index. (D) Venn diagram of all three groups at OTUs level. (E) NMDS based on Unweighted UniFrac algorithm. (F) Colony composition of the three groups at the phylum level. (G) Colony composition of the three groups at the family level. The number of samples per group is $n = 5$. Significance calculated using unpaired t test (A–C). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

illustrates that the species diversity of the B10 group was dramatically greater than that of the CDI group and was no longer significantly distinct from that of the Control group (Figure 6C).

The Venn diagram shows the number of common and endemic species among the three groups, visualizing the overlap of characteristics among the samples (Figure 6D). The number of species shared by the three groups was only 39, accounting for only a small proportion of each group; the proportion of species shared by the CDI and B10 groups was 18.1% and 12.3%, respectively. NMDS on the basis of the Unweighted UniFrac algorithm showed variations among the three microbiota groups. Additional NMDS analysis for CDI and B10 groups showed a Stress value of 0.1059 along with PERMANOVA as well as ANOISM of 0.009 and 0.011, respectively, indicating a significant composition discrepancy between the CDI and B10 groups (Figure 6E).

At the phylum level, there was great dissimilarity in the composition of the microbiota of the three groups (Figure 6F). In descending order of percentage, the Control group was mainly composed of Firmicutes (51.05%), Bacteroidota (36.86%), Verrucomicrobiota (7.15%), Desulfobacterota (1.33%) and Actinobacteriota (1.19%). By contrast, CDI caused a major decrease in Firmicutes (30.11%) and Actinobacteriota (0.03%), and a serious increase in Proteobacteria (17.59%) and Desulfobacterota (4.62%). Although after B10 treatment, there was some rise in Bacteroidota (43.51%) and a remarkable rise in Verrucomicrobiota (13.92%); whilst Firmicutes (21.01%), Proteobacteria (16.69%) and Desulfobacterota (3.91%) all showed a decreasing trend. When analysed at the family level, when compared to the CDI group, therapy with B10 restored to some extent Muribaculaceae, in the intestine which have the ability to utilize mucin monosaccharides, Bacteroidaceae, and Oscillospiraceae, considered as potential probiotics, and significantly increased the relative abundance of Akkermansiaceae, while simultaneously decreased the level of Lachnospiraceae (Figure 6G).

The LEfSe analysis was then used to compare the distinct species of colonic microbiota between the three groups, and all species with Linear discriminant analysis (LDA) scores ≥4 are shown (Figure 7A,B; Table S3). Among the species enriched in the Control group were *Alistipes*, a group proven to be protective against colitis, Muribaculaceae, which can utilize mucin monosaccharides, and the Prevotellaceae along with the Eubacterium coprostanoligenes group; the B10 group showed enrichment of *Akkermansia*, *Bacteroides*, and unclassified Enterobacteriaceae. Contrarily, numerous harmful bacteria, such as *C. difficile*, *Escherichia Shigella*, *Robinsoniella*, *Klebsiella*, unclassified Desulfovibrionaceae were abundant in the CDI group; also concentrated were *Parabacteroides gordonii*, unclassified *Lachnoclostridium*, and, an uncultured Bacteroidales bacterium. Thus, these results show that treatment with B10 allows the structure of the entire intestinal microbiota to be restored to a healthy balance, even though the species richness is still less than that of normal individuals, by restoring the balance of the microbiota and inhibiting the growth of many pathogenic microorganisms, including *C. difficile*, to compensate for the dysbiosis of the CDI-induced colony.

B10 suppresses the harmful bacteria and restores keystone species

The differences between the three groups at the family level were analysed using ANOVA and showed all species whose abundance in the B10 group was greater than 0.01% and multigroup (*p*) was less than 0.05 (Figure 8A). B10 treatment generally brought the colony structure closer to that of healthy individuals, that is the Control group, and in particular the depleted four families Marinifilaceae, Rhodocyclaceae, Streptococcaceae and unclassified Gastranaerophilales due to CDI were restored, and the levels of Prevotellaceae, unclassified Bacteroidales, Akkermansiaceae and Bacteroidaceae were elevated significantly, whilst the levels of Ruminococcaceae, unclassified Bacilli, and Morganellaceae were significantly diminished. To locate the key species in the CDI group versus the B10 group, both groups were analysed at the family level with the random forest algorithm (Figure 8B). Among the top 15 species with MeanDecreaseGini, only Morganellaceae, Lachnospiraceae and Peptostreptococcaceae had higher relative abundance in the CDI group than in that of the B10 group, while all others were more abundant in the B10 group; meanwhile, Streptococcaceae, Oxalobacteraceae, Flavobacteriaceae, Saccharimonadaceae, Selenomonadaceae and Eubacteriaceae were exclusive to the B10 group, while depleted in the CDI group.

Spearman's rank correlation analysis was performed at the genus level and screened data with correlations greater than 0.2 and *p*-values less than 0.05 were used to construct correlation networks to determine the interaction relationships between different species. Intra-group analysis of CDI revealed a broad negative correlation of *Akkermansia* and *Bacteroides*, while these two are strongly positively correlated (Figure 8C). The intra-group analysis of B10, a further step, revealed the interaction of *Akkermansia* and *Bacteroides* with other species, as well as the multiple inhibitory effects of unclassified Enterobacteriaceae (Figure 8D). Also, *Klebsiella* and unclassified Desulfovibrionaceae, which are enriched in the CDI group, were uncovered in relation to other species that can be suppressed by species such

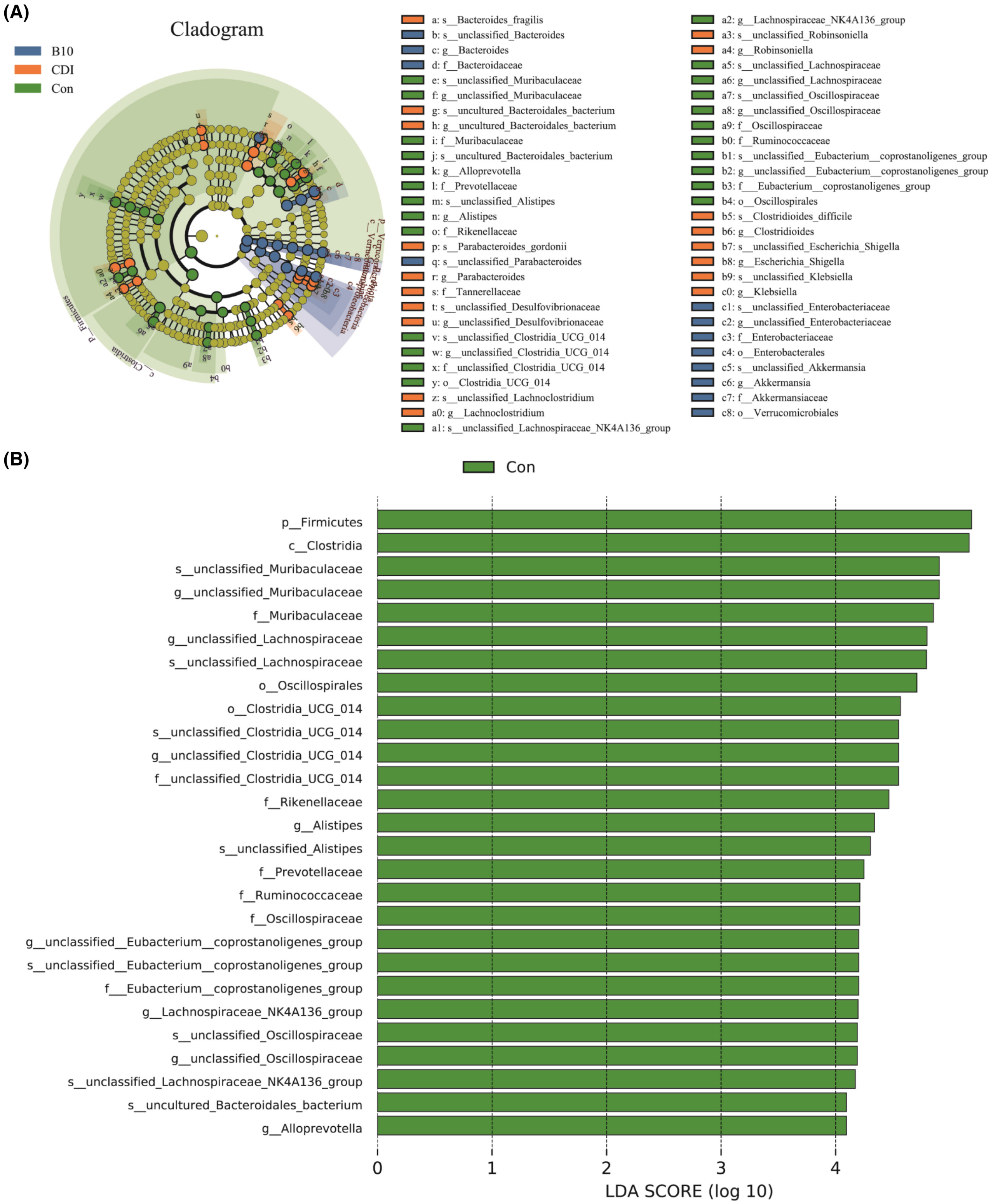

FIGURE 7 LEfSe analysis results. (A) LEfSe cladogram. (B) The taxa with Linear discriminant analysis scores ≥4.0 between all three groups, suggesting stronger discriminatory features that may have functional or taxonomic relevance, see Table S3 for details.

as *Ligilactobacillus*, *Streptococcus*, and *Lactobacillus*. To compare the changes completely and accurately in species relationships brought about by B10 treatment, the two sets of data were analysed together. *Staphylococcus*, positively correlated with both *Akkermansia* and *Bacteroides*, showing an ability to inhibit growth of many potentially harmful species, including *Parabacteroides* (Figure 8E).

All indicators that presented significance before and after therapy in relation to the species (genus level) of

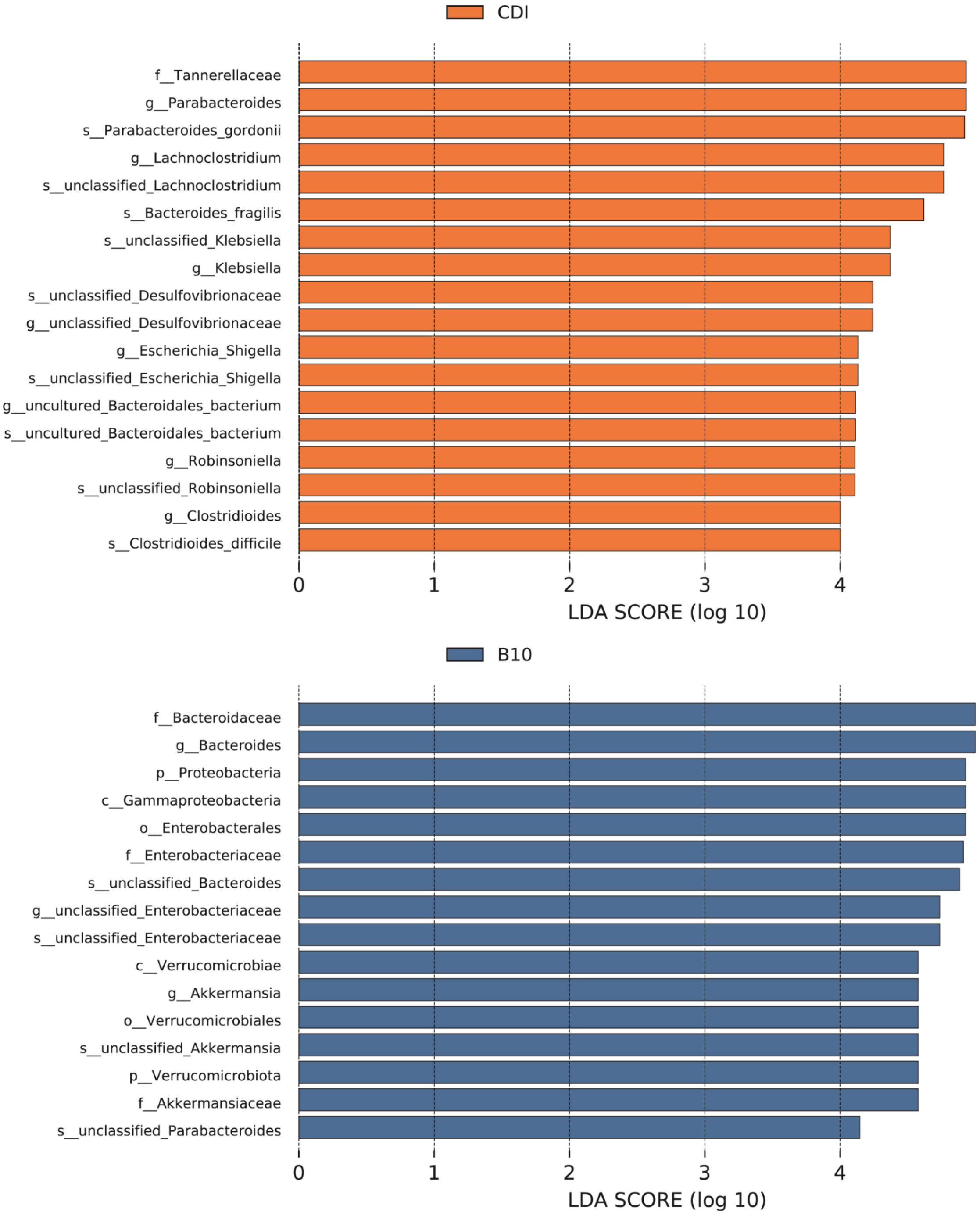

FIGURE 7 (Continued)

the colonies were integrated (Figure 9). Obviously, species that were negatively correlated with inflammatory factors and TcdB were mostly positively correlated with Claudin-1 expression and vice versa. Colorectal length, on the other hand, did not show the same correspondence with other indicators, and among all these species with relative abundance greater than 0.01%, only *Providencia*, a member of Morganellaceae, exhibited significantly negative correlation with colorectal length. Here, we defined as positive those species that were negatively correlated with inflammatory factors and TcdB or positively correlated with Claudin-1: *Streptococcus*, *Odoribacter*, *Akkermansia*, *Bacteroides*, *Staphylococcus*, unclassified Enterobacteriaceae and unclassified Muribaculaceae, respectively. As can be seen, these species with positive significance were either directly supplemented by B10 or recovered to higher amounts after treatment compared to the CDI group. While those with negative significance, such as unclassified Bacilli, *Robinsoniella*, *Escherichia Shigella* are mostly CDI-group enriched species, including the previously mentioned *Providencia*. Therefore, one can

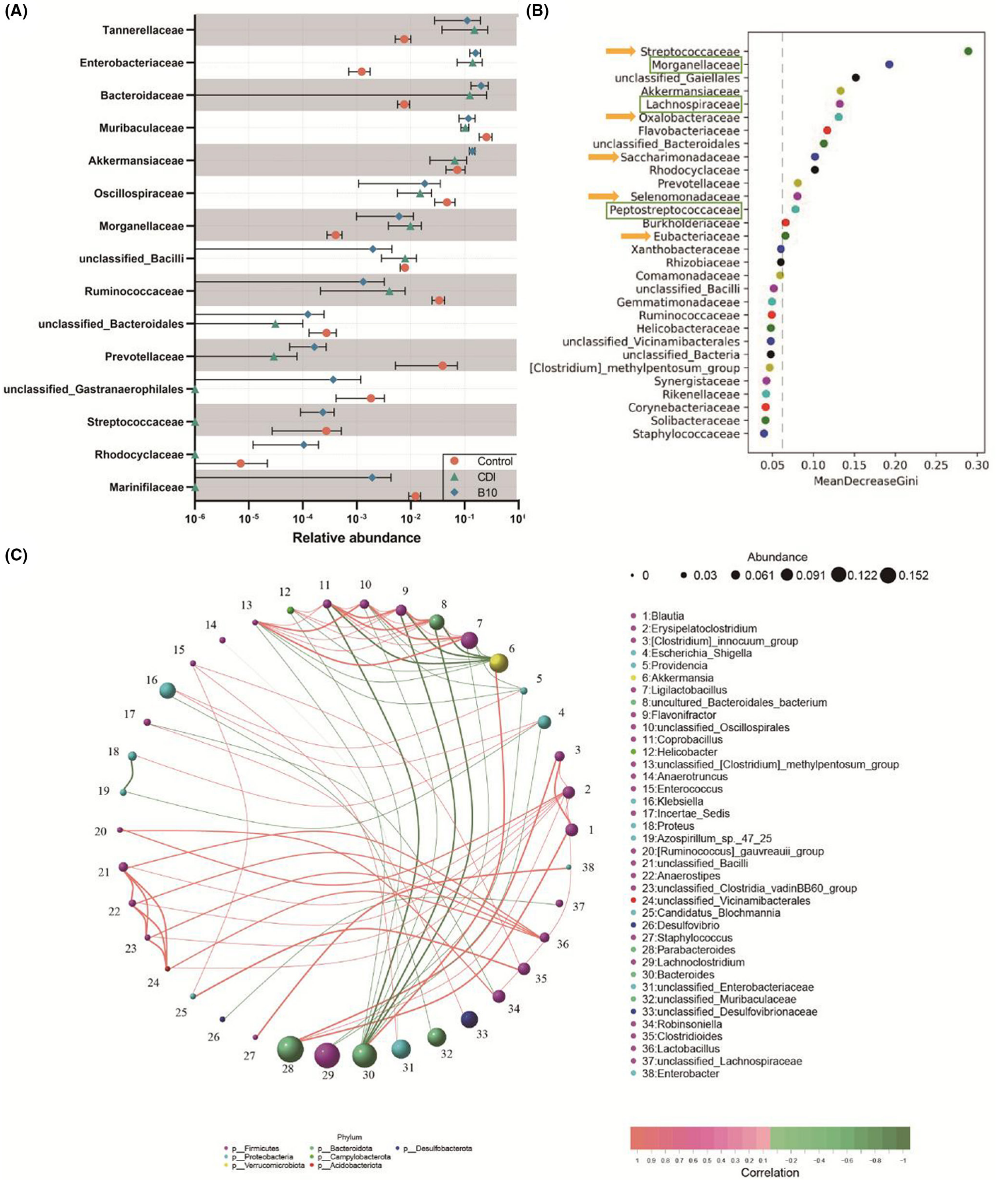

FIGURE 8 B10 recovered numerous species and exhibited a wide range of interactions. (A) Species with relative abundance greater than 0.01% and multigroup (p) less than 0.05 in the B10 group at the family level, significance calculated using ANOVA. (B) Random forest analysis of the B10 group versus the *Clostridioides difficile* infection (CDI) group, with yellow arrows labelling species endemic to the B10 group and green rectangles labelling species with higher relative abundance in the CDI group. (C) Correlation network within the CDI group. (D) Correlation network within the B10 group. (E) Correlation network constructed by joint analysis of both CDI and B10 groups.

be sure that the administration of B10 inhibited numerous potentially threatening species, whilst reviving the levels of beneficial bacteria, even some that had been completely exhausted in the CDI group, thus restoring the intestinal microbiota to a balanced state for therapeutic purposes.

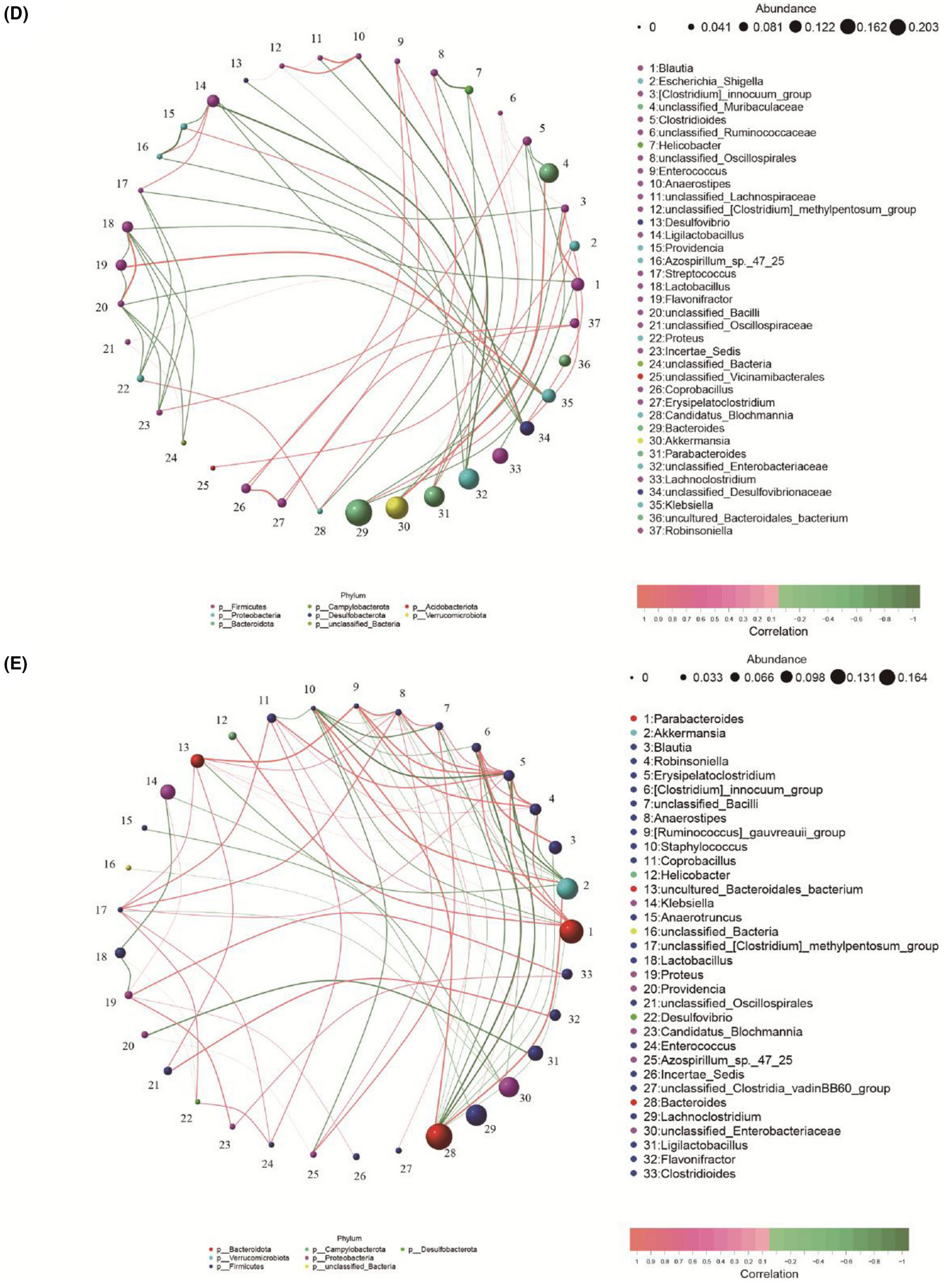

FIGURE 8 (Continued)

DISCUSSION

Although vancomycin is still the first choice for the treatment of CDI, the high recurrence rate of CDI makes it a priority to prevent the recurrence of CDI. It has been shown that antibiotics against *C. difficile* may lead to further destruction of the intestinal colonies and thus increase the chance of recurrence of the infection (Pochapin, 2000). Therefore, once antibiotic treatment is stopped, the intestinal microbiota needs to

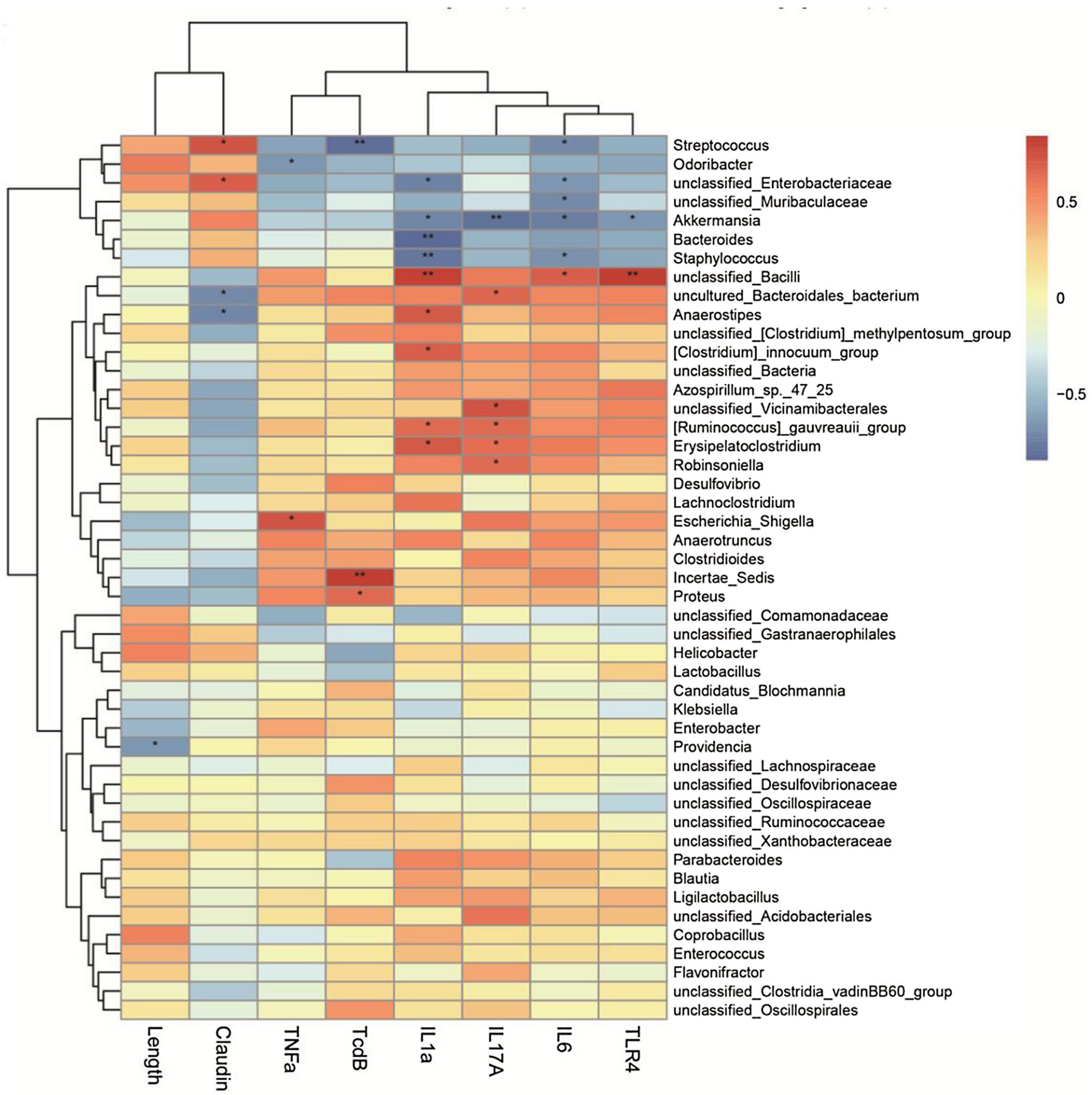

FIGURE 9 Correlation of individual species with *Clostridioides difficile* infection-related indicators at the genus level.

be restored immediately to ensure colonization of species that are resistant against *C. difficile*. To address this problem, FMT is gradually replacing antibiotic therapy as the mainstream therapy. Likewise, there is a need to face some of the problems that have been exposed, such as the transfer of antibiotic-resistant microorganisms due to FMT and resulting deaths caused by this, and the potential relationship with diseases such as peripheral neuropathy and rheumatoid arthritis (Wang et al., 2019). Therefore, there are now also FMT-based, further ensuring the safety of WMT, which are needed to be understood, explored and tackled (Zhang et al., 2020).

Our results showed that the SMC B10 is effective for the treatment of CDI in vitro. It was assumed that the gavage treatment was initiated after the onset of symptoms of CDI, that is, therapeutic administration, rather than prophylactic administration before the occurrence of symptoms. It can be found that B10 significantly improved the survival rate of diseased mice, even to the level of prophylactic administration, meaning that there was no mortality. At the same time, B10 significantly improved the physiological condition in the cecum, restored the mucus layer to reduce the chance of reinfection of the host with *C. difficile* and reduced the inflammatory response as well as increased the expression level of tight junction proteins. Furthermore, it can be reasonably speculated that B10 inhibited the proliferation of many potentially harmful bacteria that produce LPS, and downregulated the expression of inflammatory factors through multiple pathways including this one, in combination with the changes in mRNA expression levels and bacterial community structure. More accurately, B10 treatment led to the tendency of the intestinal microbiota to develop to that of healthy individuals, and the culture directly suppressed the growth of *C. difficile*, while simultaneously replenishing the numbers of *Akkermansia* and *Bacteroides*, restoring the levels of a wide range of core intestinal bacteria and extensively restraining the levels of potentially harmful bacteria.

In this study, a great boost in the relative abundance of *Akkermansia* and *Bacteroides* in the gut after B10 supplementation can be visualized. A previous study showed that prophylactic supplementation of *A. muciniphila* effectively mitigated the effects of CDI by reducing the levels of various inflammatory factors including IL-17A, IL-1α, IL-6, etc. (Wu, Xu, Gu, Chen, et al., 2022; Wu, Xu, Gu, Wang, et al., 2022). Similarly, there is also a study of prophylactic supplementation of *B. fragilis* to achieve a therapeutic effect. They proved that *B. fragilis* prophylactic treatment can ameliorate morbidity and mortality in a CDI mouse model by modulating gut microbiota and alleviating barrier destruction (Deng et al., 2018). A defined community of gut bacterial species exhibited robust colonization resistance against pathogenic *E. coli*, and the defined bacterial community enabled a mechanistic interrogation of species and genes on microbiome-associated phenotypes (Cheng et al., 2022). In combination with these studies, it appears that the keystone species play an essential role in the process of microbiota homeostasis. In the area of CDI treatment, there is also the product RBX2660 which has successfully completed phase III clinical. Results demonstrated that 70.6% of participants treated with RBX2660 remained free of CDI recurrence through 8 weeks (Khanna et al., 2022).

Prophylactic administration of *Lactobacillus* is often considered an effective way to prevent and treat CDI (Rätsep et al., 2017; Wu, Xu, Gu, Chen, et al., 2022; Wu, Xu, Gu, Wang, et al., 2022), but analysis of interactions revealed a positive correlation between *Lactobacillus* and some potentially harmful bacteria, and *Lactobacillus* did not fit this studies definition of a positive species, even though it also inhibited some other harmful bacteria and the culture directly suppressed the growth of *C. difficile*. There is a need for more adequate studies to understand the specific role of *Lactobacillus* in therapeutic administration. And, secondary metabolites in the gut were not quantified, going only by IL-17A levels and prediction of metabolic pathways. The many species that were depleted by CDI and re-emerged after B10 treatment were likewise not specifically investigated, which also needs to be further explored.

In conclusion, probiotic bacterial strains were selected to construct the SMC B10, and therapy with B10 significantly improved symptoms and tissue damage in mice infected with *C. difficile*. At the same time, the colony structure and metabolic pathways were ameliorated to prevent the recurrence of *C. difficile*. Thus, the SMC constructed with probiotic strain with antibacterial and anti-quorum sensing activities may be a promising approach for the treatment of CDI.

AUTHOR CONTRIBUTIONS

Jinqiu Liu: Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); writing – original draft (equal). **Wei Zhu:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Duncan James Lessing:** Visualization (equal); writing – review and editing (equal). **Weihua Chu:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

No potential conflict of interest was reported by the author(s).

DATA AVAILABILITY STATEMENT

The raw data for the 16S rRNA gene sequence have been deposited in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession number: PRJNA923450. The other data of this study are available on request from W.C.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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