Mucosa-reparing and microbiota-balancing therapeutic effect of *Bacillus subtilis* alleviates dextrate sulfate sodium-induced ulcerative colitis in mice

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Abstract. Gut microbiota composition of patients with ulcerative colitis (UC) is markedly altered compared with healthy individuals. There is mounting evidence that probiotic therapy alleviates disease severity in animal models and patients with inflammatory bowel disease (IBD). *Bacillus subtilisis*, as a probiotic, has also demonstrated a protective effect in IBD. However, the therapeutic mechanism of its action has yet to be elucidated. In the present study, a dextrose sulfate sodium (DSS)-induced UC mouse model was used to investigate the role of *B. subtilis* in the restoration of gut flora and determine its effective dose. Mucosal damage was assessed by performing alcian blue staining, cytokine levels were analyzed by ELISA and microbiota composition was investigated using 454 pyrosequencing to target hypervariable regions V3-V4 of the bacterial 16S ribosomal RNA gene. The results demonstrated that a higher dose *B. subtilisis* administration ameliorated DSS-induced dysbiosis and gut inflammation by balancing beneficial and harmful bacteria and associated anti- and pro-inflammatory agents, thereby aiding intestinal mucosa recovery from DSS-induced injuries. These findings indicate that choosing the correct dose of *B. subtilis* is important for effective UC therapy. The present study also helped to elucidate the mechanisms of *B. subtilis* action and provided preclinical data for *B. subtilis* use in UC therapy.

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Introduction

Ulcerative colitis (UC), which is a subtype of inflammatory bowel disease (IBD) (1), is a chronic and debilitating condition that results in serious intestinal injuries. UC typically occurs as a result of inflammatory dysfunction (2). Patients with UC often exhibit intestinal barrier dysfunction, as well as microbiota and bacterial dysbiosis (3).

Although UC is common, its etiology remains poorly understood (4). In a previous study, epithelial barrier impairment was demonstrated to be associated with low-grade inflammation and dysbiosis as potential causative factors, and are associated with the severity of UC (2). Furthermore, in patients with UC, an increase in gut permeability has previously been associated with the altered expression levels or distribution of tight junction proteins, including occludin and zonula occludens-1 (ZO-1) (5). Therefore, increased intestinal permeability and the occurrence of dysbiosis may be the cause of UC-symptoms (6). The evidence that gut microbiota may have a role in the pathophysiology of UC provides a rationale for probiotic use, which has exhibited beneficial effects (7). However, the therapeutic mechanism of action for the effect of probiotics in UC has yet to be elucidated.

Probiotics are defined as live organisms that exert a health benefit on the host through diverse mechanisms. *Bacillus subtilis* is a type of probiotic tolerated by humans and animals (8). *B. subtilis* is hypothesized to affect the composition or function of the commensal, bacterial and host epithelia. Furthermore, it also influences immunological responses and restricts bacterial and lipopolysaccharide (LPS) translocation, and decreases visceral sensitivity (8). In recent clinical trials, probiotics have been widely used to treat disorders of the intestine (7). As enhancement of the intestinal barrier has been associated with the repair of mucosal injuries, the role of *B. subtilis* treatment in maintaining gut barrier integrity was investigated in the present study, due to its potential usage in the alleviation of UC mucosal injuries.

Changes in the gut microbiota have been associated with IBD, including alterations in the relative abundance of bacteria that are both beneficial and detrimental to gut health, and a

decrease in the diversity of the microbiota (9,10). Although previous reports have demonstrated the protective effect of *B. subtilis* in gut protection (11,12), the impact of *B. subtilis* administration on gut microbiota alteration remains unknown. The present study aimed to elucidate the role of *B. subtilis* in the restoration of mucosa, determine its effective dose, and provide preclinical data for *B. subtilis* usage in UC therapy.

Materials and methods

Modeling of colorectal colitis in mice and treatment. Male C57 mice (body weight, 23±1 g; 6 weeks old) were obtained from the Animal Center, Nanjing Drum Tower Hospital (Nanjing, China) and the *in vivo* experiment was performed in the same facility. Mice were maintained under controlled conditions (25˚C, 55% humidity, 12 h light/dark cycle) and fed standard laboratory food. Mice were administered 3% (wt/vol) dextrose sulfate sodium (DSS) (molecular weight, 35,000-44,000; MP Biomedicals, Inc., Aurora, OH, USA) via drinking water for seven days. Additionally, mice were treated daily with different reagents via gavage (catheter diameter, 1.2 mm), including normal saline (NS; n=8) or *B. subtilis* (R179; Beijing Hanmi Pharm Co., Ltd., Beijing, China) at a high ($1x10^9$ CFU/mouse/day; n=8) or low ($1x10^8$ CFU/mouse/day; n=8) dosage until the end of the study. On day eight, mice were weighed and then sacrificed via ether exposure (200 mg/l; Shanghai National Medicine Group, Shanghai, China) in an airtight container in a biosafety cabinet. Colons were harvested, measured and fixed in 4% formalin for subsequent histological examination. Animal experiments were approved by the Ethics Committee of Medical Research, Huashan Hospital of Fudan University (Shanghai, China).

Assessment of colitis. Following the initiation of DSS treatment, daily changes in body weight and clinical signs of colitis, such as rectal bleeding, diarrhea and piloerection, were examined. The disease activity index consisted of scoring for rectal bleeding (0-4), as previously reported (13). Hemoccult SENSA (Beckman Coulter, Inc., Brea, CA, USA) was used to examine rectal bleeding.

Periodic acid-Schiff/alcian blue staining. Alcian blue staining was performed according to a previous report (14). Tissue sections (6 μm thick) were immersed in 100% ethanol for 10 min, rinsed in water for 10 min, immersed in 3% acetic acid for 2 min and subsequently stained in 1% alcian blue 8GX in 3% acetic acid (pH 2.5) for 2.5 h. To remove non-specific staining, 3% acetic acid and water was used to rinse the sections for 10 min. Slides were subsequently oxidized in 1% periodic acid in water at room temperature for 10 min, washed in water for 5 min, immersed in Schiff's reagent for 10 min, rinsed in water for 5 min and three times in 0.5% sodium metabisulphite prior to a final wash in water. To reveal O-acetylated oligosaccharides, sections were treated with 0.1 M KOH for 30 min and 1 mM periodic acid prior to the Schiff reagent.

Immunofluorescence. Frozen tissue sections (6 μm thick) were immunostained with 1:100 primary antibodies against ZO-1 (clonality, H-300; cat. no. sc-10804) and claudin (clonality, D-4; cat. no. sc-137121; species: mouse, rat, human, equine, canine, bovine, porcine) (both Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Images were analyzed using a BIOREVO immunofluorescence microscope (Keyence Corp., Osaka, Japan). Each result was obtained from at least three separate experiments. Six mice per group were prepared for each experiment.

Measurement of intestinal permeability. Intestinal permeability was determined according to a previously described method (15). DSS-treated mice with high/low-dose *B. subtilis* or control saline (n=5; 4 days) were fasted for 12 h prior to oral gavage of disaccharide permeability probes [100 mg/ml lactulose and 50 mg/ml mannitol (both Sigma-Aldrich; Merck Millipore, Darmstradt, Germany) dissolved in 2 ml water] and urine was collected 12 h later. Urine volume was measured and the concentrations of lactulose and mannitol were determined by high-performance liquid chromatography with an NH2 column (Bischoff Chromatohraphy, Leonberg, Germany) and acetonitrile (70%) based elution. The ratio of the amount of probe in urine to the amount administered as lactulose or mannitol recovery rate was calculated accurately. Intestinal permeability was evaluated as a ratio of lactulose recovery rate to mannitol recovery rate.

Measurement of serum cytokines and endotoxin. On the 4th day following DSS treatment, mice were anesthetized with ether (200 mg/l; Shanghai National Medicine Group) in an airtight container within a biosafety cabinet and blood was collected from the retrobulbar venous plexus using pyrogen-free heparinized syringes. Cytokine [interleukin (IL)-10, IL-12 p70, IL-17A, and IL-23] levels were analyzed by ELISA according to the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN, USA). Plasma endotoxin was measured using a Limulus amebocyte lysate pyrogen test kit (Xiamen Houshiji, Ltd., Xiamen, China; cat. no. KC48).

Short-chain fatty acid (SCFA) assay. Fresh mice fecal samples were collected from the cages, weighed and stored at -80˚C. Fecal samples were mixed with distilled water and centrifuged (2,500 x g). The supernatant was removed, filtered and mixed with ether and sulfuric acid. Following centrifugation (2,500 x g), the ether layer was collected and measured in an Agilent 6890N gas chromatograph machine (Agilent Technologies, Inc., Santa Clara, CA, USA) to determine the total SCFA concentrations.

Microbiological analysis of mice fecal samples. Microbiota composition was assessed by 454 pyrosequencing (GS FLX TI technology, Genoscreen, Lille, France) targeting the V3-V4 region of the bacterial 16S rRNA gene (V3, forward 5'-TAC GGRAGGCAGCAG-3' and V4 reverse 5'-GGACTACCA GGGTATCTAAT-3'). Sequences were binned for a minimal sequence length of 300 pb, a minimal base quality threshold of 30 cycles and a maximum homopolymer length of 6 cycles. Resulting sequences were assigned to different taxonomic levels, from phylum to genus using the Ribosomal Database Project (16). Sequences were further clustered into operational taxonomic units (OTUs) or phylotypes at 97% of identity using the Quantitative Insights into Microbial Ecology pipeline and CD-HIT (17,18). OTUs were assigned to their closest taxonomic neighbors and relative bacterial species using Seqmatch

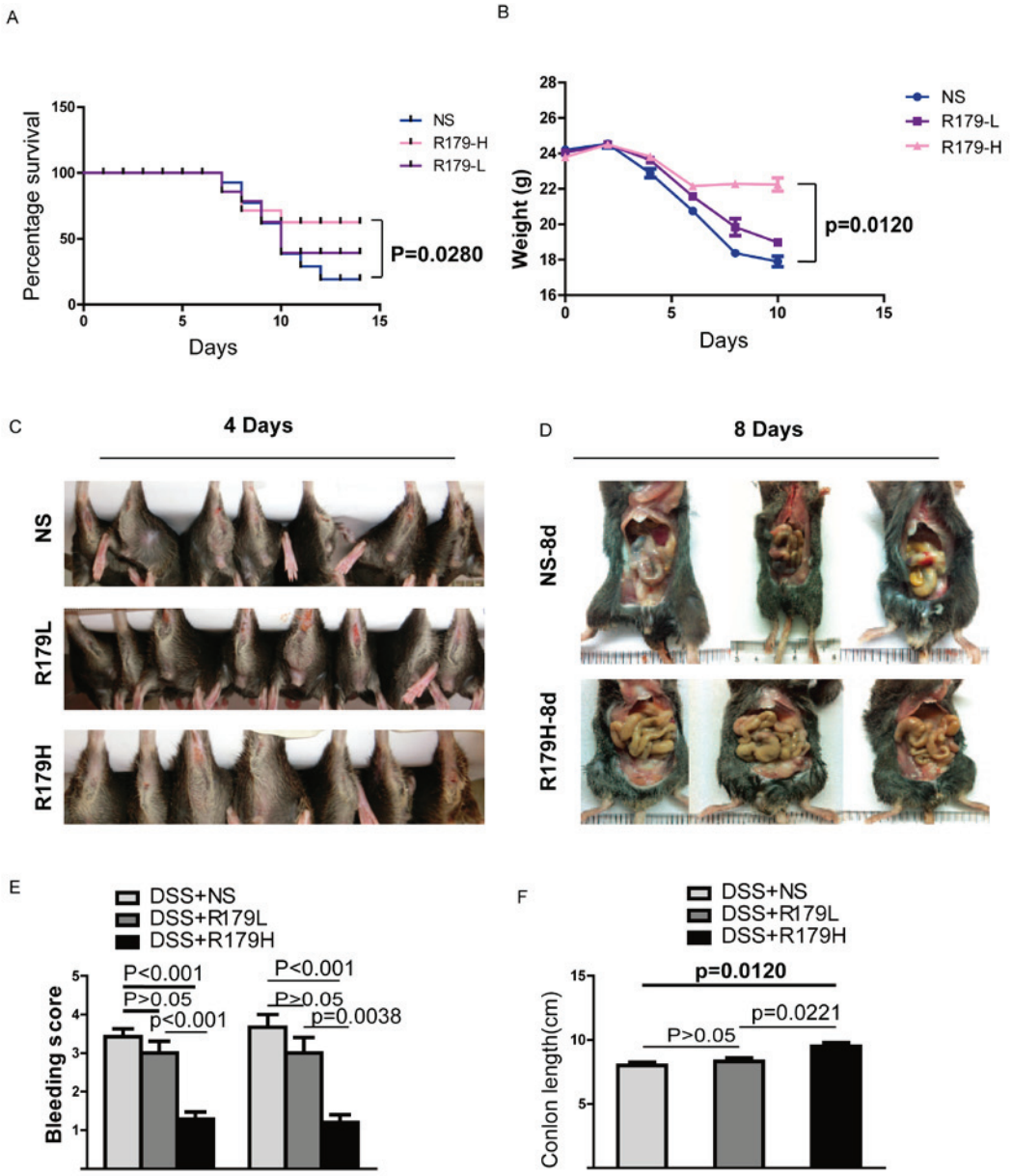

Figure 1. *Bacillus subtilis* alleviates DSS-induced lethality and intestinal injuries in mice. (A) Percentage survival rate over time; (B) change in body weight over time; representative image of (C) degree of gut damage and (D) colon length after mice were treated for 8 days; (E) bleeding scores after mice were treated for 4 days; and (F) colon length after mice were treated for 8 days. DSS, dextrose sulfate sodium; NS, normal saline; R179-L, low-dose *B. subtilis*; R179-H, high-dose *B. subtillis*.

(Michigan State University, East Lansing, MI, USA) and Blastall (National Centre for Biotechnology Information, Bethesda, MD, USA).

Statistical analysis. Data were expressed as the mean ± standard error of the mean. Differences were analyzed using Student's *t*-test, Chi-square test, or one-way analysis of variance with Tukey's post-hic test for multiple group comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

B. subtilis alleviates DSS-induced lethality and intestinal injuries in mice. To examine the role of *B. subtilis* in the amelioration of UC *in vivo*, mice were initially exposed to a lethal dose of 4% DSS (20 ml/d), a pharmacological agent used to induce UC that also causes severe secondary symptoms. Mice were subsequently treated orally with either a high- or low-dose of *B. subtilis* preparation, or phosphate-buffered saline for control at eight days post-administration. The results indicated that the high dose of *B. subtilis* solution protected mice from the lethal effect of DSS-induced UC (Fig. 1A). At the end of the study, ~50% survival of probiotic-treated mice was observed. In contrast, only ~1/3 of mice who received normal saline survived for the same period as the high-dose probiotic-treated group and this difference was significant (P=0.0280; Fig. 1A). In addition, a high-dose of *B. subtilis* administration significantly ameliorated weight loss compared

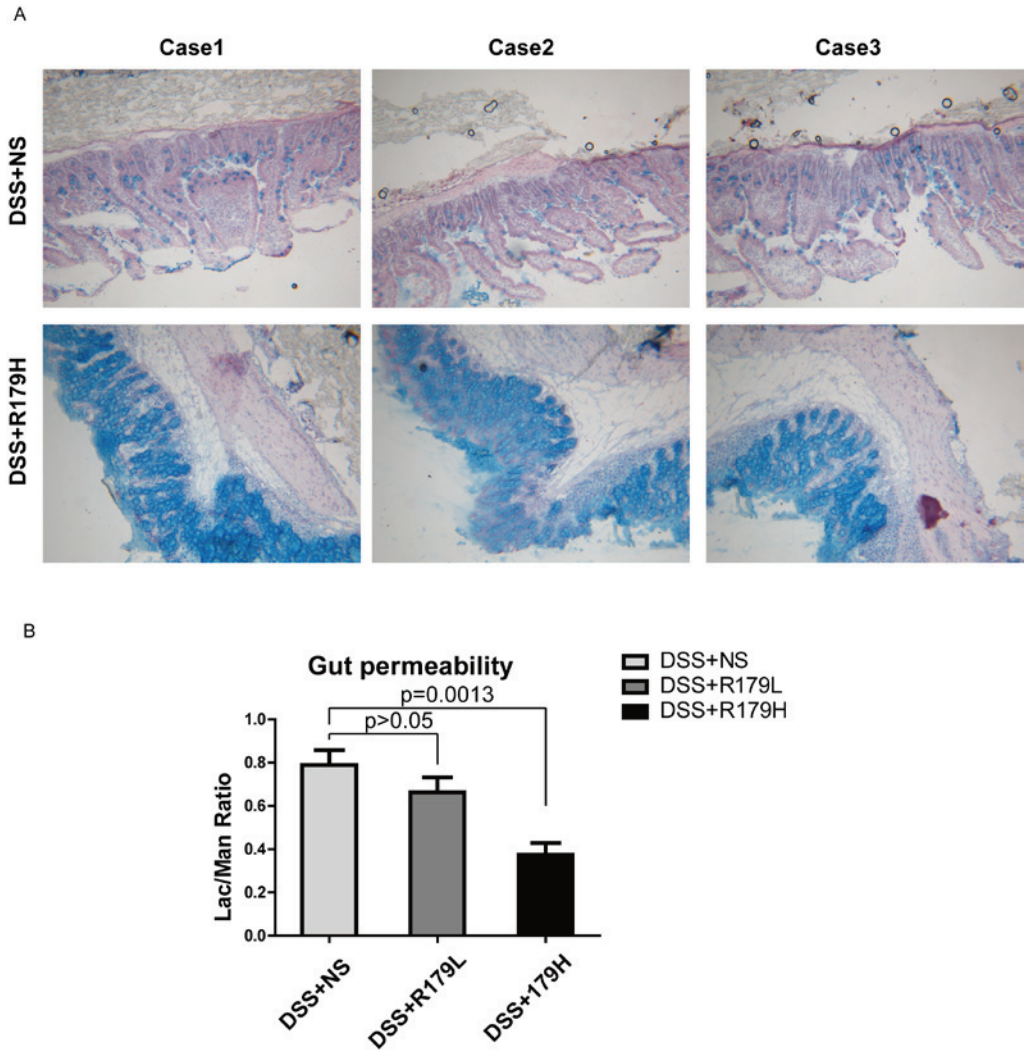

Figure 2. *Bacillus subtilis* protects mouse intestinal mucosal from DSS-induced damage. (A) Mucin was detected by alcian blue staining after mice were treated for 4 days. (B) Detection of intestinal permeability after mice were treated for 4 days. DSS, dextrose sulfate sodium; NS, normal saline; R179L, low-dose *B. subtilis*; R179H, high-dose *B. subtillis*.

with the normal saline group (P=0.0120; Fig. 1B). As DSS can promote intestinal damage, such as hematochezia and intestinal bleeding, the anuses and intestinal tracts of the three groups were examined. For mice in the high-dose *B. subtilis*-treated group, the anus and intestinal tract exhibited reduced bleeding and anabrosis than the control and low dose groups (Fig. 1C and D). Mice in the control and low-dose groups suffered more severe colon necrosis and shorter colons compared with the high-dose *B. subtilis*-treated group (Fig. 1E and F). These results indicate that high-dose *B.* subtilis administration alleviates DDS-induced colon damage and that *B. subtilis* induces a dose-dependent effect.

B. subtilis protects against DSS-induced intestinal mucosal damage in mice. DSS induces UC by causing serious intestinal mucosal damage (19). In accordance with the findings mentioned, low or high doses of *B. subtilis* may attenuate the symptoms of DSS-induced UC. In light of this, it was hypothesized that *B. subtilis* may produce its effect by protecting the intestinal mucosa from damage and by reinforcing its repair. To test this hypothesis, alcian blue staining was conducted to determine how the intestinal mucosa reacted to the administration of *B. subtilis* following DSS treatment. As shown in Fig. 2A, mice treated with high-dose *B. subtilis* exhibited increased expression levels of mucins compared with the control group, which indicated repair of the colon mucosa. These results suggest that the high dose of the *B. subtilis* probiotic promoted the restoration of intestinal mucosa.

To further test this hypothesis, intestinal permeability was measured, as DSS-induced intestinal mucosa damage may lead to an increase in intestinal permeability. The results of the present study demonstrated that intestinal permeability was recovered in the *B. subtilis*-treated group (Fig. 2B). This further supports the hypothesis that treatment with *B. subtilis* may protect intestinal mucosa from DSS-induced damage and attenuate the inflammatory reaction.

B. subtilis helps to restore tight junctions. The tight junction complex of the intestinal mucosa is considered to be the first 'firewall' for gut immunity (20). ZO-1 and claudins are two large families of the tight junction complex. To further explore whether *B. subtilis* was able to repair DSS-induced damage to the tight junctions, the intestines of mice treated with or without *B. subtilis* were harvested and probed with

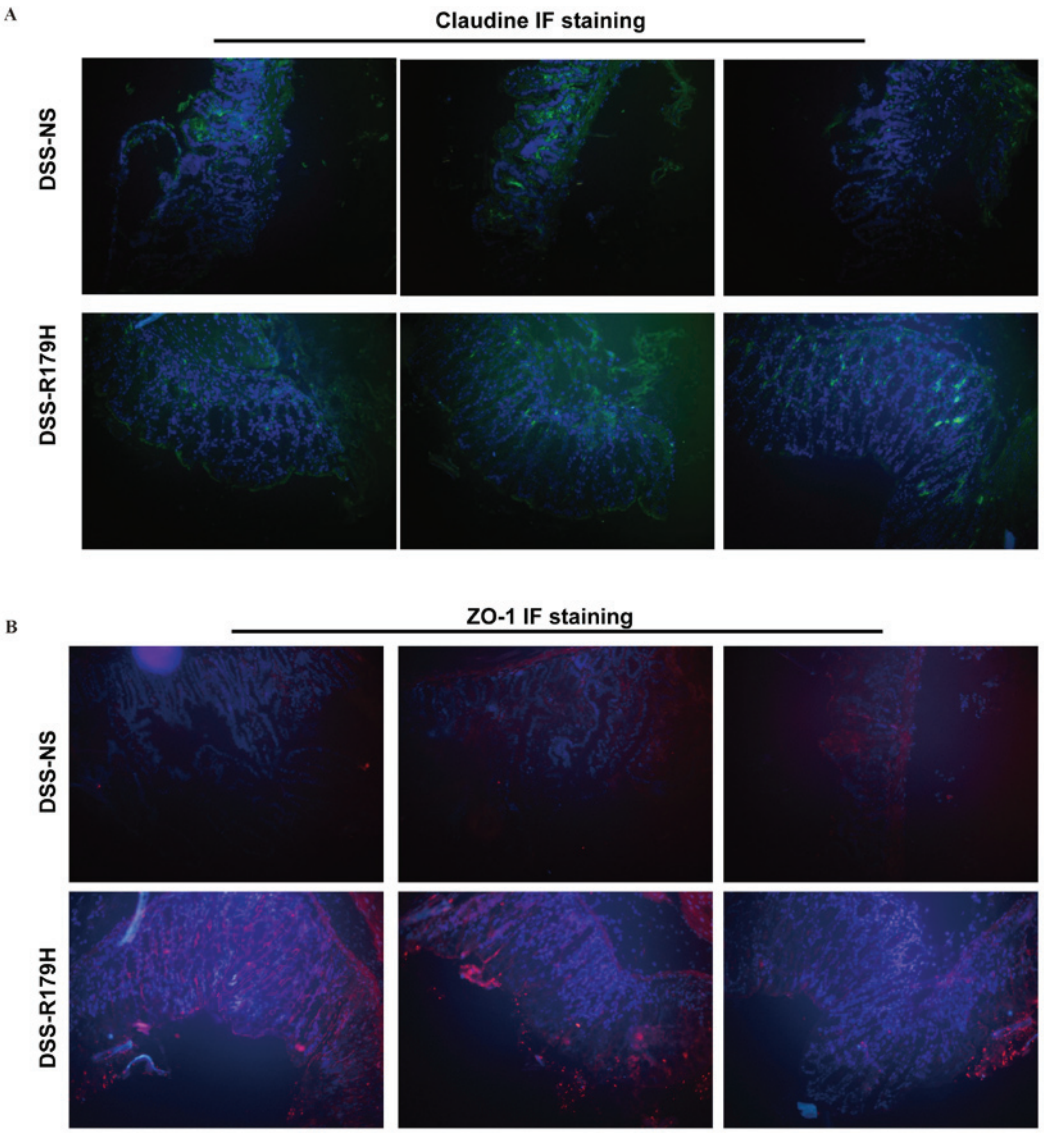

Figure 3. *Bacillus subtilis* helps restore tight junctions damaged by DSS. IF staining with the primary antibody (A) claudin and (B) ZO-1. IF, immunofluorescence; DSS, dextrose sulfate sodium; NS, normal saline; R179H, high-dose *B. subtillis*; ZO-1, zona occludens-1.

ZO-1 and claudins. The samples were then observed under a confocal laser scanning microscope, and the results demonstrated that the two tight junction-associated markers increased following high-dose *B. subtilis* treatment (Fig. 3A and B). This indicated that *B. subtilis* was involved in the repair process of DSS-induced mucosal damage and restored the mucosal tight junction complex.

B. subtilis administration alleviates systemic inflammation upon DSS treatment. Restoration of intestinal permeability may be related to the relief of the inflammation reaction (21). Therefore, the effect of *B. subtilis* administration on gut inflammation was explored using ELISA. An increase in plasma cytokines has previously been reported, including IL-12, IL-17 and IL-23, whereas IL-10 decreased in IBD (22). In the present study, the mean plasma levels of IL-12, IL-17 and IL-23 in the high-dose *B. subtilis* group were significantly reduced (P=0.0411, 0.0087 and 0.0152, respectively) and the mean IL-10 plasma levels were significantly increased (P=0.0450) (Fig. 4) compared with the NS group. However, low-dose *B. subtilis* treatment produced a less-marked effect.

B. subtilis administration balances anti-and pro-inflammatory factors in the gut of mice. Gut microbiota is a primary source of LPS endotoxin, which is a damage-associated pathogen that promotes gut inflammatory reactions and systemic inflammation (23,24). Plasma LPS of mice was tested and a difference in LPS was detected between the high-and low-dose *B. subtilis*-treated groups and the control group. These results indicated a significant decrease of LPS concentration in the probiotic group with the administration of high-dose *B. subtilis* compared with the control group (P<0.001; Fig. 5A).

SCFAs have anti-inflammatory functions via interaction with G protein-coupled receptor 43 and have been demonstrated to induce pro-inflammatory cytokines in various models of colitis (25,26). The concentration of total SCFAs was significantly higher in the high-dose *B. subtilis* group

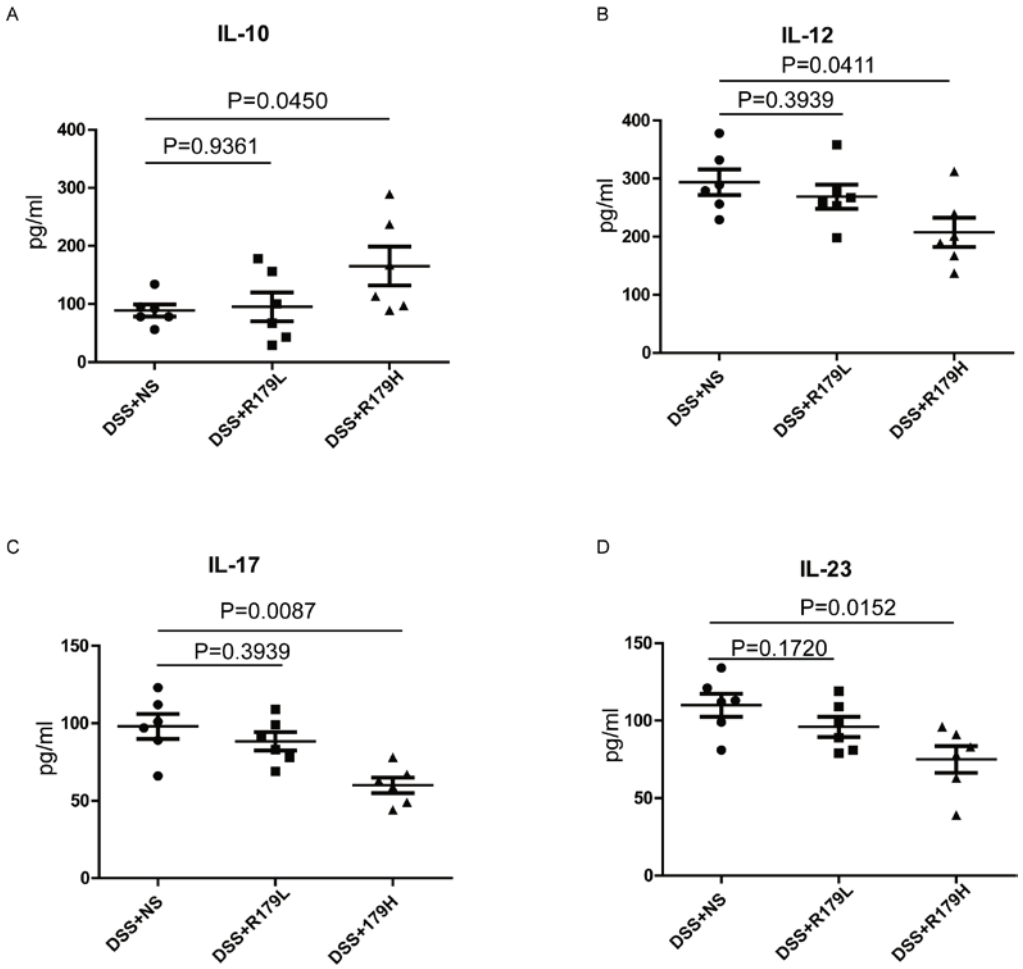

Figure 4. *Bacillus subtilis* administration alleviates DSS-induced systemic inflammation. Serum (A) IL-10, (B) IL-12, (C) IL-17 and (D) IL-23 detection by ELISA. DSS, dextrose sulfate sodium; NS, normal saline; R179L, low-dose *B. subtilis*; R179H, high-dose *B. subtillis*; IL, interleukin.

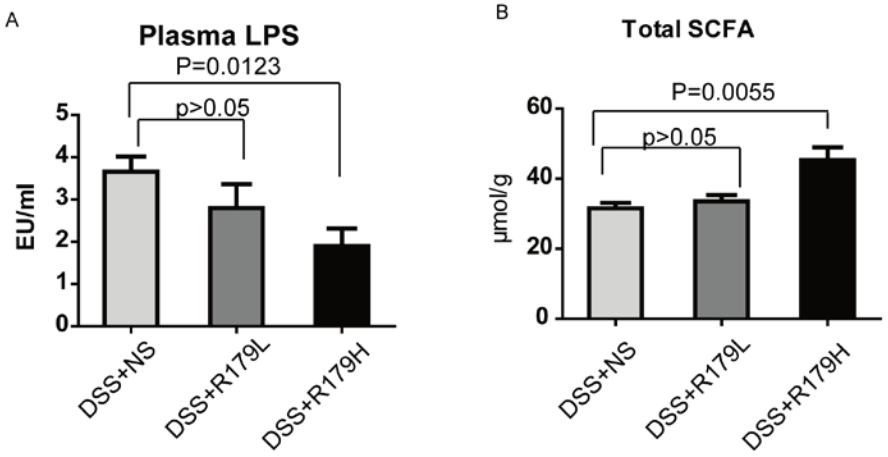

Figure 5. *Bacillus subtilis* administration balances anti-and pro-inflammatory factors in the gut. Detection of (A) plasma LPS and (B) fecal total SCFA. LPS, lipopolysaccharide endotoxin; SCFA, short chain fatty acid; DSS, dextrose sulfate sodium; NS, normal saline; R179L, low-dose *B. subtilis*; R179H, high-dose *B. subtillis*.

compared with the control group (P=0.0055; Fig. 5B), suggesting that high-dose *B. subtilis* was beneficial in maintaining SCFA content, which in turn reduced gut inflammation.

B. subtilis administration ameliorates DSS-induced dysbiosis in the gut of mice. There are 100 trillion microorganisms housed in the human body. These microorganisms are maintained as commensals on the gut mucosa, and are associated

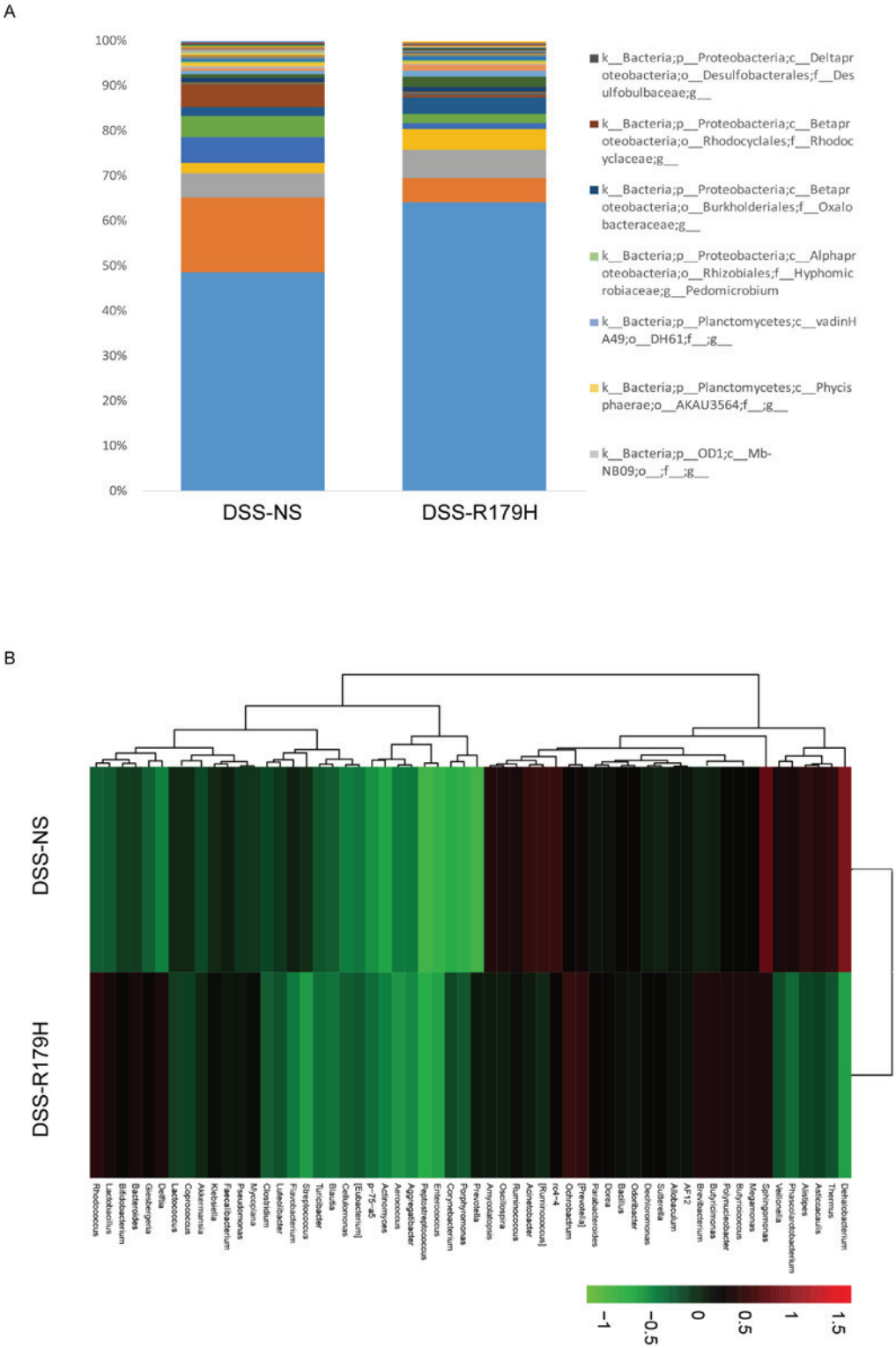

Figure 6. *Bacillus subtilis* administration ameliorates DSS-induced dysbiosis in the gut. (A) Microbiota composition at the genus level in mice after 4 days of treatment with *B. subtilis*. (B) Heatmap of differentially expressed bacterial genera for NS or high-dose *B. subtilis*-treated mice (4 days). Mice with the highest and lowest bacterial levels are presented as red and green, respectively. DSS, dextrose sulfate sodium; NS, normal saline; R179H, high-dose *B. subtillis*.

with metabolism, including maintaining the internal environment and regulating the immune system (27). Consequently, 16s-rDNA sequencing analysis was performed in the present study to examine changes in the microbiota. As demonstrated in Fig. 6, it was observed that the gut microbiota was markedly altered in the high-dose *B. subtilis*-treated group compared with the control group. Specifically, a reduction of *Acinetobacter* sp., *Ruminococcus* sp., *Clostridium* spp. and *Veillonella* sp. was detected upon high-dose *B. subtilis* treatment, whereas levels of *Bifidobacterium* sp., *Lactobacillus* sp., and *Butyricicoccus* sp. were increased. *Acinetobacter* spp., *Ruminococcus* spp. *Clostridium* sp. and *Veillonella* sp. have been shown to be overrepresented in IBD patients, whereas *Bifidobacterium* spp., *Lactobacillus* spp., and *Butyricicoccus* sp.

are decreased (28-32). These results demonstrated the role of *B. subtilis* in the amelioration of DSS-induced dysbiosis in the gut of model mice.

Discussion

In the present study, DSS was used to induce UC in a mouse model, and treatment with *B. subtilis* was revealed to markedly decrease the mortality of DSS-treated mice and protect the intestine from further damage. In addition, *B. subtilis* treatment decreased the damage caused by DSS, which supports the hypothesis that *B. subtilis* is able to repair epithelial cell injury in intestinal inflammation via immunomodulation (9). Elevated levels of IL-12, IL-17 and IL-23 have previously been found in the epithelial mucosal barrier of subjects with IBD, whereas IL-10 is known to have a protective role in alleviating gut inflammation (9,33,34). Additionally, the present study detected elevated levels of IL-10 and decreased levels of IL-12, IL-17 and IL-23 in the high-dose, but not low-dose, *B. subtilis*-treatment groups. Therefore, the present study provides evidence that *B. subtilis* regulates gut immune balance in a dose-dependent manner.

A reduction in the number of SCFA-producing bacteria can result in a degree of focal metabolic stress and vulnerability to inflammatory disease (35). Using gas chromatography, it was determined that *B. subtilis* administration increased the levels of SCFAs. In addition, a significant increase of the *Butyricicoccus* spp., which contributes to butyrate generation (36), was detected upon *B. subtilis* treatment in the present study. Previous studies have shown that the IBD phenotype was associated with lower levels of the *clostridial* cluster IV genus *Butyricicoccus* (36,37). These findings indicate that *B. subtilis* may be beneficial for the survival and expansion of *Butyricicoccus* spp. under the conditions of gut damage.

A balance of healthy gut commensal bacteria is required for the suppression of pathogenic infections (38), with increasing evidence suggesting that the restoration of normal commensals via transplant is more effective at fighting *Clostridium* sp. infection than antibiotics (39). Commercially available probiotics, including *Lactobacillus* and *Bifidobacterium* spp., are used to attenuate inflammatory activity and prevent relapses in UC (40). In the present study, it was determined that beneficial *Bifidobacterium*, *Lactobacillus*, and *Butyricicoccus* spp. increased in the high-dose *B. subtilis*-treated groups, compared with the control group. Species known to promote gut damage, such as *Acinetobacter* sp., *Ruminococcus* sp., *Clostridium* spp. and *Veillonella* sp, were found to be decreased following *B. subtilis* treatment in the present study. These results indicate the potential role of *B. subtilis* administration in restoring a healthy balance of beneficial and harmful bacteria in the gut.

In conclusion, dose-dependent *B. subtilis* administration was demonstrated to aid intestinal mucosa recovery from DSS-induced damage and protect the intestinal mucosa by balancing beneficial and harmful bacterium and their respective, associated anti- and pro-inflammatory agents. The present study elucidated the mechanisms of *B. subtilis* action and provided preclinical data for *B. subtilis* use in UC therapy.

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