Fermentation products of Danshen relieved dextran sulfate sodium-induced experimental ulcerative colitis in mice

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Research Article

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Abstract

With the increased incidence and recognition, ulcerative colitis (UC) has become a global public health problem in the world. Although many immunosuppressants and biological drugs have been used for UC treatment, the cure rate is still very low. It is necessary to find some safe and long-term used medicine for UC cure. In recent years, fermented Chinese medicine has been more and more used in the treatments of inflammatory and chronic diseases. In this manuscript, *Lactobacillus Rhamnosus* (F-B4-1) and *Bacillus Subtillis Natto* (F-A7-1) were selected to ferment enzymatic Danshen. The fermented Danshen products were gavaged in the dextran sulfate sodium (DSS)-induced UC model mice. It is suggested that after fermented Danshen with *Rhamnosus* and then with *Natto*, Danshen had the better results to attenuate symptom of DSS-induced UC. It reduced the loss of body weight and disease activity index (DAI) and inhibited the abnormally short colon lengths and the colonic damage. Moreover, it suppressed pro-inflammatory cytokine expression during DSS-induced UC. The results indicated that fermented Danshen relieved DSS-induced UC in mice. And the Danshen fermented by probiotics might be an effective drug to treat UC in the clinic in the future.

Introduction

Ulcerative colitis (UC) is a non-specific intestinal inflammatory disease characterized by chronic inflammatory reaction and intestinal mucosal epithelial damage. The major clinical characteristics of the UC are abdominal pain, blood in the stool, diarrhea, and weight loss. Thus, the quality of life of patients with UC declines [1]. UC is a global public health problem. The incidence and prevalence of UC have been increasing over time. The highest incidences of UC have been reported in Western Europe and North America (affecting >0.3% of the population) [2]. In developing countries, the incidence and recognition of UC is increasing for the past few years, such as Asia, the Middle East and South America [3]. It has been proved that untreated UC is likely to cause complications, such as peritonitis, even colorectal cancer. Because of the difficulty in curing, the increasing incidence rate, and the cancer tendency, UC has been classified as a refractory disease by the World Health Organization (WHO).

Although it has been identified that several factors contribute to UC, including genetic factors, stress, dietary habit, excessive production of inflammation-related cytokines and abnormal immune responses in the mucosal and submucosal layers of the intestine, the currently available therapies are limited [4]. Now in clinical treatment, aminosalicylates are chosen as the main drugs for mild to moderate UC. Topical and systemic steroids can be used to treat UC flares. And immunosuppressants and biological drugs are used to treat moderate to severe UC [3]. However, low cure rate and high recurrence rate are two major problems of UC treatment drugs [5]. In terms of the current treatment level and technology, UC is still a chronic, lifelong and progressive disease, which needs long-term treatment. Intermittent or transitory therapy would lead to repeated symptoms and eventually lead to structure or function damage of intestinal. While long-term or high-dose use of the UC drugs would result in many side effects, such as abdominal pain,

kidney damage, hepatotoxicity and blood disorders [6]. Thus, it is necessary to find some natural drugs from medicinal plants to cure UC.

Salvia miltiorrhiza Bunge (Danshen) is a traditional herb which has been used in China for more than 2,000 years. Danshen has a wide spectrum of pharmacological activities, such as anti-inflammatory, anticancer, cardiovascular treatment, anti-diabetes and so on [7-10]. Until, there are many reports about Danshen used in vitro or animal work. It has been reported that Danshen could significantly reduce the production of NO and the expression of iNOS in macrophages induced by LPS in vitro model. And Danshen could alleviate dextran sulfate sodium (DSS)-induced arthritis in mice[11-13]. Additionally, it has been reported that a variety of natural ingredients in Danshen could protect DSS-induced experimental UC in mice, such as cryptotanshinone, tanshinone IIA, dihydrotanshinone I [14-16]. And it has been proved that Danshen prevented the occurrence of DSS-induced colitis by inhibiting the TLT4/PI3K/ Akt /mTOR signaling pathway in mice[17]. Thus, we believed that Danshen would have an effect in DSS-induced colitis model and might be a potential and effective drug to treat UC in the clinic.

It has been reported that gut modulation by probiotics would be one potential strategy to prevent or treat UC. Over the past twenty years, many investigations have focused on the role of probiotics in the treatment of UC [18]. It is suggested that after supplemented with *Bifidobacterium animalis* subsp. *lactis* BB12 in the murine model of DSS-UC, the reduction colon length was recovered, the apoptosis in IECs (intestinal epithelial cells) was reduced and the increased level of TNF-α was reduced [19]. *Bifidobacterium longum* subsp. *infantis* BB-02 could attenuate the clinical symptoms of UC and reduce edema [20]. Not only one probiotic, the probiotic mixture had the better effects. Until now, the most known probiotic mixture with proven efficacy is VSL#3. This mixture contains 900 billion lyophilized bacteria, including four strains of Lactobacillus, three strains of Bifidobacteria and one strain of Streptococcus thermophilus [21]. The efficacy of VSL#3 was proven not only in the DSS-induced colitis mice model but also in patients with mild to moderate UC [22]. Nowadays, probiotics have gradually become an adjuvant therapy for UC.

In this manuscript, the screened *Lactobacillus Rhamnosus* (F-B4-1) and *Bacillus Subtillis Natto* (F-A7-1) were used to ferment Danshen. The effects of the fermentation products on UC were investigated. It is proved that after fermented by probiotics, Danshen had the better results to attenuate symptom of DSS-induced UC by anti-inflammatory effect. Therefore, the Danshen fermented by probiotics might be an effective drug to treat UC in the clinic in the future.

Methods

Chemicals and reagents

Human and mouse IL-6, TNF-α and IL-1β ELISA kit were purchased from Beijing dakowei Biotechnology Co., Ltd. LPS (*Escherichia coli* 055:B5) from Sigma–Aldrich (St. Louis, MO) was dissolved in ddH_2O as a stock solution at a concentration of 1 mg/mL. Danshen was purchased from Shandong Institute of Traditional Chinese Medicine. Acid cellulase, acid pectinase, whey protein powder, peptone, MRS (lactic

acid bacteria) liquid medium, MRS (lactic acid bacteria) solid medium, nutrient liquid medium (Dried grass), LB liquid medium (Staphylococcus aureus, Escher ichia coli), LB solid medium (Staphylococcus aureus, Escherichia coli), liquid medium for Salmonella (Salmonella typhimurium, Salmonella enteritis) and salmonella solid culture medium (Salmonella typhimurium, Salmonella enteritis) were from Solebol Technology Co. LTD.. Dextran sulfate sodium (DSS, MW 40000) was obtained from Shanghai Aladdin Biochemical Technology Co., LTD (Shanghai China).

Cell culture and treatment

Caco-2 cells (presented by professor Yanqing Li from Qilu hospital of China) were cultured in Dulbecco's Modified Eagle's Medium-Hight glucose (DMEM-H, Gibco, 12800-017) supplemented with 10% fetal bovine serum (FBS; v/v) (Hyclone, SV30087.02). Cells were kept at 37 °C in a humidified atmosphere of 5 % CO_2 under standard conditions. When caco-2 cells were grown to 80% confluency, cells were stimulated with LPS. Sample treatments were performed 12 h after cells exposure to LPS.

Cell viability analysis

Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide) assay [23]. Cells were seeded into 96-well plates and treated with LPS. After 12h LPS treatment, cells were treated with or without different samples. Then cells were incubated with 0.5% MTT for 4 h. The medium was removed and 100 μl of 0.04 M dimethylsulfoxide solution (DMSO) was added. The absorbance of the reaction product in solution at 570 nm was measured using a SpectraMax ABS microplate spectrophotometer (Molecular Devices, USA). The percentage of living cells was calculated by the ratio of OD.

Antimicrobial spectrum analysis

Four pathogens (*Staphylococcus aureus, Escherichia coli, Salmonella typhimurium and Salmonella enteritidis)* were selected and inoculated with LB medium (L3522, Sigma-Aldrich) and Salmonella medium (peptone 5 g, beef extract 3g, NaCl 50g, pH 7.0-7.2) respectively at 37℃ for 12h. Then absorb the pathogen bacteria suspension (200 ul) into agar culture-medium (20 ml) and mix. The mixture was placed at room temperature for 1 h. Punch holes in the AGAR plate with a hole punch with a diameter of 8mm. Add different bacteria solutions (50 ul) into the holes and cultured at 37 ℃ for 48 h to observe the bacteriostasis ability. The liquid medium was as the blank control.

Gastric and intestinal fluid tolerance

The bacterial strain was inoculated in 5mL MRS liquid medium for 24h at 37℃. After centrifuged at 3000 rpm for 10min, the supernatant was discarded and rinsed with PBS buffer (pH 7.0) for 3 times. After suspended the bacterial in 5mL sterile saline, 1mL bacterial suspension was mixed with 9mL artificial gastric juice (NaCl 0.2g, pepsin 0.35g, ddH_2O 100ml, pH 2.5) or artificial intestinal juice (trypsin 0.1g, $NaHCO_3$ 1.1g, NaCl 0.2g, bile salt 1.8g, ddH_2O 100ml, pH 8.0) and cultured at 37℃ for 3h or 4 h. Then,

the culture medium was diluted with normal saline. 1mL diluent was mixed with 30mL MRS solid medium and cultured at 37℃ for 48h. The survival rate was calculated by counting [24].

The treatments of Danshen

All plant experiments complied with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The Danshen was divided into three different treatments, boiled, enzymolysis and fermentation. (1) *Boiled* Ultramicro mill was used to crush Danshen into powder above 1500 mesh. The ratio of the powder and distilled water was 1:9. After the uniform stirring, the water was extracted by boiling at 100℃ for 30 minutes. And then the water was filled up. The sample was centrifuged at 10000 r/min and the supernatant was reserved. (2) *Enzymolysis* The Danshen was ultra-fine pulverized and dissolved in sterile water (1:9, pH 4.8-5.0) after ultraviolet irradiation. The enzymolysis was did at 50 ℃ for 2 h by adding acid pectinase and acid cellulase (acid fiber and sour fruit amount accounted for 1 ‰ of solid). Then sugar (2%), peptone (5 ‰ of total volume) and protein powder (2.5 ‰ of total volume) were added. The samples were pasteurization (85 ℃ for 30min) to form raw material culture medium. (3) *Fermentation* After enzymolysis, Denshen was fermented by four different manners. FDS47 (Fermented Danshen Sequence 47): Fermented Danshen with *Lactobacillus Rhamnosus* (F-B4-1) (shaking table) at 37℃ for 12h until the concentration of the reducing sugar was stable (pH 6.5-7.0). The number of colonies of *Lactobacillus Rhamnosus* was counted (1.2 x 10^9 CFU/ml). Then it was further fermented with *Bacillus Subtillis Natto* (F-A7-1) (static culture) at 37℃ for 12h until the concentration of the reducing sugar was stable. The numbers of colonies of *Lactobacillus Rhamnosus* and *Bacillus Subtillis Natto* were counted (2x10^8 CFU/ml). FDS74 (Fermented Danshen Sequence 74): Fermented Danshen with *Bacillus Subtillis Natto* (F-A7-1) (shaking table) at 37℃ for 12h until the concentration of the reducing sugar was stable (pH 6.5-7.0). The number of colonies of *Bacillus Subtillis Natto* was counted (8 x 10^8 CFU/ml). Then it was further fermented with *Lactobacillus Rhamnosus* (F-B4-1) (static culture), at 37℃ for 12h until the concentration of the reducing sugar was stable. The numbers of colonies of *Lactobacillus Rhamnosus* and *Bacillus Subtillis Natto* were counted (6 x 10^8 CFU/ml). FDT47-sha (Fermented Danshen Together 47 Shake): Fermented Danshen with *Lactobacillus Rhamnosus* (F-B4-1) and *Bacillus Subtillis Natto* (F-A7-1) together (shaking table) at 37℃ for 12h until the concentration of the reducing sugar was stable (pH 6.5-7.0). The numbers of colonies of *Lactobacillus Rhamnosus* and *Bacillus Subtillis Natto* were counted (1.53 x 10^9 CFU/ml). FDT47-sta (Fermented Danshen Together 47 Stationary): Fermented Danshen with *Lactobacillus Rhamnosus* (F-B4-1) and *Bacillus Subtillis Natto* (F-A7-1) together (static culture) at 37℃ for 12h until the concentration of the reducing sugar was stable (pH 6.5-7.0). The numbers of colonies of *Lactobacillus Rhamnosus* and *Bacillus Subtillis Natto* were counted (3.3 x 10^8 CFU/ml).

All the samples were filtered through a 60 μm nylon net filter (Millipore, Bedford, MA, USA) and freeze-dried. The yields of solid content were FDS47-sha 5.8%, FDS47 5.7%, FDS74 5.7% and FDS47-sta 5.1%. The freeze-dried powder was dissolved in ddH_2O. The solution was filtered (0.2 μm, pore size) and maintained at 4℃ prior to use.

Microbial counts

The fermented Danshen liquid was vortexed to homogenize and the supernatant was obtained. For counting *L. Rhamnosus*, 0.01 mL of the diluted samples was spread onto a MRS (lactic acid bacteria) solid medium. Plates were prepared in duplicate and incubated at 37°C for 24 h. For counting *B. Subtillis Natto*, 0.1 mL of the diluted samples was spread onto a nutrient solid medium . The plates were incubated at 37 C for 24 h, and the viable cells were enumerated. Cell counts were expressed as CFU/mL.

Animal treatment

Female C57BL/6 mice (6-weeks-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.. Mice were housed under standard conditions of humidity, room temperature and dark-light cycles. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The animal experimental protocol complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and was approved by the Animal Experiment Ethnics Committee of Qilu University of Technology. All animals were divided into 5 groups (n = 7 per group). Mice were treated with saline in NC group. DSS, Boi, CBF and FDS47 groups were treated with drinking water containing 2.5% DSS for 14 days [25]. On the 8th day, mice were treated daily with saline, DSS (2.5%), Boiled Danshen (50mg/Kg), compound bacterium fluid (CBF, the mixture of F-B4-1 and F-A7-1 in 1:1 ratio) or FDS47 (50mg/Kg) by gavage respectively in the NC, DSS, Boi, CBF and FDS47 groups. All mice were weighed every day. On the 14th day of the experiment, all mice were killed by exsanguination after deep anesthesia.

Evaluation of Colitis

The disease activity index (DAI) was determined by an investigator blinded to the protocol by scoring changes in weight, hemoccult positivity or gross bleeding, and stool consistency as the protocol previously described [25].

Blood and tissue collection

Serum was prepared by centrifugation at 3000 g for 20 min at 4 ℃ and stored at −80 ℃ for biochemical analysis. The mice were killed by exsanguination after deep anesthesia. Colons were rapidly removed and their length were documented. Then the colons were snap-frozen in optimal cutting temperature (OCT) embedding medium (Tissue-Tek) for histology analysis.

Hematoxylin and eosin analyses

The distal colon were dissected and immersed in OCT embedding medium (Tissue-Tek). Serial 8-μm-thick cryosections from every 3 sections (8–10 sections per mouse) were mounted on poly-d-lysine-coated

slides. Cryosections were prepared and stained with hematoxylin and eosin (HE). Each colon was calculated from 4 consecutive 8-μm sections taken every 40 μm and covering the colon.

Enzyme-linked immunosorbent assays (ELISA)

The mouse intestinal tissues were homogenized on ice with NP40 lysis buffer (Beyotime Biotechnology, China). The homogenates were quantified using the BCA assay as reported previously (Beyotime Biotechnology, China) [26]. Cell supernatants, serum and tissue homogenates were collected respectively for the determination of IL-6, IL-1β and TNF-α concentrations according to the manufacturers' instructions.

Statistical analysis

All experiments were repeated at least 3 times independently. Data were expressed as mean ± SEM and were analyzed by one-way ANOVA with use of SPSS v11.5 (SPSS Inc., Chicago, IL). Images were processed by use of Graphpad Prism 5 (GraphPad Software, La Jolla, CA, USA) and Adobe Photoshop CC (Adobe, San Jose, USA). $P < 0.05$ was considered statistically significant.

Results

Lactobacillus Rhamnosus (F-B4-1) and *Bacillus Subtillis Natto* (F-A7-1) were selected to ferment enzymatic Danshen

Firstly, 9 different probiotics in our laboratory were selected to do the antimicrobial spectrum analysis. The data showed that F-B12-1 and F-B20-1 did not have the antimicrobial ability for all the four pathogens *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Salmonella typhimurium (S. Typhimurium) and Salmonella enteritidis (S. Enteritidis).* All of these probiotics did not have the antimicrobial ability for *S. Enteritidis.* Then the gastric and intestinal fluid tolerance analysis were did. The results showed that the viability of F-B12-1 and F-B25-1 were just 13.89% in the artificial intestinal juice (Fig. 1A). From these data we selected F-B8-1, F-B4-1, F-B9-1, F-B16-1 and F-A7-1 to examine the clonal formation unit (CFU) (Fig. 1B). It is suggested that F-B4-1 and F-A7-1 had the highest CFU.

Lipopolysaccharide (LPS) could induce inflammatory injury in caco2 cells which is a recognized cell model for UC [27]. Caco2 cells were treated with different concentrations of LPS for 6h, 12h or 24h. The levels of IL-6 were examined in the cell supernatant. The data showed that the level of IL-6 could be increased significantly after treated with 50 ug/ml LPS for 12h (**Supplemental Fig. 1**). Thus, we used 50 ug/ml LPS in our next experiments. After caco2 cells were treated with LPS for 12h, F-B8-1, F-B4-1, F-B9-1, F-B16-1 and F-A7-1 (5mg/ml) were added to examine the IL-6 concentration. It is suggested that all of these five probiotics could decrease the IL-6 concentration induced by LPS. Among these five probiotics, F-B4-1 and F-A7-1 have the best effects (Fig. 1C). Based on these results, F-B4-1 and F-A7-1 were selected for the further experiments.

In order to explore which technology has the better effect of anti-inflammation, Danshen was boiled or enzymatic hydrolyzed to examine the IL-6 concentration in caco2 cells treated with LPS. The results showed that enzymatic hydrolyzed Danshen at 0.2 mg/ml had the best effect to decrease the IL-6 concentration (Fig. 1D). Based on above results, enzymatic hydrolyzed Danshen and F-B4-1 and F-A7-1 were selected.

Effects of different fermentation methods on LPS-mediated upregulation of TNF-α, IL-6 and IL-1β

ELISA was performed to measure the levels of TNF-α, IL-6 and IL-1β in caco2 cells after treatment. The levels of TNF-α, IL-6 and IL-1β were all upregulated after treated with LPS. All of the samples from different fermentation methods could decrease the all three inflammatory factor levels (Fig. 2). Among these different fermentation methods, FDS47 (fermented Danshen with *Lactobacillus Rhamnosus* and then with *Bacillus Subtillis Natto*) had the best effects of inflammatory factor inhibition. And the effects of inflammatory factor inhibition was better than that in the enzymolysis groups (Fig. 2). Meanwhile, the CFU were examined in *Lactobacillus Rhamnosus* and *Bacillus Subtillis Natto* with different fermentation methods. It is suggested that the CFU was higher in FDS47 and FDT47-sha groups (**Supplemental Fig. 2**). Thus, fermented Danshen with *Lactobacillus Rhamnosus* and then with *Bacillus Subtillis Natto* was selected to do further research. The ELISA results showed that FDS47 at 0.2 mg/mL could inhibit the IL-6 level induced by LPS significantly. With the increased concentrations (1 mg/mL or 5 mg/mL), the IL-6 level elevated in caco2 cells treated with FDS47 for 48 h (Fig. 3).

FDS47 alleviated the clinical symptoms of DSS-induced colitis in mice

We first examined the organs (heart, spleen, kidney, lung and liver) of mice in each treatment group. There was no significant changes in all organs (**Supplemental Fig. 3**). The body weight and colon length are important indicators of colitis severity. DSS-induced colitis could decrease the body weight and shorten the colon length. The data showed that FDS47 could effectively prevented body weight loss induced by DSS (Fig. 4A). The grade of UC induced by DSS was evaluated by the disease activity index (DAI) score, which was the sum of scores given for body weight loss, stool consistency, and presence of fecal blood. A significant increase of DAI score was observed in the DSS-treated group at the 14th day compared with the normal groups ($p < 0.05$). In three treatment groups, the DAI scores were significantly decreased when compared to the DSS group at the 15th day ($p < 0.05$). FDS47 most effectively prevented DAI increase compared to Bio and CBF groups (Fig. 4B). In addition, mean colon length was the highest in the normal mice and lowest in the DSS-induced colitis mice (Fig. 4C **and D**). In all three treatment groups, the colon lengths were longer than those in the DSS group. In the FDS47 group, the colon length almost returned to the normal level (Fig. 4C **and D**).

Histologic examination of the colon revealed the degree of inflammation and epithelial damage. The colons from all of the mice in each group were examined in hematoxylin-eosin (HE) stained slides. According to the Fig. 5A, DSS treated mice displayed the most severe infiltration of inflammatory cells, disruption of surface epithelium, and loss of crypts. Intragastric administration of FDS47 showed the least severe colitis compared to the DSS treated group. Histological scores in all the mice were

determined at the 15th day. The total lesion scores in DSS group increased compared to the normal group. And the decrease of the total lesion scores were the most remarkable in the FDS47 group (Fig. 5B). All these above results indicated that FDS47 could significantly alleviate the clinical symptoms of DSS-induced colitis in mice. And the alleviate effects in FDS47 group were better than those in Bio and CBF groups.

FDS47 Decreased Pro-inflammatory Cytokines In DSS-induced Mice

To understand the mechanism that underlined the alleviation of DSS-induced colitis in mice after treatment with FDS47, the levels of pro-inflammatory (TNF-α, IL-6, and IL-1β) in serum (Fig. 6A-C) and colon tissues (Fig. 6D-F) were examined. No matter in serum or colon tissues, the levels of IL-6, IL-1β and TNF-α were significantly increased in DSS treatment group (Fig. 6). After intragastric administration of FDS47, a remarkable decrease of the three pro-inflammatory cytokines were observed. And FDS47 performed better than Boi and CBF (Fig. 6).

Discussion

Probiotics could relieve various inflammatory diseases by regulating the microbiota in gastrointestinal tract, especially in UC treatment [28]. Nowadays, many probiotics have been shown to have therapeutic effects on UC. Usually, these products are multi-component probiotic mixtures, because each strain may have additive or synergistic effects [29,30]. It is reported that the supplement of *L. plantarum* ZDY2013 and *B. bifidum* WBIN03 could remit UC through modification of gut microbiota to regulate oxidative stress and inflammatory mediators [31]. By the analysis of antibacterial spectrum, gastrointestinal fluid tolerance, bacterial viability and inflammatory factor inhibition, *Rhamnose* and *natto* were selected in this work. They could not only effectively reduce the growth of several harmful bacteria, but also have good tolerance to the gastrointestinal environment (Fig. 1A and B). Additionally, the metabolites of these two probiotics could significantly reduce the release of inflammatory factors in caco-2 cells caused by LPS (Fig. 1C). From these results, it is suggested that *Rhamnose* and *natto* could become hopeful strains in curing UC.

In China, Herbs have been used for thousands of years to cure of diseases. Until now, a large number of biologically active substances in herbs have been proved [32]. Danshen (*Salvia Miltiorrhiza*) is a very valuable herb in Chinese traditional medicine. It has been reported that Danshen has the antioxidant, anti-inflammatory and antibacterial activities [33]. The anti-inflammation efficacy of Danshen is generally played by the main biological activities in it, such as cryptotanshinone, tanshinone IIA and dihydrotanshinone I [34]. Thus, Danshen was selected as fermentation material for the treatment of UC in this work. The traditional extraction methods for Danshen were boiling water extraction and enzymatic hydrolysis. Compared with boiling water extraction, enzymatic hydrolysis could better reduce the release of cellular inflammatory factors (Fig. 1D). Thus, we selected enzymatic hydrolysis to deal with Danshen to release more biological activities.

It is reported that the therapeutic effects of fermented herbs were better than that of herb itself. KIOM-MA is a specific agent for allergic and chronic inflammatory diseases, which is composed of several plants, included *Glycyrrhizae radix, Polygoni cuspidati radix, Sophorae radix, Cnidii rhizoma*, and *Arctii fructus*. Recently, it is proved that the KIOM-MA128, the probiotics fermentation product of KIOM-MA had the improved therapeutic efficacy via the absorption and bioavailability of the active ingredients[35]. FRAM, the fermented products of Rhizoma Atractylodis Macrocephalae (RAM), also exerted a better protective effects on intestinal epithelial cells (IECs) against LPS-induced perturbation of membrane resistance and permeability [36]. The metabolic processes could be improved in herbs fermented with probiotics [37]. And in the process of fermentation, the decomposition of organic matter could be promoted by microorganisms and many new micromolecules could be produced from macromolecules [38]. It is reported that fermentation could better extract the effective ingredients in Chinese herbal medicines to treat enteritis [32]. Therefore, we used the selected probiotics *Rhamnose* and *natto* to ferment Danshen for the first time. The data showed that the fermented Danshen had the better effects than boiled Danshen itself or bacteria solution itself on UC treatment, including the colon length recover, decreased DAI score, lung damage recover and anti-inflammatory activities (IL-6, IL-1β and TNF-α) in serum and colons in DSS induced UC mice (Fig. 4–6). It is suggested that after fermentation, Danshen and probiotics might interact synergistically. However, the synergetic mechanisms, whether effective ingredients was increased or the new biological components were produced after fermentation, need to be further investigated in the next step.

At present, although it is believed that probiotics are safe under normal circumstances, there are individual reports of local or systemic infections such as pericarditis and sepsis caused by the ingestion of certain *lactobacilli, bifidobacteria* and other *lactic acid* bacteria [39]. Especially for patients with immunodeficiency, broken bowel syndrome, central duct occlusion, heart valve disease or premature infants, the risk of adverse events might be higher if probiotics are taken indiscriminately [40]. In severely UC, due to the destruction of the integrity of the intestinal barrier, there is a danger of live probiotics from the intestines and stomach to the internal organs of the body (bacterial displacement), which may lead to bacteremia [41]. Due to the dangers of probiotics during use, we choose safer metabolic fermentation products and verify its safety in in vitro cell experiments and in vivo experiments in mice.

It has been pointed out that the reduction of inflammatory cytokines in the serum and colons represents a logical target for UC therapy [42], such as TNF-α, IL-6 and IL-1β, which play leading roles in the formation of UC [3,43]. Our data suggested that after intragastric administration of the fermentation products, all these pro-inflammatory cytokine (TNF-α, IL-6 and IL-1β) levels were decreased in the serum and colons of the DSS-induced UC mice (Fig. 6). And the in vitro experiments also demonstrated it. The fermentation products inhibited the pro-inflammatory cytokine (TNF-α, IL-6 and IL-1β) levels in the caco-2 cells treated by LPS (Fig. 2). All of the results proved that the probiotics fermented Danshen products relieved the DSS-induced UC mice by blocking pro-inflammatory cytokines.

Conclusions

In summary, we screened *Rhamnose* and *Natto* from many probiotics which could reduce harmful bacteria growth, have tolerance to the gastrointestinal environment and inhibit the inflammatory factors caused by LPS to ferment enzymatic Danshen. The fermentation products has anti-colitis effects through the inhibition of pro-inflammatory factors (Fig. 7). The fermented Danshen might be further developed as an effective treatment approach to treat intestinal inflammation.

Declarations

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

Author contributions

LS designed the work, did the experiments, analyzed the data and wrote the manuscript. YS did the experiments and analyzed the data. ZY A did the experiments. PZ did the experiments. QL Y analyzed the data. CZ analyzed the data. XS acquired the data. SZ revised the manuscript. XL L revised the manuscript. KL L supplied the funds. LZ drafted the work, revised the manuscript, final approval of the version to be published and supplied the funds. All authors read and approved the final manuscript.

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Figures

A

Number	Antimicrobial spectrum analysis				Gastroenteric tolerance	
	S.aureus	*E.coli*	*S.Typhimurium*	*S.Enteritidis*	Gastric Survival	Intestinal Survival
F-B4-1	+	+	+	-	84.17%	105%
F-B8-1	+	+	+	-	111.11%	370%
F-B9-1	+	+	+	-	81.81%	73.33%
F-B16-1	+	+	-	-	138.46%	80.95%
F-B12-1	-	-	-	-	58.82%	13.89%
F-B20-1	-	-	-	-		
F-B25-1	+	-	-	-	180%	13.89%
F-A1-2	+	+	-	-	83.33%	85%
F-A7-1	+	+	-	-	116.67%	120%

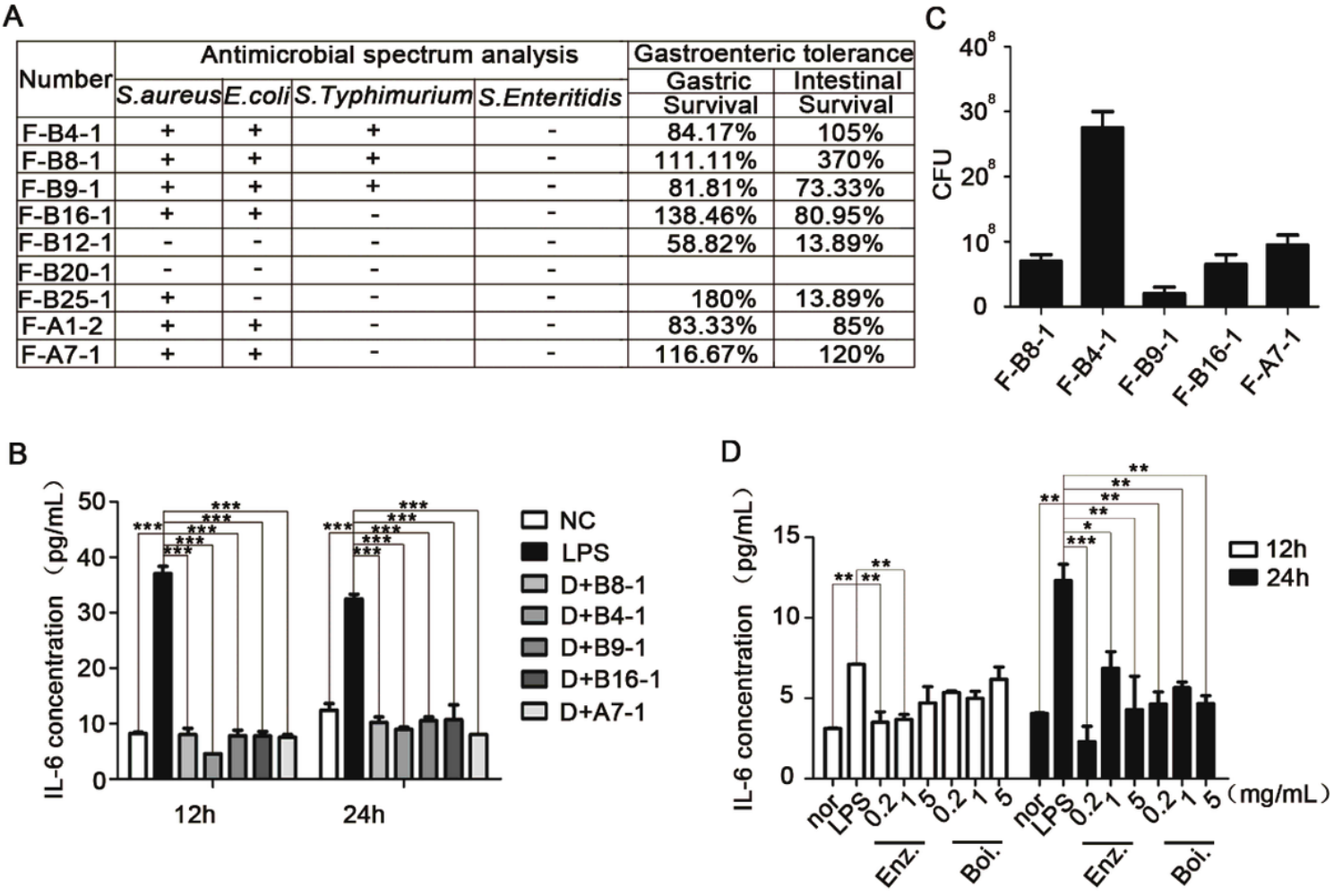


Figure 1

Screening of probiotics and comparison of two traditional extraction methods of Danshen (Salvia miltiorrhiza). (A) Antibacterial spectrum analysis of probiotics and gastrointestinal fluid tolerance. F-B4-1 (Lactobacillus Rhamnosus), F-B8-1 (Lactobacillus Plantarum), F-B9-1 (Lactobacillus Fermenti), F-B16-1 (Lactobacillus Casei), F-B12-1 (Streptococcus Thermophilus), F-B20-1 (Leuconostoc Mesenteroides), F-B25-1 (Lactobacillus Harbin), F-A1-2 (Bacillus Subtilis), F-A7-1 (Bacillus Subtillis Natto). (B) IL-6 levels were examined in the supernatant of Caco-2 cells which were pretreated with 50 ug/ml LPS for 12h, then treated with sterilized probiotic liquid for 12 h or 24 h. (C) The CFU was detected after cultured for 24 h. (D) Enz. (enzymolysis), Boi. (boiled). IL-6 levels were examined in the supernatant of Caco-2 cells which were pretreated with 50 ug/ml LPS for 12h, then treated with different concentrations of the two traditional extraction methods of Danshen for 12 h or 24 h. (Data are expressed as means ± S.E. *p < 0.05, **p < 0.01 and ***p < 0.001)

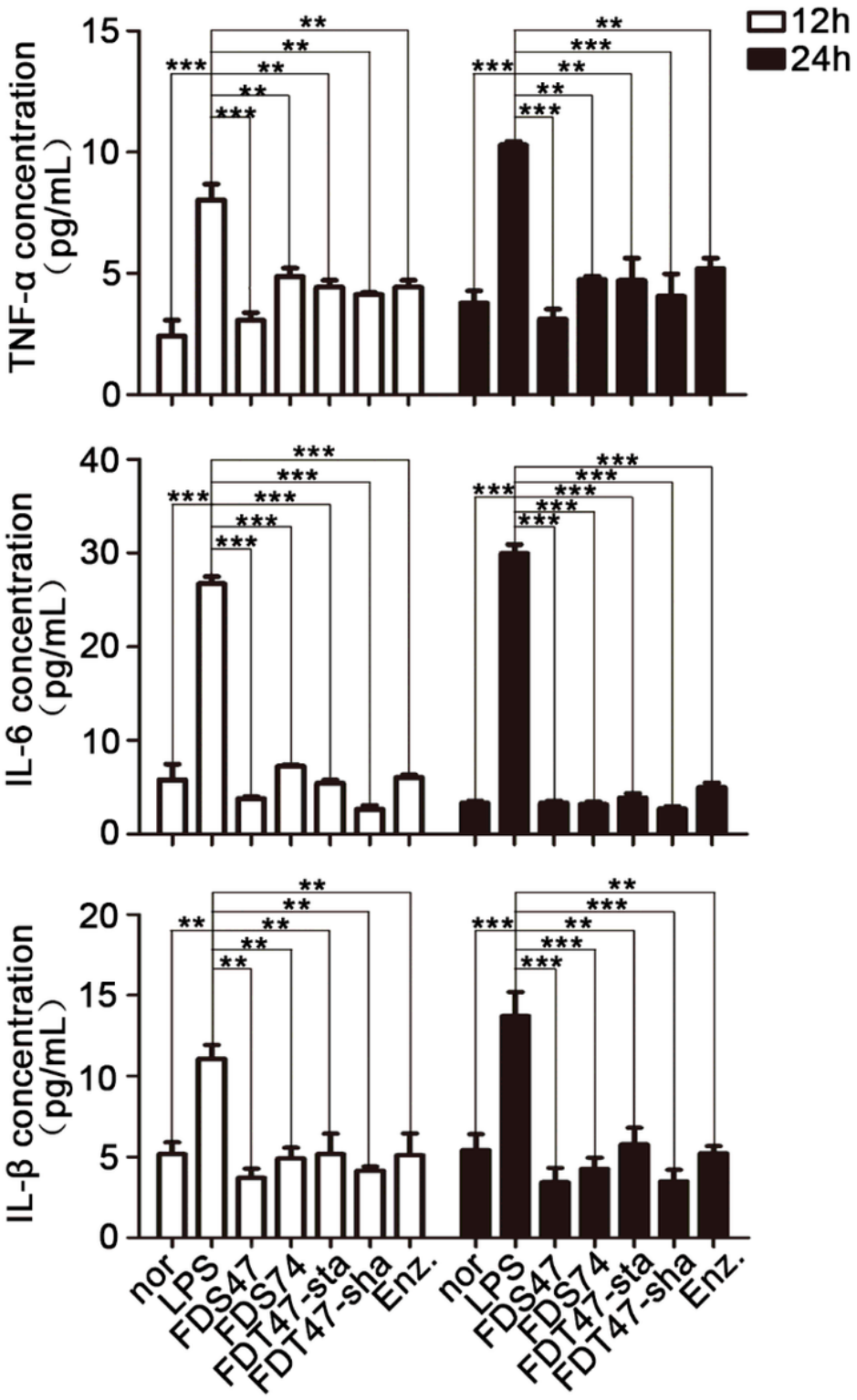


Figure 2

The levels of TNF-α, IL-6 and IL-1β in caco-2 cells after treated with different Danshen products. FDS47 (fermented Danshen sequence 47), FDS74 (fermented Danshen sequence 74), FDT47-sta (fermented Danshen together 47 shake), FDT47-sha (fermented Danshen together 47 stationary), Enz. (enzymolysis). TNF-α, IL-6 and IL-1β levels were examined in the supernatant of Caco-2 cells which were pretreated with

50 ug/ml LPS for 12h, then treated with different Danshen products for 12 h or 24 h. (Data are expressed as means ± S.E., **p < 0.01 and ***p < 0.001)

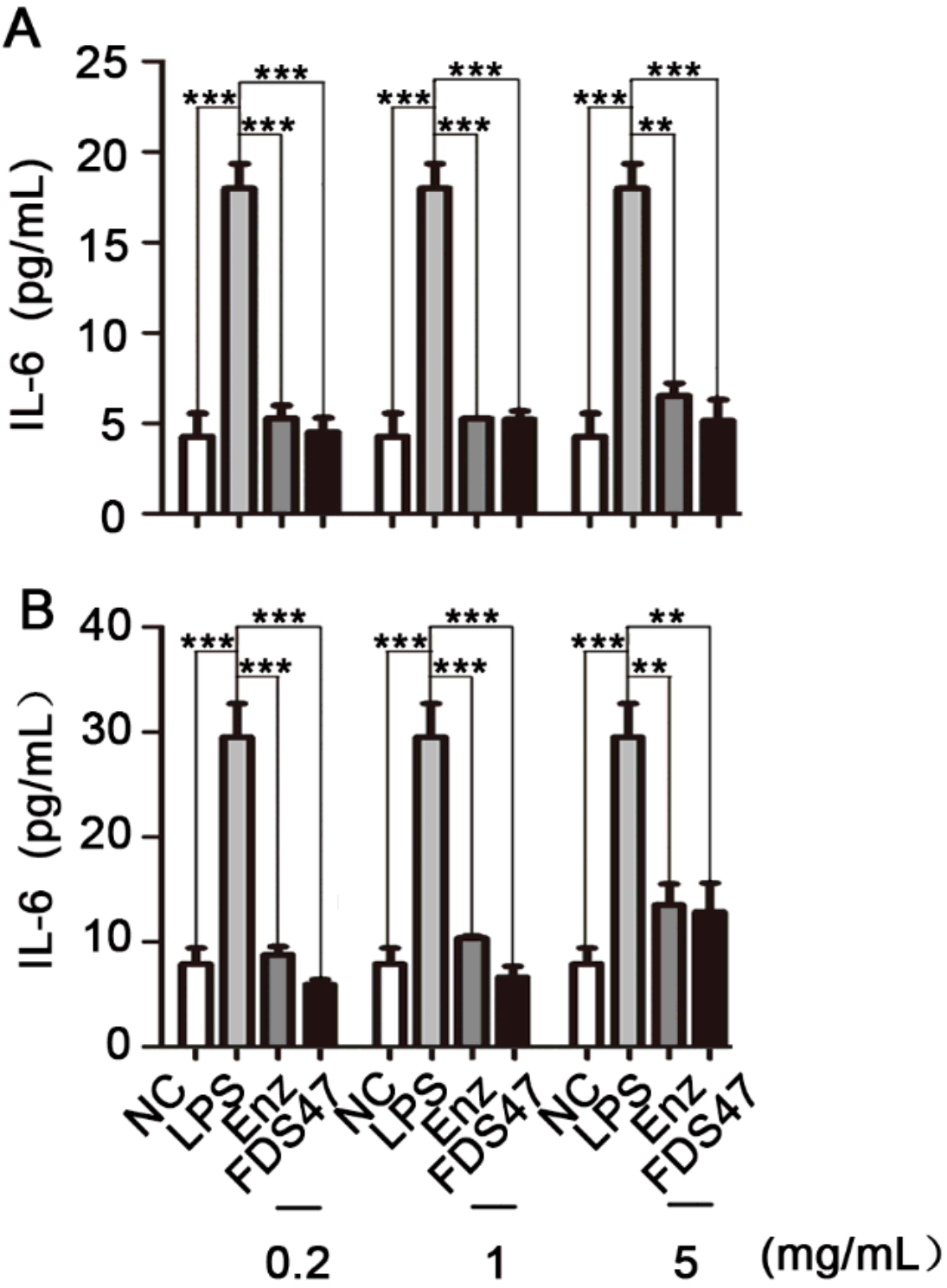


Figure 3

The IL-6 levels in caco-2 cells treated with different concentrations of Enz. and FDS47. FDS47 (fermented Danshen Sequence 47), Enz. (enzymolysis). IL-6 levels were examined in the supernatant of Caco-2 cells

which were pretreated with 50 ug/ml LPS for 12h, then treated with different concentrations of Enz. and FDS47 for 12 h (A) or 24 h (B). (Data are expressed as means ± S.E., **p < 0.01 and ***p < 0.001)

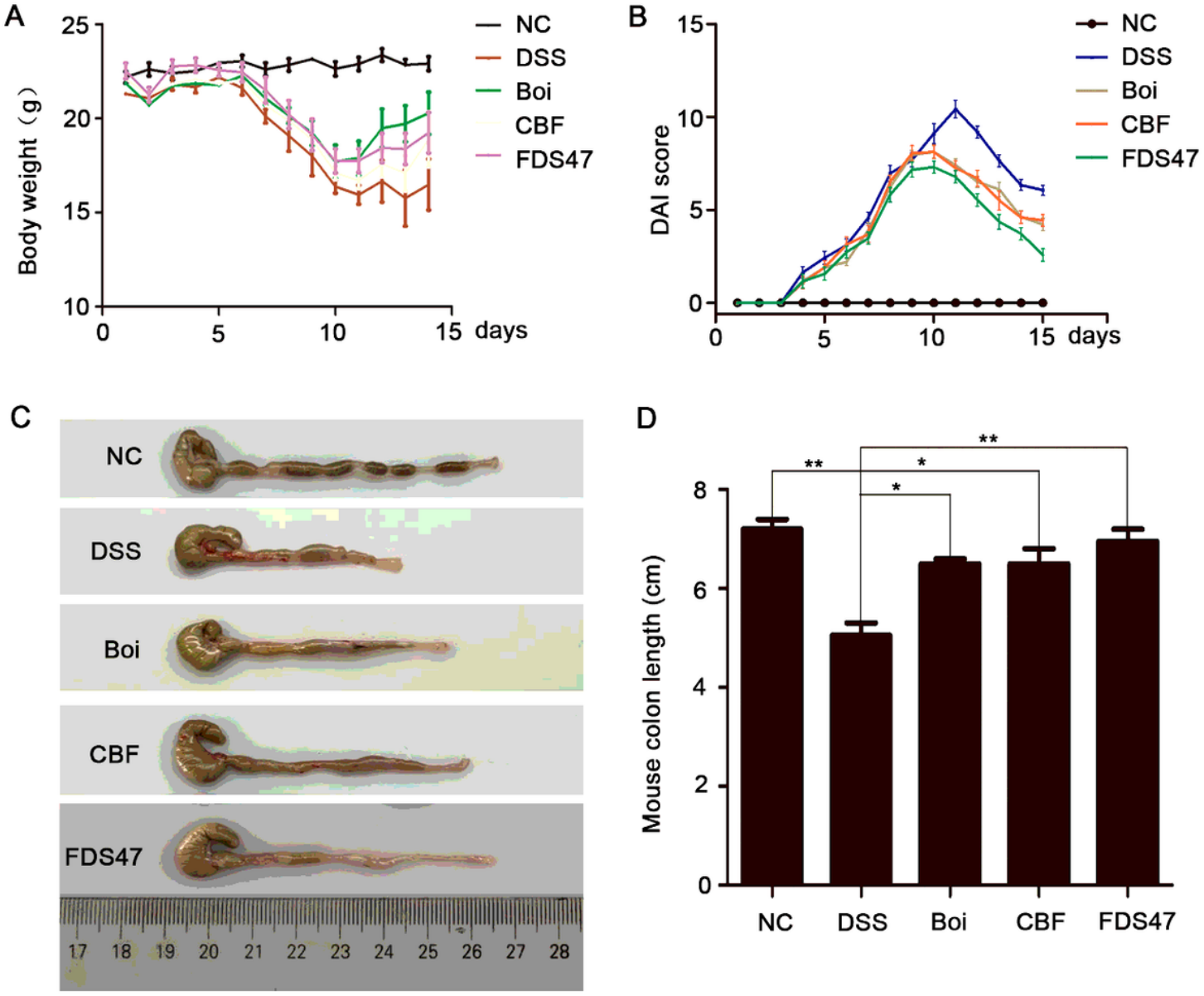
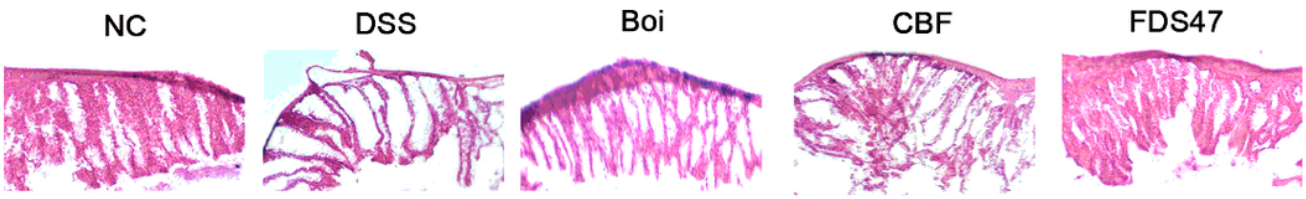


Figure 4

The effects of FDS47 on relieving DSS-induced UC mice was better than boiled Danshen itself or bacteria solution itself. NC (normal), FDS (fermented Danshen Sequence 47), Boi. (boiled), CBF (compound bacterium fluid). (A) The body weights of mice were detected every day. (B) DAI (disease activity index) score of mice was examined every day. (C) The photos of colon length (one representative colon from each group). (D) The colon lengths calculated from C. (Data are expressed as means ± S.E. *p < 0.05 and **p < 0.01)

A



B

Parameter	NC	DSS	Boi	CBF	FDS47
Inflammation					
Severity	1.2±0.1***	3.5±0.0	3±0.3	2.7±0.2	1.9±0.2**
Thickness	0.8±0.2**	2.7±0.2	2.5±0.1	2.4±0.2	1.7±0.2*
Epithelial damage					
Character	0.6±0.2*	1.9±0.2	2.1±0.1	2.0±0.1	1.5±0.2
Extent	1.0±0.0**	2.4±0.1	2.1±0.1	2.1±0.1	1.5±0.1**
Total lesion score	3.6±0.5**	10.5±0.5	9.7±0.6	9.2±0.6	6.6±0.6*

Figure 5

Histological sections of colonic tissue stained with hematoxylin and eosin. (A) Histological sections of colonic tissue stained with hematoxylin and eosin (HE) under microscope. Boi (boiled), CBF (compound bacterium fluid) and FDS47 (fermented Danshen sequence 47). (B) Effects of DSS on colon pathology of DSS-induced UC mice. (Data are expressed as means ± S.E. *p < 0.05, **p < 0.01 and ***p < 0.001)

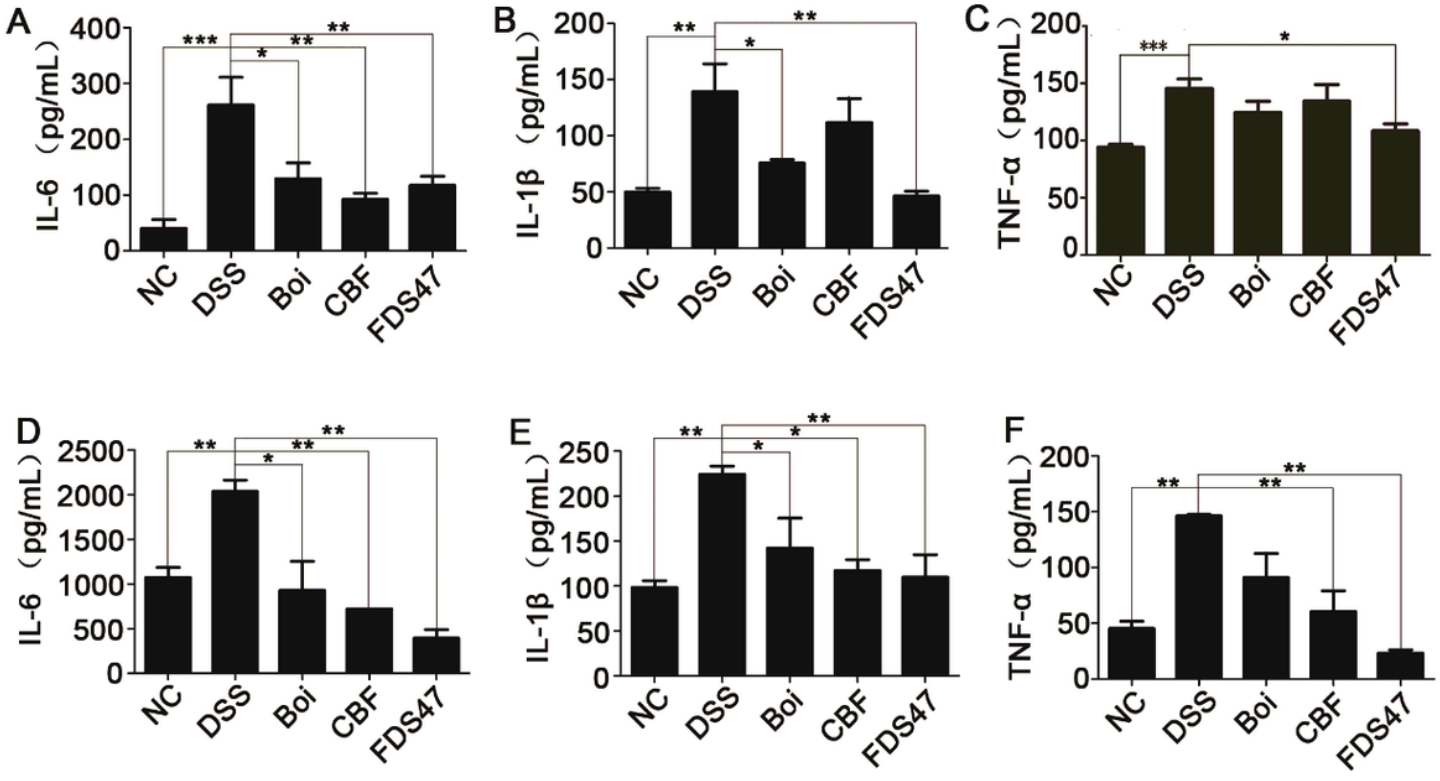


Figure 6

Effects of Boi, CBF or FDS47 on the levels of inflammatory cytokines in mice. Boi (boiled), CBF (compound bacterium fluid) and FDS47 (fermented Danshen sequence 47). The levels of IL-6 (A), IL-1β (B) and TNF-α (C) in serum of DSS- induced UC mice were detected using ELISA Kits. The levels of IL-6 (D), IL-1β (E) and TNF-α (F) in the colonic homogenate of DSS-induced UC mice were detected using ELISA Kits. (Data are expressed as means ± S.E. *p < 0.05, **p < 0.01 and ***p < 0.001)

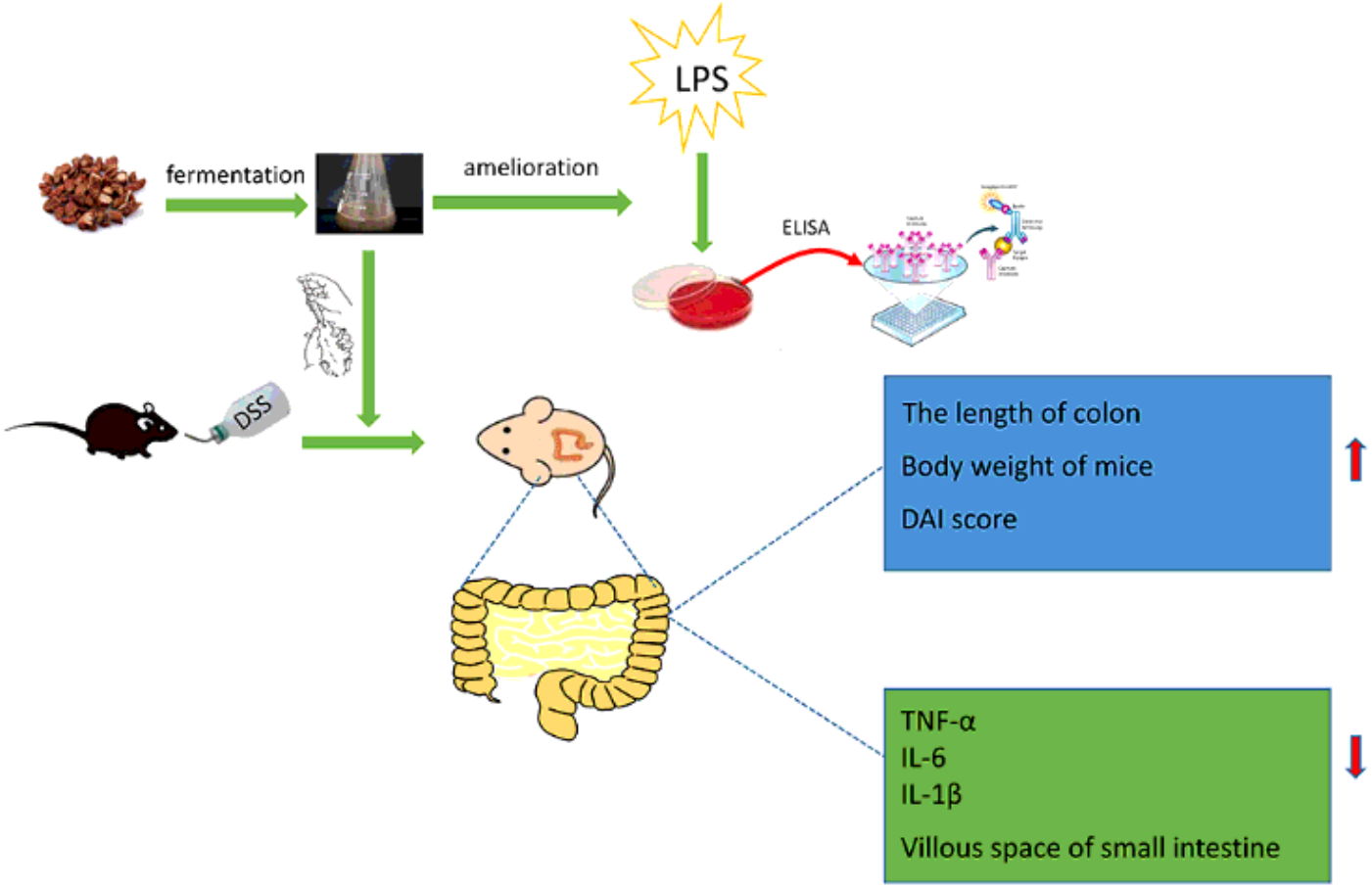


Figure 7

The diagram of the experimental procedure and results.

Supplementary Files

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