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Metal ion-procyanidins coating enhances the nutrition intervention of *Weizmannia coagulans* in colitis based on gut microbiota-brain axis

Shanshan Tie^{a, c}, Xue Zhang^a, Jiawei Ding^a, Qing Zhang^a, Yannan Chen^{b*}, Ying Wu^{a, c}, Jianrui Sun^a, Pingping Tian^a, Yuwan Li^a, Min Zhang^a, and Shaobin Gu^{a, d*}

^a College of Food and Bioengineering, Henan University of Science and Technology, Luoyang 471023, China

^b College of Food Science and Engineering, Shandong Agricultural University, Taian 270018, China

^c Henan Engineering Research Center of Food Material, Henan University of Science and Technology, Luoyang 471023, China

^d Henan Engineering Research Center of Food Microbiology, Luoyang 471000, China

ABSTRACT: The purpose of this study was to design and prepare *Weizmannia coagulans* BC99 delivery system, and analyze its intervention effect on ulcerative colitis from gut microbiota-brain axis. The selection of Fe³⁺-procyanidins (PC) coatings was used to load BC99, and the prepared BC@Fe³⁺-PC system was rod-shaped, with a low particle size of 1687.48 nm. The coating could effectively improve the survival rate of BC99 under pH and bile salt conditions, and allow BC99 to pass through the gastrointestinal tract. BC@Fe³⁺-PC administration could effectively reduce the score of disease activity index of colitis mice, inhibit the level of oxidative stress and inflammation, and regulate the diversity, composition and abundance of intestinal microorganisms related to inflammation, anxiety and depression. Moreover, the number of neuronal cells, the expression of neurotransmitters, and the level of short-chain fatty acids increased. In summary, BC99 delivery system effectively alleviated the comorbidity of colitis with anxiety and depression by modulating gut microbiota-brain axis.

Keywords: *Weizmannia coagulans* BC99; Procyanidins; Gut microbiota-brain axis; Ulcerative colitis; Depression

1. Introduction

Ulcerative colitis (UC) is an autoimmune intestinal disease caused by genetic, environmental, microbial and immune factors, with chronic and lifelong recurrence tendency [1, 2]. The typical clinical symptoms of UC include weight loss, abdominal pain, diarrhea, rectal bleeding, and fecal incontinence [3]. Although the exact etiology and pathogenesis of UC are unclear, the current UC disease models generally focus on the study of intestinal-related mechanisms, including oxidative stress, immune system and gut microbiota disorders [4, 5]. As the fluctuation of the disease courses, patients will also experience symptoms related to brain physiological dysfunction, such as fatigue, anxiety and depression. Therefore, it is difficult to find and solve the potential causes of UC only by focusing on the mechanism of inhibiting intestinal immunity.

Gut microbiota-brain dysregulation has been recognized by the scientific community as a crucial pathway to the understanding of intestinal diseases [6]. This represents the complex bidirectional signaling

*Corresponding author
ynchen04@163.com; shaobingu@haust.edu.cn

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system between gut microbiota and brain, actively communicating through the neuroendocrine system, vagus nerve, and hypothalamic-pituitary-adrenal axis pathways [7-9]. The reciprocal relationship between the two is essential to maintain immune regulation, energy balance, mental health, and even the brain function of the host. For UC patients, the adverse information such as the secretion of inflammatory cytokines, the damaged intestinal barrier, and imbalance of gut microbiota in the colon can be fed back to the brain through gut-brain axis, resulting in emotional and behavioral disorders such as nervousness, anxiety, and depression [10, 11]. The specific mechanisms involved are as follows: (i) neurotransmitters, i.e., γ -aminobutyric acid, norepinephrine, glutamate and dopamine can directly transmit signals from the intestine to the brain through neural networks; (ii) Inflammatory cytokines secreted by immune cells in the intestinal tract can regulate the brain; (iii) Metabolites produced by gut microbiota, such as short-chain fatty acids (SCFAs) and indole-3-propionic acid can also enter blood and brain to affect their behavior [12, 13]. These substance changes in the systemic circulation may breach the blood-brain barrier, leading to the occurrence of neuroinflammation and neuronal damage [14]. Considering the substantial prevalence of emotional and behavioral disorders in UC patients, nutritional intervention from the gut microbiota-brain axis may be an effective way to prevent colitis.

Probiotics are essentially living microorganisms that can bring many health benefits to the human body if ingested in an effective amounts [15]. Their health effects include improving gut microbiota balance, maintaining the intestinal microenvironment, enhancing immune response, lowering serum cholesterol, regulating epithelial and colonic function, thus reducing the occurrence of intestinal diseases [16, 17]. *Weizmannia coagulans* (*W. coagulans*), as an environmentally friendly probiotic, has the properties of spore formation, lactic acid production, and regulation of intestinal microenvironment. In 2016, *W. coagulans* was listed in “List of Available Bacteria in Food” by the National Health and Family Planning Commission of China [18, 19]. Using *W. coagulans* instead of anti-inflammatory or immunosuppressive drugs can effectively avoid the side effects of long-term use, which may be an effective alternative to maintain the balance of gut microbiota and alleviate colitis. However, how to maintain the viability of probiotics in complex gastrointestinal and pathological microenvironments is extremely challenging. The harsh physiological conditions, such as gastric acid and bile salt in the digestive tract, can seriously damage the vitality of probiotics, and the pathological environment of colitis can make it difficult for probiotics to colonize the intestinal tract [20, 21]. In addition, the constant peristalsis of the gastrointestinal tract also accelerates the excretion of probiotics, resulting in a low survival rate and limited intestinal colonization of probiotics [22, 23]. Interestingly, oral probiotic delivery systems such as nanoarmor, micro/nanoparticles, liposomes, biofilms and hydrogels, have been one of the promising approaches to overcome these limitations.

Polyphenols, such as procyanidins (PC), as secondary metabolites of plants, have good antioxidant, anti-inflammation, anti-bacteria, intestinal regulation, and other biological activities [24]. PC has poor absorption rate in the stomach and small intestine, and unabsorbed PC can be catabolized into more active microbial metabolites by microbiota in the colon [25]. More importantly, there are abundant pyrogallol groups

in the molecular structure of PC, which have good mucoadhesion to the intestinal surface for promoting the colonization of probiotics. Metal ions, such as Fe^{3+} and Ca^{2+} , as one of the essential components to maintain the normal life activities of the organism, play an indispensable role in the growth, development and metabolism of organisms by participating in various physiological processes [26, 27]. The pyrogallol groups of PC can react with different metal ions, and self-assemble to form a metal-phenolic network structure with good biocompatibility.

Herein, we designed and developed single-encapsulated *W. coagulans* BC99 formulation using a metal ion-PC nanocoating strategy, and analyzed their nutritional intervention effect on colitis. Oral route is one of the commonly used ways for delivering probiotics due to its advantages of high safety, convenient administration, non-invasiveness and patient compliance. After oral administration, this metal ion-PC nanocoating with potent antioxidant and resistance to the extreme stomach environment could protect the loaded BC99, and achieve their intestinal delivery. Subsequently, the nutritional intervention effect of BC99 formulation on colitis was comprehensively analyzed based on gut microbiota-brain axis from the perspectives of inhibiting oxidative stress and inflammatory response, regulating intestinal barrier, remodeling gut microbiota, and alleviating cognitive impairment. This provides a simple protective strategy for oral delivery of probiotics, showing excellent potential in nutritional interventions in colitis.

2. Materials and methods

2.1 Materials

Procyanidins (PC) were purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd (Tianjin, China). FeCl_3 , CaCl_2 and MgCl_2 were bought from Tianjin Damao Chemical Reagent Company (Tianjin, China). *W. coagulans* BC99 was provided by Henan University of Science and Technology. Dextran sulfate sodium (DSS) salt was bought from Dalian Meilun Biotechnology Co., Ltd (Dalian, China).

2.2 Activation of BC99 strain

BC99 was inoculated in liquid medium at 37 °C for 24 h, and then treated 3 times to active BC99. Subsequently, probiotic cells were collected after centrifugation, and washed twice with purified water. The obtained BC99 cells were resuspended in normal saline solution at a final concentration of 1.50×10^8 CFU/mL.

2.3 Effects of metal ions and procyanidins on the growth of BC99

Before loading BC99, the effect of metal ions and PC on the vitality of BC99 were studied. CaCl_2 , FeCl_3 , MgCl_2 , and PC were co-incubated with BC99 probiotic for 16 h, and analyzed the effect of metal ions (0, 10, 20, 50, 75, 100 $\mu\text{g/mL}$) and PC (0, 10, 50, 100, 150, 200, 250, 300 $\mu\text{g/mL}$) on the OD_{600} value of probiotics.

2.4 Preparation and characterization of metal ion-procyanidin complexes

To verify whether the selected metal ions and PC could chelate to load BC99, the above concentration of metal ions and PC that could promote the vitality of BC99 were selected to react at room temperature.

The detailed operation steps were as follows: PC (0.05 mL) and 0.05 mL of CaCl_2 (or FeCl_3 , MgCl_2) were added to 0.90 mL HEPES buffer, and vortexes were carried out immediately for 3 s after addition. Subsequently, the three types of metal ion-PC complexes were incubated at room temperature for 4 h. The particle size of PC, Ca^{2+} -PC, Fe^{3+} -PC, and Mg^{2+} -PC complexes was analyzed by laser particle size analyzer (Better size 2600, Better, China). The microscopic morphology and molecular interaction were recorded by scanning electron microscope (SEM, TM3030Plus, Hitachi High-Tech, Japan) and Fourier transform infrared spectroscopy (FTIR, Vertex70, Bruker, Germany).

2.5 Synthesis and characterization of BC99 delivery system

In this study, the metal-phenolic coordination method was used to load BC99. Briefly, 50 μL of CaCl_2 (or FeCl_3 , MgCl_2) and 50 μL of PC were added to the obtained suspension of BC99 (900 μL). After vortex treatment, these solutions continuously stirred for 30 min. According to the different types of metal ions added, the obtained BC99 delivery systems were named as BC@Ca^{2+} -PC, BC@Fe^{3+} -PC, and BC@Mg^{2+} -PC, respectively. Transmission electron microscope (TEM), SEM and laser particle size analyzer were used to characterize the micromorphology and particle size of BC99, BC@Ca^{2+} -PC, BC@Fe^{3+} -PC and BC@Mg^{2+} -PC.

2.6 Stability experiment

BC99, BC@Ca^{2+} -PC, BC@Fe^{3+} -PC and BC@Mg^{2+} -PC were inoculated in liquid medium at pH 2.0 and 6.8 for 2 h, then 0.1 mL samples were spread on the plate and cultured at 37 °C for 48 h. The acid tolerance of probiotics was reflected in the intuitive colony photos and the number of viable bacteria. The bile salt stability test was using 0.3 mg/mL bile salt instead of different pH, and incubated with the free and loaded BC99 for 2 and 4 h.

2.7 Simulated digestion experiment

To evaluate the protective efficacy of metal ion-PC complex against BC99, the activity of BC99 was monitored under simulated digestion conditions. The detailed operation process referred to the steps reported by Zhang *et al.*, i.e., free BC99 and BC@Fe^{3+} -PC incubated with the formulated simulated gastric and intestinal fluids [23]. The obtained samples treated using the coating plate method, and the number of viable bacteria in each group was counted after 48 h incubation.

2.8 Hemolysis test

Blood samples from Kunming mice were collected and processed according to the method previously reported by Tie *et al* [28]. Using NaCl and TritonX-100 as control, the blood compatibility of BC99, Fe^{3+} -PC and BC@Fe^{3+} -PC was evaluated by photography and UV spectrophotometer.

2.9 DSS-induced colitis mouse model

The male Kunming mice (4 weeks old, 20 ± 22 g) were obtained from Henan SCBS Biotechnology Co., Ltd (Henan province, China). All animal trails conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU, and the guidelines

and animal protocol [SCXK(Yu)2020-0065] were approved by the ethical committee of Henan University of Science and Technology.

Colitis was induced for 7 consecutive days with 5 wt% DSS aqueous solution instead of drinking water. Forty mice were randomly divided into 4 groups, namely Con, DSS, BC99, and BC@Fe³⁺-PC - treated groups. After 1 week of conventional dietary adaptation, mice were orally administered with 0.3 mL of BC99, BC@Fe³⁺-PC (BC99 3.0×10⁸ CFU/mL, PC 150 µg/mL) or PBS solutions for 14 consecutive days, respectively. Subsequently, 5 wt% DSS solution was used instead of drinking water, and the health status of mice was monitored daily during this period, focusing on the important indicators of body weight changes, food intake, fecal bleeding, and fecal consistency. The mice euthanized at the end of the experiment, and blood, feces, brain, colon, spleen and other organs were collected.

2.10 Behavioral experiment

Open field and forced swim experiments were used to record the anxiety and depression-like behavior of mice on the seventh day of DSS induction. In the open field experiment, mice were placed in collection box with a length and width of 52 cm, and the movement process of mice within 6 min was recorded by a camera system. In the forced swim experiment, mice were placed in the same device with water temperature of 25 °C and water depth of 30 cm, and the swimming and immobility behaviors of mice were evaluated during 5-min swimming period.

2.11 Histology staining

Colon, brain, heart, liver, spleen, lung, kidney and testis were fixed with paraformaldehyde, embedded in paraffin and stained with Hematoxylin & eosin (H&E) according to the standard procedure. The histological evaluation of colon was carried out according to the degree of inflammation, ulceration and crypt injury [29], and brain tissues were analyzed according to the neuronal structure and cell number of cerebral cortex, hippocampal cornu ammonis (CA) 1, CA3 and dentate gyrus (DG) regions. Staining results were observed by Leica CMS GmbH fluorescence microscope (Wetzlar, Germany).

2.12 Detection of oxidative stress, inflammatory cytokines and neurotransmitter levels

The colon tissue samples were weighed and homogenized with a 9-fold volume of 3% saline solution in an ice bath. The supernatant was obtained after centrifugation at 5000 g for 10 min, and the levels of glutathione (GSH), superoxide dismutase (SOD), myeloperoxidase (MPO) and malondialdehyde (MDA) were detected using the corresponding assay kits. Inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-10 in serum was analyzed. Brain was treated in the same manner as colon tissue, and the concentration of 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (γ-GABA), dopamine (DA) and brain-derived neurotrophic factor (BDNF) were detected by the assay kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.13 16S rRNA sequencing

The mouse feces collected on the last day were analyzed using 16S rDNA sequencing technology, and small fragment libraries were selectively constructed by sequencing. The primary processing of sample involved whole-genome DNA extraction, PCR amplification, product purification, amplicon quantification and in silico sequencing. Subsequently, the composition of microbiota, relative abundance, inter-group differences, similarity data, and functional prediction were analyzed.

2.14 Detection the level of short-chain fatty acids

The levels of SCFAs in mouse feces were analyzed by Thermo TSQ 9000 gas chromatography. After stool samples were milled, homogenized, centrifuged, filtrated and other treatment, the content of SCFAs was recorded based on the method reported by Zhai *et al.* using chromatographic grade acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid as standards [30].

2.15 Statical analysis

The experiments were repeated at least 3 times, and results were expressed as mean $\pm$ standard deviation. One-way ANOVA was performed on the data using SPSS 19.0 software, where $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 The biocompatibility of metal ions and procyanidins

To evaluate the effect of nanocoating materials used on *W. coagulans* BC99, the biocompatibility of different concentrations of Ca^{2+} , Fe^{3+} , Mg^{2+} and PC was studied. Generally, the higher the concentration of BC99 in the bacterial solution, the higher the absorbance value. According to the different absorbance of bacteria solution, it can reflect the change of its bacterial concentration, and then analyze the influence of metal ions and PC on the growth of BC99. As shown in Fig. 1A, with the increase of Ca^{2+} concentration, the absorbance value of BC99 bacteria shows an increasing first and then decreasing trend. When the content of Ca^{2+} in the medium was 50 $\mu\text{g/mL}$, the absorbance value of BC99 bacteria solution reached the highest, increasing by 10.46%. Some studies have shown that Ca^{2+} plays an vital role in transport, nutrition and metabolism process of probiotics [31]. The presence of Ca^{2+} as salts could promote the survival, activity and growth of probiotics, which was consistent with the results of this experiment.

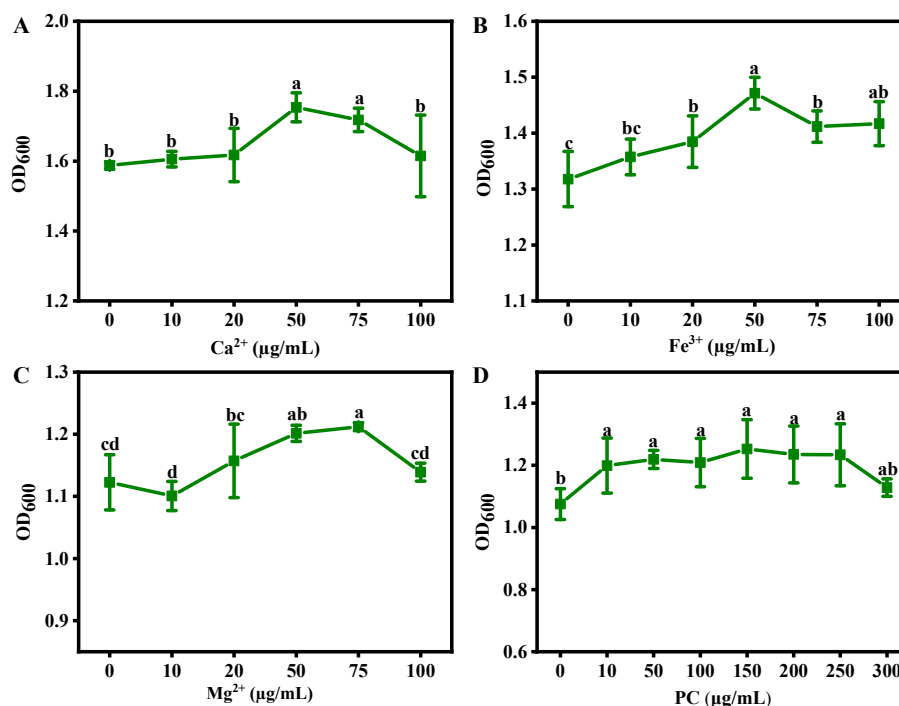


Fig. 1. Effect of different concentrations of (A) Ca^{2+} , (B) Fe^{3+} , (C) Mg^{2+} and (D) procyanidins (PC) on the vitality of BC99.

The absorbance value of BC99 incubated in a medium containing different concentrations of Fe^{3+} and Mg^{2+} also showed a similar trend, and OD value of BC99 was relatively high when the content of metal ions was 50 $\mu\text{g/mL}$ (Fig. 1B-C). In addition, the vitality of BC99 was not inhibited by different metal ions in the range of 0-100 $\mu\text{g/mL}$. PC, as a kind of polyphenols, can also promote the proliferation of BC99. The OD₆₀₀ of BC99 was relatively higher at a PC concentration of 150 $\mu\text{g/mL}$ (Fig. 1D). Banerjee *et al.* reported that polyphenolic extracts in native form, or from different sources like grape seeds and red fruits, had antibacterial effects against pathogens, but possessed growth-promoting effects on probiotics [32]. These indicated that the metal ions and PC used for embedding probiotics had good biocompatibility.

3.2 Characterization of coordination of metal ions and procyanidins

Before embedding, the coordination reaction between metal ions (50 $\mu\text{g/mL}$) and PC (150 $\mu\text{g/mL}$) that could promote the growth of BC99 was studied. As shown in Fig. 2A, metal ions are directly mixed with PC in HEPES buffer solution, and the molecular structure of PC containing pyrogallol functional groups can provide complexation sites for metal ions, thus promoting the self-assembly of metal-phenol network. The solution colors of Ca^{2+} , Fe^{3+} , and Mg^{2+} mixed with PC were light brown, black and light brown, respectively (Fig. S1A). The result of SEM images showed that the morphology of PC was irregular flake structure. The morphology of Ca^{2+} -PC, Fe^{3+} -PC, and Mg^{2+} -PC complexes changed greatly, and they were spherical or network structures (Fig. 2B). The particle size of free PC ranged from 10.0 to 12.5 μm , and the particle sizes of Ca^{2+} -PC, Fe^{3+} -PC, and Mg^{2+} -PC complexes were less than 10.0 μm (Fig. 2C). The size of Fe^{3+} -PC complex was relatively low, 235.34 nm, which was related to the shrinkage of the network volume after the coordination of PC and iron ions. Polydispersity index (PDI) can be used to reflect the dispersion of particles in the solution. Generally, PDI value is lower than 0.3, which means that the distribution of particle in the

solution is uniform. The PDI value of Fe^{3+} -PC complex was 0.17, indicated that Fe^{3+} -PC complex had good dispersion property. Moreover, the particle size distribution of all samples was normally distributed and relatively narrow (Fig. S1B). FTIR results showed that -OH absorption peak of Ca^{2+} -PC, Fe^{3+} -PC, and Mg^{2+} -PC complexes shifted from 3419 cm^{-1} to 3406 , 3426 , 3417 cm^{-1} , and C=O absorption peak red-shifted from 1604 cm^{-1} to 1608 , 1608 , 1606 cm^{-1} , respectively, compared with that of PC (Fig. 2D). These changes indicated that carboxyl and hydroxyl groups had an important effect in the reaction of PC and different metal ions.

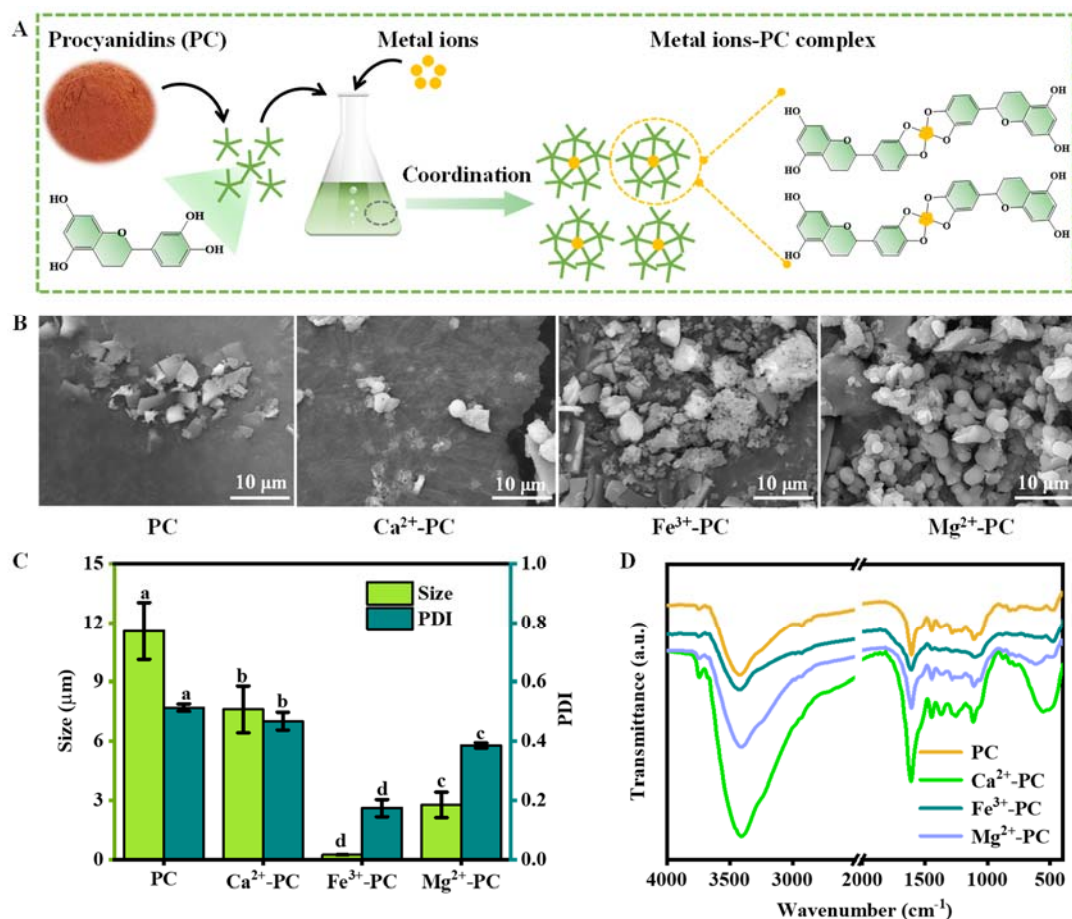


Fig. 2. Coordination chelation and characterization of metal ions and procyanidins (PC). (A) Schematic diagram of coordination reaction between metal ions and PC, (B) SEM images, (C) particle size, and (D) FTIR spectra of PC, Ca^{2+} -PC, Fe^{3+} -PC, and Mg^{2+} -PC complexes.

3.3 Characterization of BC99 delivery system

The BC@XX-PC delivery system was synthesized by metal-phenolic coordination method, and the detailed operation process was shown in Fig. 3A. PC and metal ions were added into BC99 bacteria suspension, respectively, and BC@XX-PC delivery system was obtained by magnetic stirring for 30 min. The morphology of delivery system was observed by TEM, the results showed the presence of light-colored coatings around BC@ Ca^{2+} -PC, BC@ Fe^{3+} -PC and BC@ Mg^{2+} -PC compared with free BC99 (Fig. 3B). Subsequently, SEM observed that the lyophilized delivery systems were all rod-shaped like BC99, but their morphology were uneven due to irreversible aggregation (Fig. S2). Particle size analysis results showed that compared with free BC99 probiotic, the particle size of BC99 loaded with Ca^{2+} -PC, Fe^{3+} -PC, and Mg^{2+} -PC

complex increased by 139.96, 209.09 and 126.86 nm, respectively, and all showed normal distribution (Fig. 3C and Fig. S3). These results proved that PC-metal ion complexes were successfully coated on the surface of BC99 probiotic.

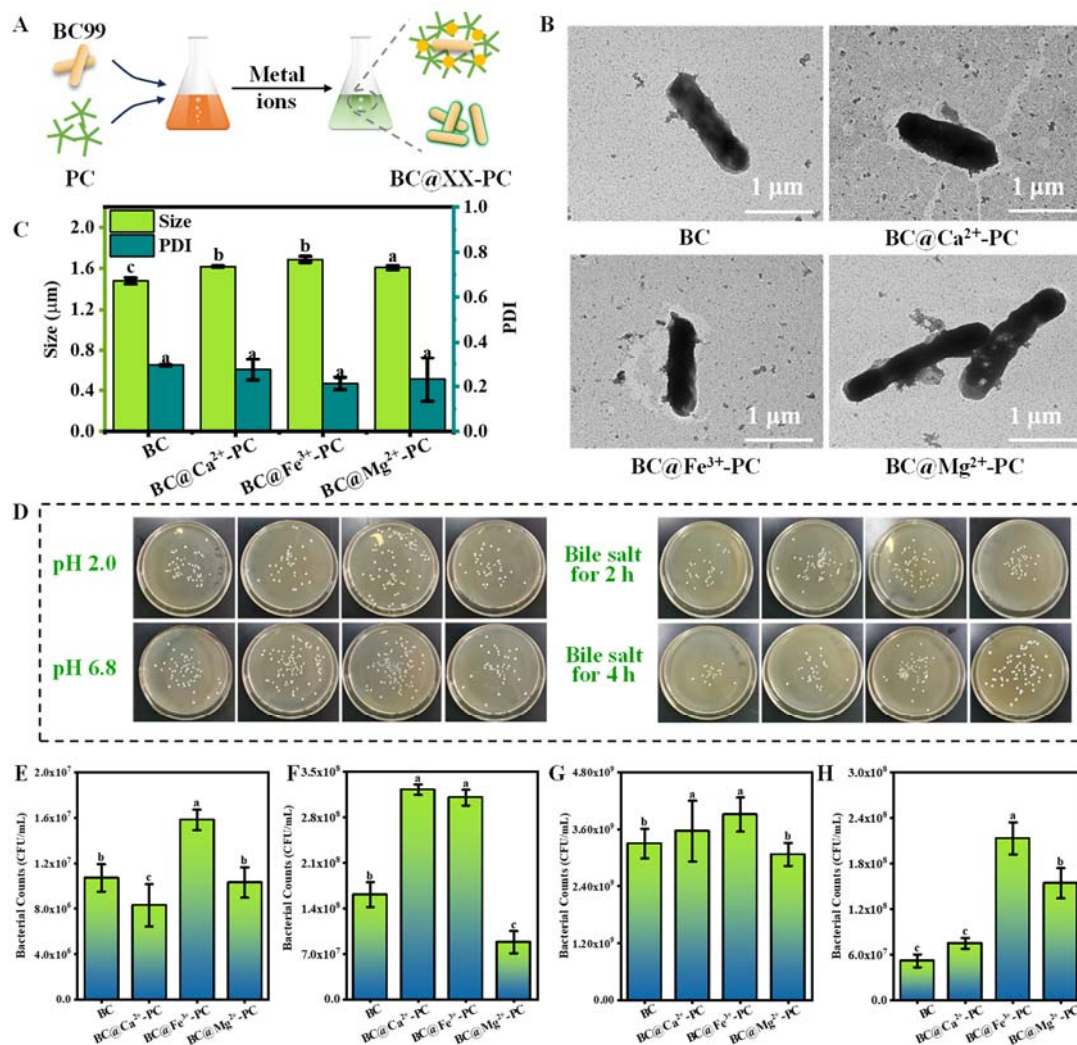


Fig. 3. Preparation and characterization of BC99 delivery system. (A) Schematic diagram of the preparation, (B) TEM images, (C) particle size and PDI index of BC, BC@Ca²⁺-PC, BC@Fe³⁺-PC and BC@Mg²⁺-PC. (D) The optical photographs of free and loaded BC99 resistant to adverse environments. Note: from left to right, BC, BC@Ca²⁺-PC, BC@Fe³⁺-PC and BC@Mg²⁺-PC. Bacterial numbers of free and loaded BC99 treated in (E) pH 2.0 for 2 h, (F) pH 6.8 for 2 h, (G) bile salts for 2 h and (H) bile salts for 4 h, respectively. Note: BC represent for BC99 probiotic.

3.4 Free and loaded BC99 resist the harsh physiological environment

Generally, probiotics can successfully survive and be transferred to the intestinal tract after oral administration, so as to better exert their beneficial effects. However, oral probiotics face some biological challenges, that is, the acidic and bile salts environment will affect the survival rate of probiotics [33]. Whether the activity of probiotics can be maintained after metal ion-phenol coating is the focus of attention. As shown in Fig. 3D-E, BC99 samples with the same bacterial concentration are incubated at pH 2.0 for 2 h, and the result observed in the bacterial growth plate shows that compared with free BC99, the bacterial number of BC99 loaded with Fe³⁺-PC complex are significantly improved, reaching 1.6 × 10⁷ CFU/mL, which is 1.48 times that of free BC99. Similar experimental results were also obtained by using pH 6.8 instead of pH 2.0, and the survival rate of BC@Ca²⁺-PC and BC@Fe³⁺-PC treatment increased by 1.61 × 10⁸ and 1.49 × 10⁸

CFU/mL compared with free BC99, respectively (Fig. 3F). However, Mg^{2+} -PC complex loading failed to improve the pH stability of BC99. This indicated that self-assembled Ca^{2+} or Fe^{3+} -phenol complex could improve BC99 viability under harsh environmental conditions. Moreover, after incubation in medium containing 0.3 mg/mL bile salt for 2 h, the viable bacteria number of BC99 group was 3.3×10^9 CFU/mL (Fig. 3G). The viable bacteria of BC@Ca^{2+} -PC and BC@Fe^{3+} -PC was slightly higher than that of the free BC99 group, but the difference was relatively low, which was related to the shorter incubation time of bile salt solution. As the incubation time was extended to 4 h, the concentration of viable bacteria in all BC99-loaded groups was much higher than free BC99 (Fig. 3H), indicating that BC99 had stronger tolerance to bile salt solution after embedding. Comparing the survival rate of different metal ions, Ca^{2+} , Fe^{3+} and Mg^{2+} , with PC complexes against BC99 probiotic under pH and bile salt conditions, it was found that Fe^{3+} -PC coating showed a better protective effect on BC99. The Fe^{3+} -PC coating could act as an “armor” to protect BC99 from the complex gastrointestinal environment. In the subsequent experiments, the biological effect of BC@Fe^{3+} -PC could be analyzed emphatically.

3.5 Enhanced the simulated digestion stability of BC99

To further evaluate the protective effect of Fe^{3+} -PC coating on BC99, free and loaded BC99 were treated in simulated gastric fluid (SGF) for 2 h and then moved to simulated intestinal fluid (SIF) for continued digestion for 2 h. The viable count of BC99 decrease by 5.66 log CFU/mL after digestion in SGF solution, and the viable count of BC99 loaded in Fe^{3+} -PC coating still maintain a high activity of 9.16 log CFU/mL, which was 63,924 times that of free BC99 (Fig. 4A). Centurion *et al.* used the coordination interaction between the catechol groups of polydopamine and Mn^{2+} for nano-embedding of lactic acid bacteria [34]. Subsequently, free and PDA-coated probiotics were incubated in SGF (pH 2.5) solution for 45 min, and the protection of PDA coating could enable the survival rate of probiotic up to 1.4 times compared to free probiotics. In this study, the incubation time was as long as 2 h at pH 2.0, and the Fe^{3+} -PC coating could effectively maintain the survival rate of BC99. After being transferred to SIF solution, BC@Fe^{3+} -PC still had a viable cells number of 8.90 log CFU/mL, which was 5.73 log CFU/mL higher than that of free probiotic BC99. Gao *et al.* used different types of polyphenols chelated with FeCl_3 for loading *Lactobacillus rhamnosus* GG (LGG), which were dispersed in SIF containing bile or trypsin (pH 6.8), respectively. Compared with free LGG, metal ion-polyphenol coatings could effectively improve the survival rate of LGG, and the number of surviving bacteria was about 2 log units higher than that of free LGG [35]. This proved that Fe^{3+} -PC coating effectively improved the survival rate of probiotic in the gastrointestinal tract.

3.6 Blood compatibility

Hemolysis study was carried out to analyze the biocompatibility of BC@Fe^{3+} -PC. As shown in Fig. 4B, the blood samples of TritonX-100 group have a bright red color compared with that of normal saline (NaCl), indicating hemolysis occurs due to the rupture of red blood cells. The hemolysis rate of erythrocytes co-incubated with free BC99, Fe^{3+} -PC coating, and BC@Fe^{3+} -PC were all less than 5%, indicating that the prepared BC@Fe^{3+} -PC had good biocompatibility.

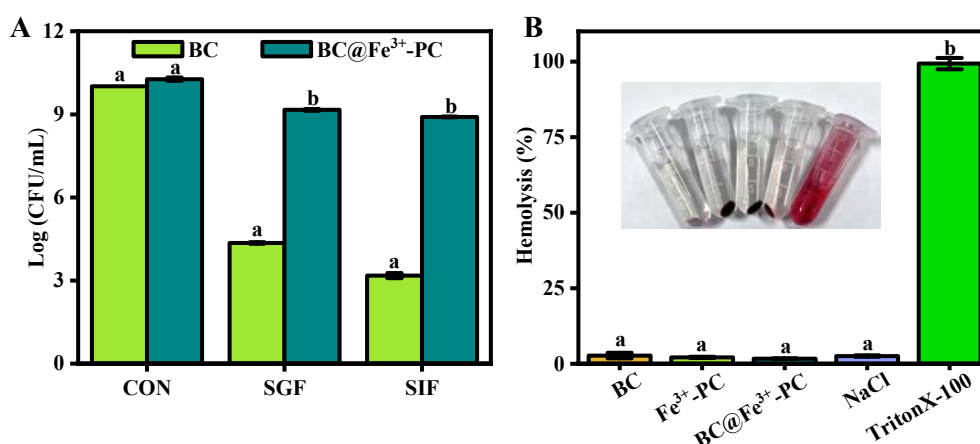


Fig. 4. (A) The viable bacteria numbers of BC and BC@Fe³⁺-PC after 2 h incubation in control (CON), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). (B) Hemolysis rate of BC, Fe³⁺-PC, BC@Fe³⁺-PC, NaCl, and TritonX-100 treated red blood cells. Note: BC represent for BC99 probiotic.

3.7 Nutritional intervention evaluation of BC99 delivery system on DSS-induced colitis

According to the experimental design, mice were divided into healthy Con, DSS, BC, and BC@Fe³⁺-PC treated groups. From the 15th day of intervention, 5% DSS was used instead of normal drinking water for 7 days to assess the effect of BC99 delivery system on DSS-induced colitis (Fig. 5A). After drinking 5% DSS aqueous solution for 7 days (from day 15 to day 21), the body weight of mice in DSS group decreased from 43.53 to 36.59 g, and the food intake also decreased significantly (Fig. 5B-C). However, BC@Fe³⁺-PC intervention could effectively alleviate the body weight loss caused by DSS. Disease activity index (DAI), determined by weight loss, stool characteristics and stool bleeding, was one of the critical indicators to analyze the injury severity of UC. The DAI score of DSS group, was markedly higher than that of Con group, BC99 probiotic and BC@Fe³⁺-PC intervention groups had a lower DAI score than the DSS group (Fig. 5D). Notably, the colon length of mice in BC99 probiotic and BC@Fe³⁺-PC intervention groups increased by 5.45% and 28.36%, compared to that of DSS group (Fig. 5E-F). Moreover, BC99 delivery system could also alleviate DSS-induced splenomegaly in mice (Fig. 5G).

Histopathological assessment of colon was performed using 3 parameters, in terms of inflammatory severity, crypt damage and ulceration. As shown in Fig. 5H, compared to Con group, the colonic mucosa of DSS-induced colitis mice showed severe tissue destruction, incomplete crypt structure and inflammatory infiltration. A similar tissue structure occurred in the BC99-treated group. After BC@Fe³⁺-PC intervention, these intestinal mucosal injuries and inflammatory infiltration were significantly alleviated. In addition, the changes of heart, liver, spleen, lung, kidney and testis tissue sections of different treatment groups were compared (Fig. S4), and no abnormalities such as inflammation, necrosis and bleeding were found, which proved that the prepared BC99 delivery system had good oral biological safety.

Subsequently, the intervention effect of BC99 delivery system on colitis was studied from oxidative stress and inflammatory cytokines. The contents of GSH and SOD in mice decreased after DSS induction compared with Con group, and BC@Fe³⁺-PC intervention could significantly reverse this phenomenon (Fig. 5I). Compared with DSS-treated group, the content of GSH and SOD increased by 25.32% and 67.40%, respectively. Conversely, MDA and MPO, as indicators of lipid peroxidation product of oxidative stress and

neutrophils infiltrating colonic mucosa, respectively, were significantly reduced after BC@Fe³⁺-PC intervention. Moreover, the induction of DSS significantly increased pro-inflammatory cytokine TNF- α content, and decreased anti-inflammatory cytokine IL-10 content in colon tissue. After the intervention of BC99 delivery system, the concentration of TNF- α decreased by 21.95% and IL-10 increased by 15.86%. This indicated that BC@Fe³⁺-PC could effectively alleviate the oxidative stress and inflammatory cytokines caused by DSS.

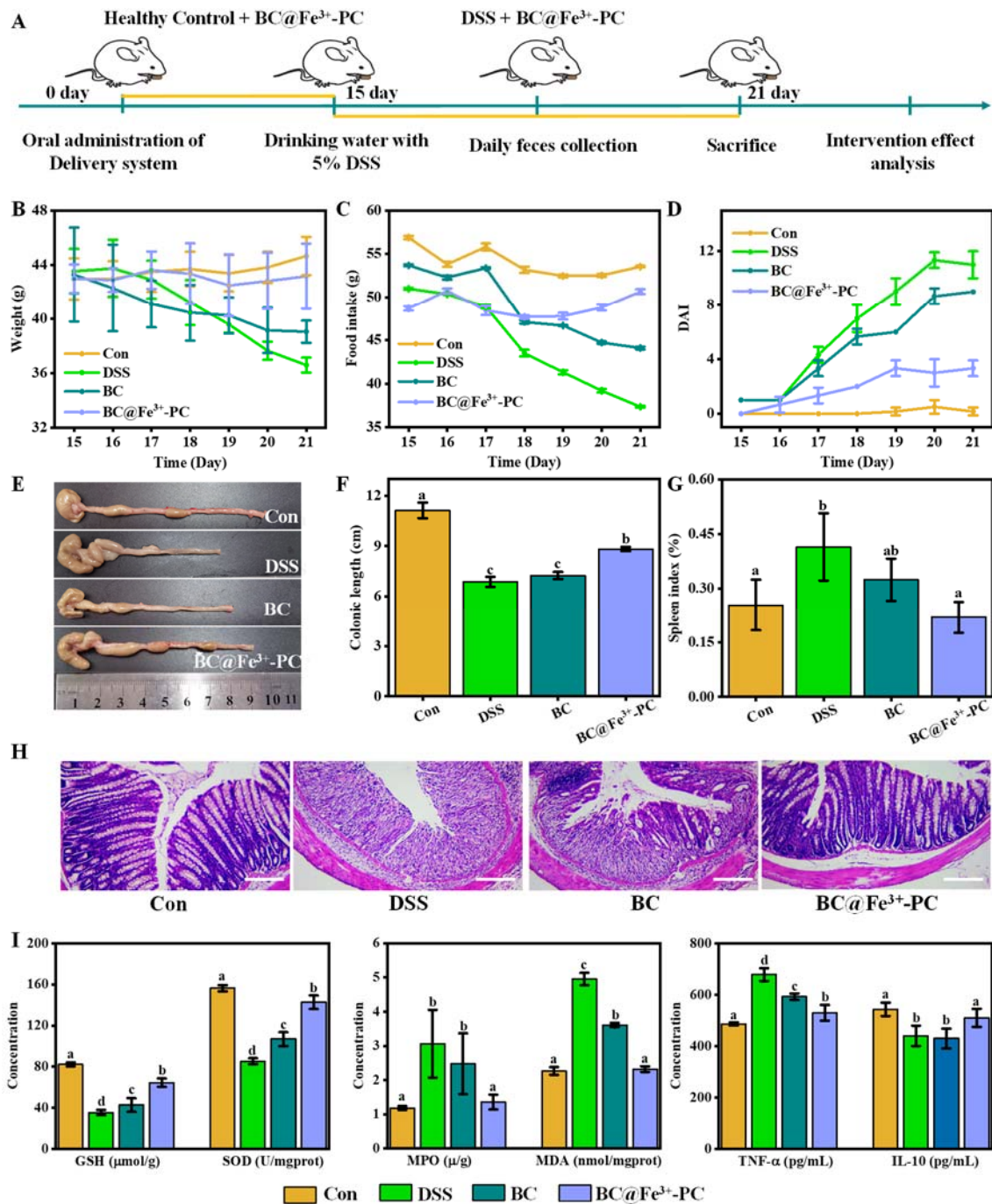


Fig. 5. The interventional effect of oral delivery of BC@Fe³⁺-PC on DSS-induced colitis mice. (A) Scheme illustrating the design of animal experiment, (B) body weight curve, (C) food intake, (D) disease activity index (DAI), (E) colon photograph, (F) colon length corresponding to (E), (G) spleen index of Con, DSS, BC and BC@Fe³⁺-PC treatment groups. (H) Representative images of H&E in mouse colon tissue, scale bar = 200 μ m. (I) The level of glutathione (GSH), superoxide dismutase (SOD), myeloperoxidase (MPO), malondialdehyde (MDA), and the inflammatory cytokines content of tumor necrosis factor (TNF)- α and interleukin (IL)-10 levels in different treatment groups. Different letters indicate significant difference ($P < 0.05$). Note: Con: control, DSS: dextran sulfate sodium, BC: BC99 probiotic.

3.8 Modulation of BC99 delivery system on anxiety and depression symptoms in DSS-induced colitis

Using DSS to induce colitis in mice is currently the most widely used chemically induced UC model, and DSS cannot cross the blood-brain barrier due to its high molecular weight. However, DSS causes increased intestinal permeability and macrophage activation, leading to anxiety, depression-like behavior, and decreased hippocampal neurogenesis in UC mice [36]. To further study the effects of probiotic delivery system on anxiety and depression, the behavior, brain tissue sections and neurotransmitter levels of mice were analyzed.

Open field test and forced swim test were used to assess the spontaneous activity in mice. The results of open field test showed that compared with Con group, the total movement distance of DSS group was significantly reduced by 67.97% ($P < 0.05$). After intervention with BC99 and BC@Fe³⁺-PC, the total distance of mice recovered by 8.48% and 53.39% (Fig. 6A-B). Meanwhile, BC@Fe³⁺-PC group showed an increase time spent in the central zone (Fig. S5). In the forced swim test, the immobility time of mice changed significantly after BC@Fe³⁺-PC intervention, decreased by 95 s (Fig. 6C). These results proved that DSS-induced colitis did lead to the development of anxiety and depression symptoms, and the anxiety and depression-like behavior of mice after BC@Fe³⁺-PC intervention was alleviated. As shown in Fig. 6D, H&E staining is used to observe the tissues of cerebral cortex, hippocampal CA1, CA3, and DG regions. It was found that compared to Con group, the number of neuronal cells in the cerebral cortex of DSS group decreased, and the neuronal arrangement in the hippocampal CA1 and CA3 was disorganized and loose. After the intervention of BC@Fe³⁺-PC, the number of neuronal cells increased, showing the ability to alleviate the damage of cerebral cortex and hippocampus in mice. Chang *et al.* [36] used rattan pepper polysaccharide to intervene DSS-induced colitis, and showed a similar phenomenon in the brain. Moreover, neurotransmitters are closely related to anxiety and depression-like behaviors of colitis. By analyzing the levels of neurotransmitters, for example 5-HT, γ -GABA, DA and BDNF, the intervention effect of BC99 delivery system on anxiety and depression in UC mice could be reflected. 5-HT, also called as serotonin, is an important neurotransmitter that affects gut movement and brain function, and it is also the first clear chemical evidence of gut microbiota-gut-brain axis [37]. Most 5-HT in the body is synthesized, stored, and released by enterochromaffin cells in intestinal mucosa, while a small fraction (~5%) of 5-HT is synthesized in the central nervous system [38]. As shown in Fig. 6E, 5-HT level in the brain of DSS-induced colitis mice is reduced compared to Con group, and significantly upregulated after intervention with BC@Fe³⁺-PC. Meantime, other neurotransmitters, γ -GABA, DA and BDNF, in the brain were also detected, which could reflect the homeostasis of the central nervous system. The levels of γ -GABA, DA and BDNF showed a similar trend (Fig. 6F-H). In summary, the intervention of BC99 delivery system could effectively alleviate the anxiety-depression of colitis mice.

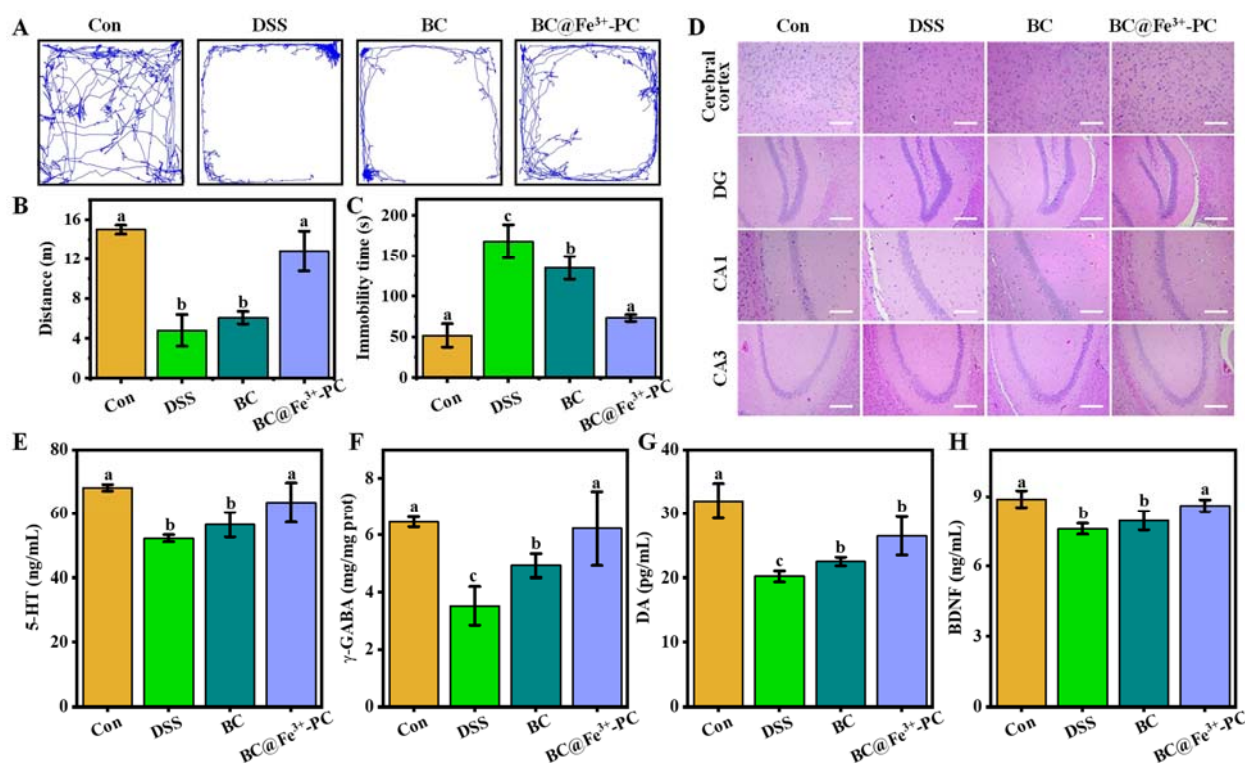


Fig. 6. The interventional effect of BC@Fe³⁺-PC on anxiety and depressive symptoms in colitis mice. Behavioral test, (A) movement tracking images and (B) total distance of mice in the open field experiment, (C) Immobility time in the forced swim test. (D) Representative images of H&E in mouse brain tissue, scale bar = 200 μ m. The level of neurotransmitters, (E) 5-hydroxytryptamine (5-HT), (F) γ -aminobutyric acid (γ -GABA), (G) dopamine (DA), (H) brain-derived neurotrophic factor (BDNF) in different treatment groups. Different letters indicate significant difference ($P < 0.05$). Note: Con: control, DSS: dextran sulfate sodium, BC: BC99 probiotic.

3.9 Regulation of BC99 delivery system on gut microbiota in DSS-induced colitis

As a complex dynamic system, gut microbiota is composed of trillions of microorganisms, for example bacteria, fungi, viruses and archaea that colonize the human intestinal tract, and possess an crucial function in maintaining the homeostasis of the body [39]. The increasing evidence also suggests that gut microbiota has become an important role in the connection between gut and brain, so the concept of “gut-gut microbiota-brain axis” is proposed [40, 41]. Studying the changes of intestinal nerve and central nervous system development, gastrointestinal function, emotion and behavior from the perspective of gut microbiota, provides a new perspective for intervening mood disorders or gastrointestinal diseases. Intestinal dysbiosis, i.e., compositional or functional changes of gut microbiota, is a hallmark of UC mice. To assess the intervention effect of BC99 delivery system on DSS-induced UC mice, the gut microbiota of cecal contents of mice were sequenced by 16s RNA technique. Rarefaction curves and species accumulation boxplot are the effective tools for investigating the species composition and predicting species abundance in samples. As shown in Fig. S6A-B, the curves of different treatment groups tend to be flattened with the increase of sequencing number, indicating the species abundance, uniformity and number of sequenced samples were appropriate, and the sequencing data were accurate and reasonable.

As shown in Fig. 7A, α diversity indexes, such as chao1 index, did not change markedly among all groups, indicating that the species richness of mice did not undergo significant changes after various

treatments. However, Non-Metric Multi-Dimensional Scaling (NMDS) plots showed that BC@Fe³⁺-PC intervention significantly changed the microbiota composition of DSS-induced colitis mice, which was similar to that of Con group (Fig. 7B). Venn diagram showed that there were 201 operational taxonomic units (OUTs) in the four groups, of which the special number of OTUs for Con group, DSS group, BC group and BC@Fe³⁺-PC treatment group was 238, 187, 282 and 366, respectively (Fig. 7C). These endemic species were different, and the presence or absence of a certain type generally had a greater impact on keeping the homeostasis of gut microbiome than the number of such bacteria. The differences of microbiota in different treatment groups were further explored by the analysis of microbial composition and relative abundance.

At phylum level, gut microorganisms were primarily composed of *Firmicutes*, *Bacteroidota*, *Verrucomicrobiota*, *Desulfobacterota* and *Proteobacteria*, and their richness accounted for more than 90% of the microbial community (Fig. 7D-E). After DSS treatment, the microbial level of colitis mice showed obvious imbalance, and the ratio of *Firmicutes* to *Bacteroidetes* (F/B) increased, which was consistent with the change trend of F/B value in UC patients reported by Stojanov *et al* [42]. F/B value has an important impact on maintaining the homeostasis of normal intestinal environment (Fig. 7F). BC@Fe³⁺-PC delivery system intervention could significantly increase the number of *Firmicutes* that involved in host metabolism and alleviate the progression of UC, reduce the level of *Bacteroidetes* and reverse F/B value [43]. *Desulfobacterota* was usually considered a potential pathogenic microorganism, and the intervention of free or loaded BC99 could inhibit the increase of *Desulfobacterota* levels caused by DSS.

Meanwhile, at the genus level, the levels of probiotics such as *Akkermansia*, *Ligilactobacillus*, *Lachnospiraceae_NK4A136_group*, *Blautia*, *Parabacteroides*, *Erysipelatoclostridium*, *[Clostridium]_innocuum_group* were reduced after DSS treatment, and the relative abundance of harmful bacteria *Bacteroides*, *Desulfovibrio* and *Staphylococcus* increased. However, the intervention of BC@Fe³⁺-PC delivery system could mitigate these changes in gut microbiota induced by DSS to varying degrees (Fig. 7G, S7). *Lachnospiraceae_NK4A136_group* (a prominent genus of *Lachnospiraceae*) and *Blautia* (a bacteriocin-producing anaerobic bacteria) could increase the production of SCFAs and reduce inflammatory levels in colitis models, as well as the levels of these probiotics were significantly improved after the intervention of loaded BC99 (Fig. 7H) [44]. *Akkermansia*, as a mucin-decomposing bacterium in the body, can inhibit the infiltration of lipopolysaccharide, and *Erysipelatoclostridium* can increase the synthesis of deoxycholic acid in the intestinal tract, both of which play an important role in regulating gut microbiota [45, 46]. Compared with DSS group, BC@Fe³⁺-PC intervention could also increase the levels of these probiotics, but there was no significant difference. In addition, *Bacteroides* and *Desulfovibrio* belonged to the conditional pathogenic bacteria, such as, enterotoxigenic *Bacteroides fragilis* in *Bacteroidetes* can exacerbate the permeability of intestinal epithelial cells and disrupt the epithelial barrier function by secreting enterotoxin, which all have an important function in the occurrence and development of UC [47, 48]. The loaded BC99 probiotic showed an inhibitory effect on these two pathogens of DSS group. As shown in Fig. 7I, heatmap shows the species abundance and community composition at the genus level, and BC@Fe³⁺-PC delivery

system is relatively similar to that of Con group. The ternary phase diagram in Fig. 7J shows the distribution of the top 10 species ranked by mean abundance at the genus level. The results further proved that the abundance of conditional pathogens in DSS treatment group was higher, while the content of *Lachnospiraceae_NK4A136_group* in BC@Fe³⁺-PC group was relatively high.

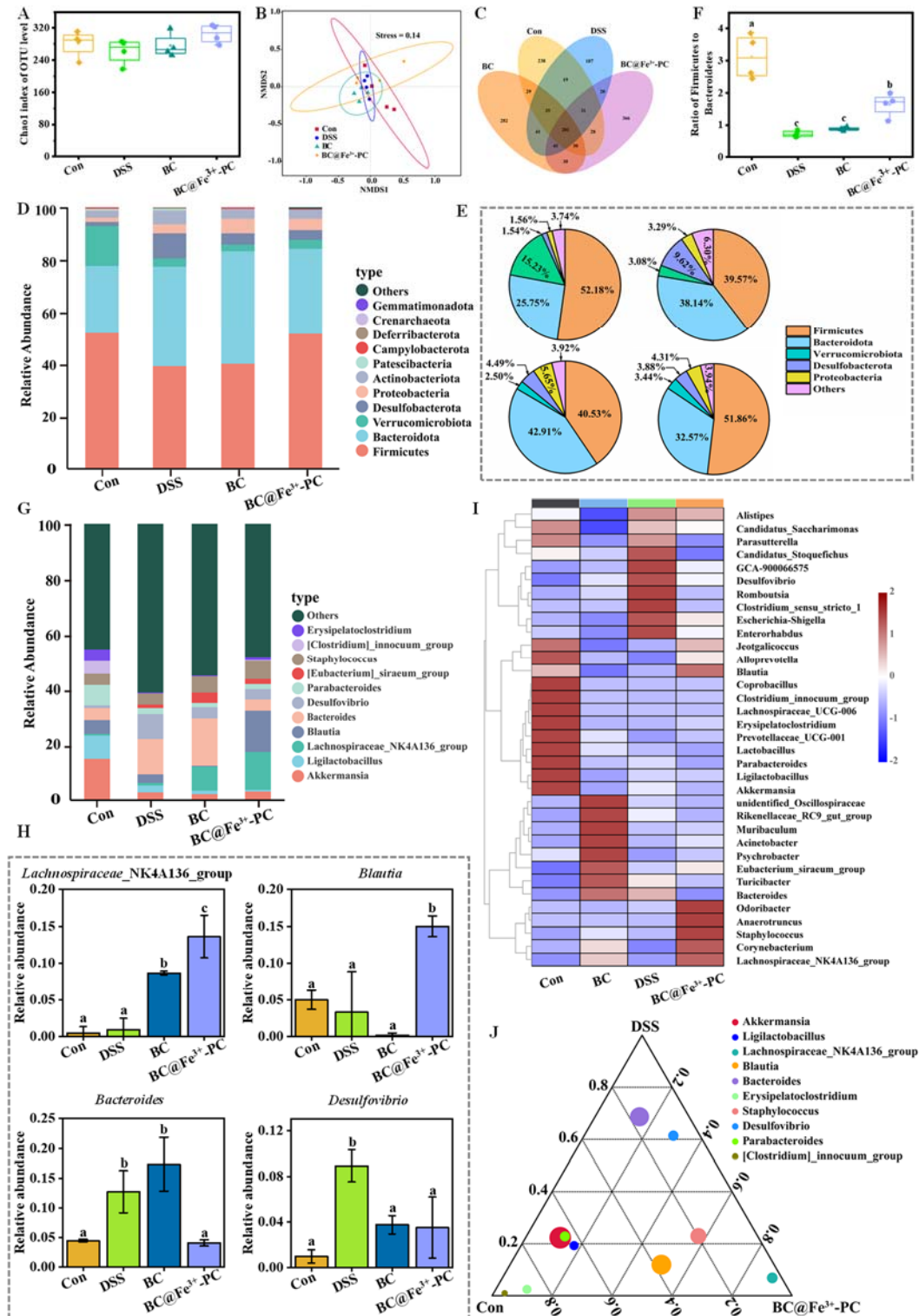


Fig. 7. Diversity and composition analysis of gut microbiota. (A) Chao1 index and (B) NMDS plots of gut microbiota. (C) Venn diagram of bacteria at the OUT level. (D) The community histogram of gut microbiota, (E) relative abundance content, and (F) the ratio of *Firmicutes* to *Bacteroidetes* at phylum level. (G) The relative abundance histogram of microbiota, and (H) the relative abundance content of *Lachnospiraceae_NK4A136_group*, *Blautia*, *Bacteroides* and *Desulfovibrio*. (I) Microbial heatmap of the top 35 OTUs and (J) ternary phase diagrams of Con, DSS and BC@Fe³⁺-PC delivery system-treated groups at the genus level.

Moreover, LDA effect size (LEfSe) commonly used to identify and interpret high-dimensional biomarkers, thus helping to identify statistical biomarkers among different groups. In the Con group, the genus of *Anaerostipes* that could convert inositol into propionic acid or acetic acid was significantly enriched, and *Romboutsia* related with intestinal inflammation, was significantly increased in DSS-treated group (Fig. 8A-B) [49]. However, the abundance of bacteria increased after BC99 probiotic intervention. In BC@Fe³⁺-PC delivery system group, the abundance of *Clostridiales bacterium_CIEAF_020* (strongly resistance to environmental changes) and *Odoribacter* (butyrate-producing bacterium with anti-inflammatory properties) were significantly increased [50]. Taken together, the results indicated that delivery system intervention could cause significant changes in the composition and diversity of intestinal microbiota in colitis mice.

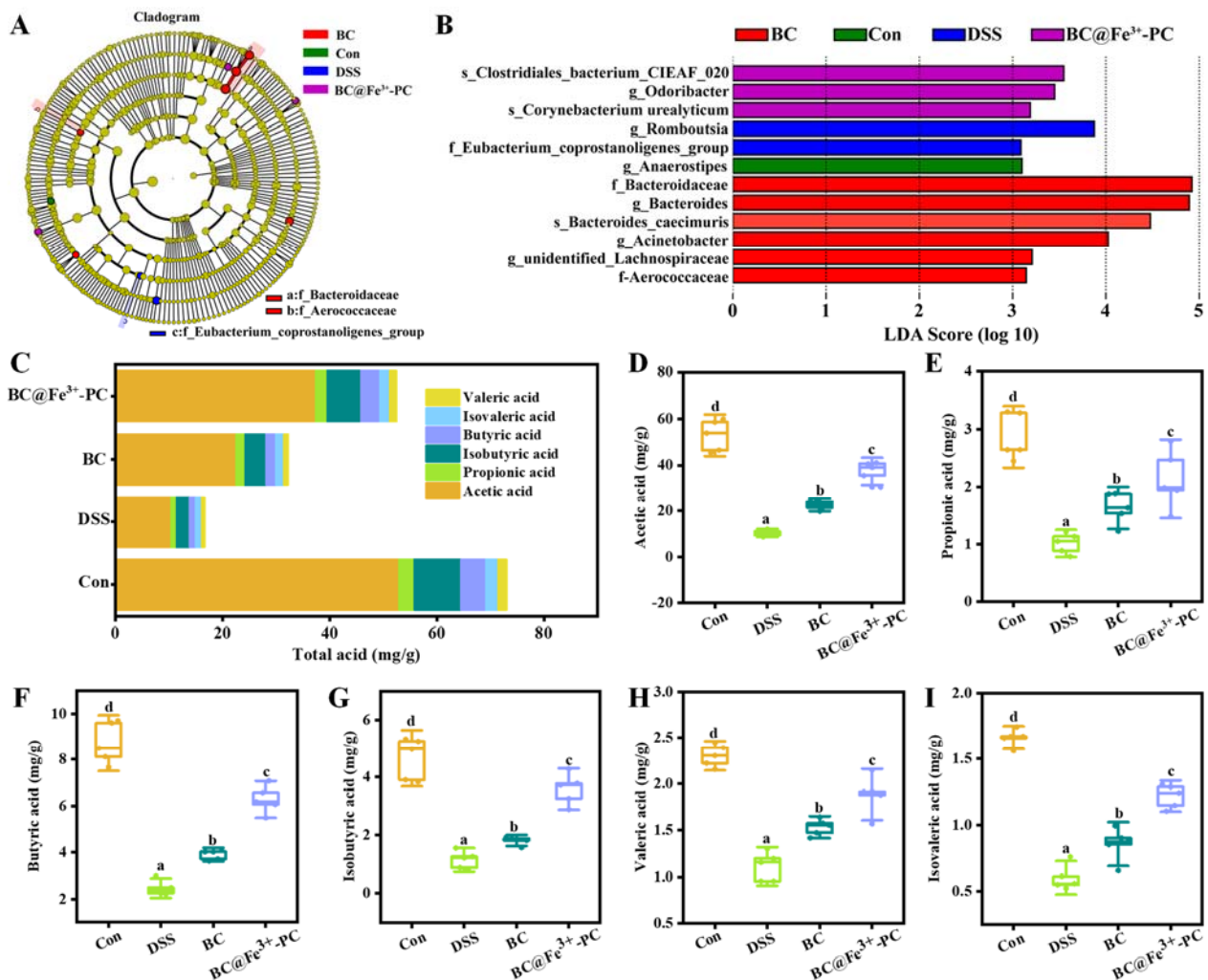


Fig. 8. LEfSe analysis results of gut microbiota and the level of short-chain fatty acids in Con, DSS, BC and BC@Fe³⁺-PC groups. (A) Taxonomic cladogram of the main constituent taxa of gut microbiota. (B) The distribution histogram identifies the significant abundance of strains with LDA greater than 3. (C) Total acid, (D) acetic acid, (E) propionic acid, (F) butyric acid, (G) isobutyric acid, (H) valeric acid, (I) isovaleric acid contents in different treatment group. Different letters indicate significant difference ($P < 0.05$). Note: Con: control, DSS: dextran sulfate sodium, BC: BC99 probiotic.

3.10 PICRUST2 function prediction analysis of gut microbiota

PICRUST2 was used to predict the metagenomic function of the main bacterial taxonomy, and the results showed significant differences in microbiota function and metabolic pathways among Con, DSS, BC and

BC@Fe³⁺-PC groups. As shown in Fig. S8A and Table S1, compared with Con group, branched-chain amino acid synthesis, purine/pyrimidine metabolism, and energy metabolism and other pathway functions are significantly altered in colitis mice after DSS treatment. This indicated that when DSS-induced intestinal barrier damage in mice, the energy supply of gut microbiota was imbalanced, resulting in further disorder of intestinal ecology. After BC99 intervention, the difference in fatty acid synthesis function narrowed compared with DSS group, which might improve intestinal homeostasis by repairing intestinal mucosal lipid barrier (Fig. S8B). The intervention of BC@Fe³⁺-PC delivery system regulated the deoxynucleotide biosynthesis pathway more obviously (Fig. S8C). However, it is still necessary to further combine metabolomics to clarify how the functional changes of gut microbiota can accurately regulate the signals of gut microbiota-brain axis through metabolites, and provide a new target for UC and its accompanying anxiety-depression symptoms.

3.11 Increased the level of short-chain fatty acids in colitis mice

As the main gut microbiota metabolites in colitis, SCFAs can cross the blood-brain barrier and enter the brain to regulate the balance of Th17 and Treg cells. The content of SCFAs showed that, the level of total SCFAs was markedly reduced in DSS-treated group compared to Con group (Fig. 8C). In particular, acetic acid, which was the most abundant in SCFAs, was reduced after DSS treatment. The intervention of free and loaded BC99 could increase the level of SCFAs, especially BC@Fe³⁺-PC significantly increased the content of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid (Fig. 8D-I). This was positively correlated with the abundance increase of SCFA-producing beneficial bacteria, indicating that the increased level of SCFAs after the intervention of BC99 delivery system could further affect the microbial community and maintain the homeostasis of the intestinal microenvironment.

3.12 Correlation analysis

The correlation analysis of gut microbiota, intestinal oxidative stress, inflammation and brain neurotransmitters, and other biochemical indicators in DSS-induced colitis mice was performed.

To explore the correlation between related expressions such as gut microbiota, gut immunity, anxiety and depression, Pearson correlation analysis was performed on the collected data. As shown in Fig. 9, the levels of antioxidant (GSH and SOD), anti-inflammatory cytokine IL-10, neurotransmitters, SCFAs in gut and brain of mice were positively correlated with *Akkermansia*, *Parabacteroides*, *[Clostridium]_innocuum_group*, *Erysipelatoclostridium*, *Lachnospiraceae_UCG-006*, and negatively correlated with *Bacteroides*, *Desulfovibrio*, *Turicibacter*. The level of MPO, MDA and TNF- α were positively correlated with *Bacteroides* and *Desulfovibrio*. Therefore, BC@Fe³⁺-PC could inhibit the expression of oxidative stress and pro-inflammatory cytokines, increase the contents of anti-inflammatory cytokines, neurotransmitters and SCFAs by regulating the diversity and abundance of intestinal microbiota, thus effectively alleviating colitis and anxiety-depression like symptoms.

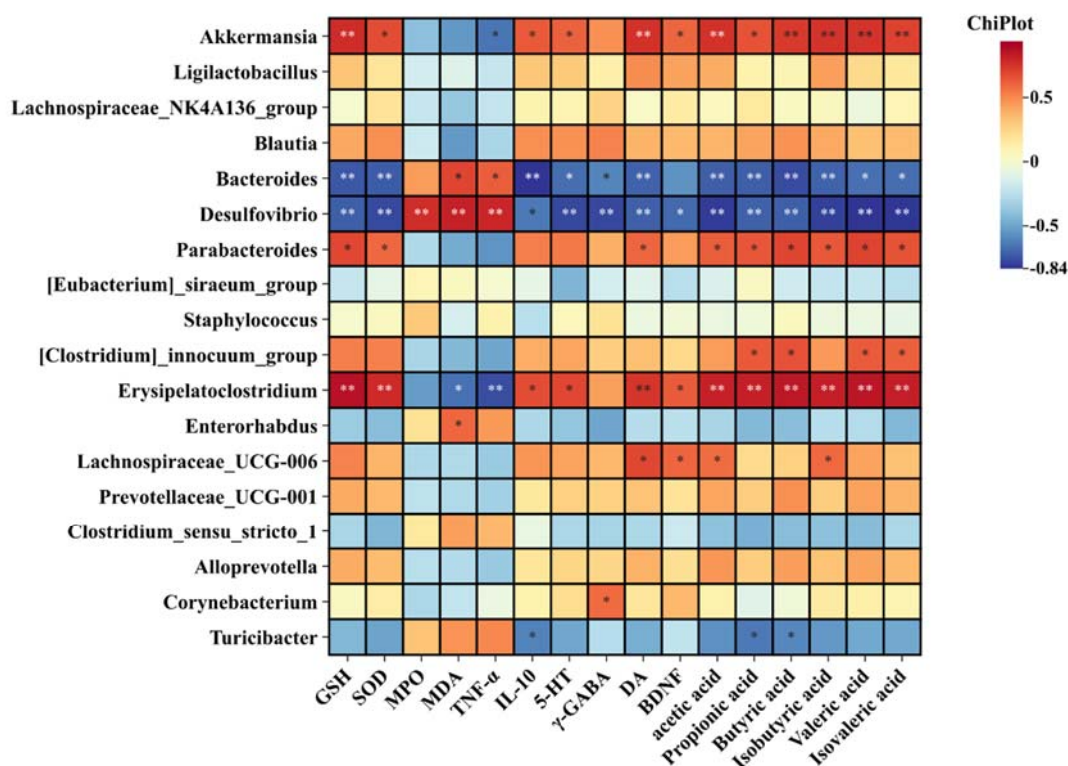


Fig. 9. Pearson's correlation heatmap among the abundance of gut microbiota, oxidative stress, inflammatory cytokines, neurotransmitters and SCFAs. The color of the boxes indicated the levels of the correlation indexes, * $P < 0.05$, ** $P < 0.01$. Note: GSH: glutathione; SOD: superoxide dismutase; MPO: myeloperoxidase; MDA: malondialdehyde; TNF- α : tumor necrosis factor- α ; IL-10: interleukin 10; 5-HT: 5- hydroxytryptamine; γ -GABA: γ -aminobutyric acid; DA: dopamine; BDNF: brain-derived neurotrophic factor.

4. Conclusions

In summary, this study successfully designed and prepared BC@Fe³⁺-PC delivery system with PC-metal ion coating to regulate DSS-induced colitis in mice through the gut microbiota-brain axis. The adopted PC and metal ions have good biocompatibility with BC99, and under the protection of metal ion-PC complex coating, it could effectively improve the stability of BC99 under pH and bile salt conditions, and pass through the gastrointestinal tract. Subsequently, DSS-induced colitis model was constructed, and BC@Fe³⁺-PC effectively alleviated oxidative stress, improved inflammation cytokines level, and regulated the diversity, composition, and abundance of gut microbiota related to inflammation, anxiety and depression. Moreover, BC@Fe³⁺-PC could also reverse anxiety and depression-like behaviors, brain neurotransmitters and short-chain fatty acid levels in mice.

Importantly, this Fe³⁺-PC coating method can also be used for the embedding of other probiotics besides BC99, and the prepared probiotic formulation can effective nutritional intervene colitis accompanied by anxiety and depression from the gut microbe-brain axis. Because of single embedding, simple preparation method and preventive effect on diseases, probiotic nano-formulation has become one of the focuses in the field of probiotic embedding. In future studies, adding prebiotics to nano-coatings or co-loading probiotics and active ingredients with nano-coatings, may further endow probiotics with beneficial functions, so that probiotics can regulate diseases more accurately or personally by multi-omics evaluation. Moreover, probiotic

nano-formulations with good biocompatibility have the potential to be transformed into clinical practice. After subsequent animal safety evaluation and clinical trials, they are expected to become a safe and efficient new therapeutic preparation, and provide a powerful intervention plan for colitis and its associated anxiety-depression.

Conflicts of 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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Supplementary data

Supplementary data contains the solution color and particle size distribution of PC complex and BC99 delivery system, animal experimental results, please see the supplementary data.

Data availability

Data will be made available on request.

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