Amelioration of the DSS-induced colitis in mice by pretreatment with 4,4’-diaponeurosporene-producing Bacillus subtilis

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Abstract. Inflammatory bowel disease (IBD) is a chronically relapsing inflammatory disorder of the gastrointestinal tract. Current IBD treatments have poor tolerability and insufficient therapeutic efficacy, thus, alternative therapeutic approaches are required. Recently, a number of dietary supplements have emerged as promising interventions. In the present study oral administration of a carotenoid (4,4’-diaponeurosporene)‑producing Bacillus subtilis markedly ameliorated dextran sulfate sodium salt‑induced mouse colitis, as demonstrated by a reduction in weight loss and the severity of bleeding, which indicated that 4,4’‑diaponeurosporene may have beneficial effects on treatments for colitis. This preliminary study indicated that 4,4’‑diaponeurosporene may function synergistically with probiotics to provide a novel and effective strategy to prevent colitis.

Introduction

Inflammatory bowel disease (IBD) in humans, including Crohn’s disease and ulcerative colitis, is a complex chronic inflammatory disorder (1). The incidence of these diseases varies widely between different countries, but overall has increased greatly in recent years, making IBD a major public health problem now (2). Though its etiopathogenesis is ambiguous, there exist a growing awareness that oxidative stress and the resulting inflammation play an important role in the development of IBD (3–6).

Current IBD treatments include aminosalicylates, corticosteroids, inhibitors of tumor necrosis factor ‑ α (TNF‑α), antibiotics and immunosuppressants (7). However, these agents have poor tolerability and insufficient therapeutic efficacy; therefore, the need for alternative therapeutic approaches is increasing (8,9). It is now clear that probiotic intervention is able to prevent pouchitis and has found to be effective in inducing and maintaining remission in ulcerative colitis, although reports might be conflicting, as they involve different mixtures of probiotic strains, different protocol designs, various doses, different read-outs and different clinical settings or patient types (10,11). The Gram‑positive spore‑forming probiotic, Bacillus subtilis (B. subtilis), has been well demonstrated to have probiotic potential (12,13). Spore‑forming bacteria may offer many interesting advantages compared to non‑sporeformers, as they are heat‑stable, resistant to low pH and other deleterious conditions such as gastric and bile secretions in the intestinal environment. Moreover, spores allow long term storage of preparations without refrigeration or need for encapsulation processes (14). Notably, a number of studies have indicated the anti‑inflammatory function of some B. subtilis strains in IBD (15,16).

Carotenoids, a subfamily of the isoprenoids containing more than 700 members, are currently used for food colorants and nutritional supplements (17). Carotenoids can act as antioxidants with the potential to remove free radicals, either by a direct reaction with radicals, resulting in the formation of harmless products, or by disrupting radical chain reactions, avoiding further damage of cellular compounds, such as membrane lipids. It was noted that patients with ulcerative colitis have extremely low concentrations of serum and mucosa carotenoids (such as lutein, zeaxanthin, α- and β-carotene) as compared to that of healthy individuals (18,19). Moreover, several studies have showed that dietary carotenoids inhibited colitis and colitis‑associated colon carcinogenesis in mice (20–22).

In previous study, we had constructed a carotenoid (4,4’-diaponeurosporene)-producing B. subtilis (B.s-Dia) by transforming a plasmid which containing carotenoid synthase gene, crn, and carotenoid dehydrogenase gene, crtn, into B. subtilis strain WB800 (23). The aim of this study was to address the protective effect of carotenoid‑producing B. subtilis on murine experimental colitis. Our results indicated that oral administration of B.s-Dia had an improved positive effect on colonic histopathological changes and length in mice dextran sodium sulfate (DSS)‑induced colitis.

Materials and methods

Mice. C57BL/6 mice (23–24 g), 8 weeks old, were purchased from the Animal Research Center of Yangzhou University.
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(Jiangsu, China). The mice were maintained under specific pathogen-free conditions for at least 1 week before use. The animal studies were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University and followed National Institutes of Health guidelines for the performance of animal experiments.

**Bacterial strains, animals.** B.s-Dia was constructed as previously described. B.s or B.s-Dia were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl/l) containing 50 µg/ml kanamycin at 37˚C.

**Induction of experimental colitis.** To inducing colitis, DSS (molecular weight 5,000; MP Biomedicals Inc., Eschwege, Germany) was added into mouse drinking water at the concentration of 5% (w/v) for 6 or 7 days. And mice were randomly equally divided into four groups (n=7 in each) as follows: Control group was just given PBS by gavage each day for 7 days and no DSS was added in drinking water. DSS group was also given PBS by gavage once a day for 7 days but followed by DSS added drinking water every day for successively 7 days. B.s+DSS or B.s-Dia+DSS group was given 1x10^9 cfu B. subtilis Wb800 or B.s-Dia once a day for 7 days and followed by DSS added drinking water every day for successively 7 days. And the dose of B.s-Dia was selected based on the results of previous studies (24-27), and that a dose of 1x10^9 cfu/ml also has been demonstrated to significantly alleviate colitis (28).

**Assessment of mouse weight and colon length.** Mouse weight was measured daily from day 0 to 7 at 9:30 a.m. every day and expressed as the relative change from day 0. The colon was isolated immediately after the last weight check. The colon was excised between the ileocaecal junction and the proximal rectum, close to its passage under the pelvisternum. The colon was placed on a non-absorbent surface and its length was measured with a ruler, in such a manner that the organ was not stretched. Mouse weight and colon length were determined as described previously (29,30).

**Histological observation and scoring.** The morphological evaluation of colon in each group was performed using hematoxylin and eosin (H&E) staining. The colons were fixed in 4% paraformaldehyde, embedded in paraffin and sliced into sections of 5 µm thickness. After H&E staining, histological analysis was performed in a blind fashion. Colitis was assessed with tissue sections and was scored in a blinded experimental set-up according to a standard scoring system: 0, no thickening of colonic tissues and no inflammation (infiltration of lymphocytes); 1, mild thickening of tissues but no inflammation; 2, mild thickening of tissues and mild inflammation; 3, severe thickening and severe inflammation (31).

**Statistical analysis.** Results were expressed as mean ± standard deviation (SD). One-way ANOVA was employed to determine statistical differences among multiple groups, and t-test was employed to determine the same between two groups. P-values <0.05 were considered statistically significant (P<0.05, P<0.01). Histological scores were analyzed by the Mann-Whitney U test.

**Results**

**DSS-induced colitis model.** In order to establish DSS-induced colitis, mice were administered 5% DSS in the drinking fluid for 6 days and weighed every day. DSS-treated mice drastically lost weight from the 4th day after initiation of the medication, compared with that of untreated animals (Fig. 1A). In addition, DSS-treated mice also had remarkably shortened colon (Fig. 1B), these results indicated the successful construction of mice colitis.

**Influences of B.s-Dia oral administration on mouse hemafecia and body weight.** To evaluate the effects of B.s-Dia on colitis, mice were orally administrated 1x10^7 cfu B.s-Dia every day, followed by adding 5% DSS in the drinking water for 7 days (Fig. 2A). Though, both B.s and B.s-Dia oral administration observably diminished bloody stools as showed in

Figure 1. DSS-induced colitis model. (A) Mice were administered 5% DSS in the drinking fluid for 6 days and weighed every day. (B) The colon was excised between the ileocaecal junction and the proximal rectum, close to its passage under the pelvisternum. The colon was placed on a non-absorbent surface and its length was measured with a ruler, in such a manner that the organ was not stretched. One representative of three similar independent experiments is shown. Asterisks indicate statistical significance via the one-way ANOVA test. *P<0.01 vs. the PBS-treated group. DSS, dextran sodium sulfate; Crl, control.
Fig. 2. Influences of B.s-Dia oral administration on mouse hemafecia and body weight. (A) The graphical representation of mouse treatment, taken B.s-Dia as example. (B) Anus of mice from different groups were photographed. (C) Mice were administered 5% DSS in the drinking fluid for 7 days and weighed every day. One representative of three similar independent experiments is shown. Asterisks indicate statistical significance via the one-way ANOVA test (*P<0.01 vs. PBS+DSS-treated group. n=7. DSS, dextran sodium sulfate; B.s, *Bacillus subtilis*; B.s-Dia, 4,4'-diaponeurosporene-producing *Bacillus subtilis*).

Fig. 3. Influences of B.s-Dia oral administration on colon length. The colon of mice from different groups was isolated and placed on a non-absorbent surface and its length was measured with a ruler. One representative of three similar independent experiments is shown. Asterisks indicate statistical significance via the one-way ANOVA test (*P<0.05). n=7. DSS, dextran sodium sulfate; Crl, control; B.s, *Bacillus subtilis*; B.s-Dia, 4,4'-diaponeurosporene-producing *Bacillus subtilis*.

Fig. 2B, B.s-Dia was more effective in this process compared with B.s. Importantly, mice in the B.s-Dia group had significantly reduced weight loss than that in the DSS or B.s group on day 7 (Fig. 2C).

**Influences of B.s-Dia oral administration on colon length.**
A shorter colon length and bleeding are considered as a hallmark of experimental colitis (32,33). Our result revealed that oral administration of B.s-Dia significantly increased colon length in experimental colitis, while B.s oral administration had no significantly effect on colon length (Fig. 3). Nevertheless, both B.s and B.s-Dia reduced the severity of bleeding in colon wall.

**Influences of B.s-Dia on histological damage induced by DSS.**
To further evaluate the effects of B.s-Dia on DSS-induced colitis, we performed histopathological examination. As showed in Fig. 4A, the surface epithelium, cryptal glands, mucosa, and submucosa in the normal mice were intact. However, DSS-treated mice showed severe damage of the surface epithelium, bleeding, disruption of the cryptal glands, and thickened submucosal layer. Mice administered B.s showed relatively intact surface epithelium, but the disruption of cryptal glands and infiltration of the inflammatory cells were still observed. However, oral administration of B.s-Dia showed more protective effects and improved DSS-induced pathology (Fig. 4B).
Discussion

At present, there is no known cure for human IBD (34). Immunosuppressive therapies, for example with TNF-α antagonists (35), are currently being used as remedies against severe human diseases. Recent investigations have focused on the development of new therapeutic strategies that aim to restore the balance of the gastrointestinal microbiota to reduce or prevent intestinal inflammation. There are growing evidence that probiotic microorganisms might influence disease outcome of IBD in both animal models and humans (36).

The *B. subtilis* species has a long history of safe use and several studies have indicated its ameliorative effect on murine experimental colitis (15,28). In addition, the preventive effect of different *B. subtilis* strains on colitis is not the same (14). Because the protective effect of probiotics is related with its antioxidant and anti-inflammatory abilities (37,38). And the severity of colitis induced by the different concentrations of DSS is not the same. Accordingly, *B. subtilis* treated mice still suffer relatively severe colitis.

Carotenoids are a kind of natural pigment with antioxidant properties that prevent oxidative stress. It can also effectively ameliorate symptoms of colitis. Literature shows that β-carotene treatment ameliorated the severity of UC by modulating various molecular targets (such as nuclear factor-κB, cyclooxygenase-2, interleukin-17) and maintained the gut integrity by increasing the expression of a tight junction protein, occluding, which was decreased in the colon of mice with UC (21). Furthermore, lycopene (39), lutein and zeaxanthin (40) all have effects on colitis. In this study, we showed that 4,4'-diaponeurosporene-producing *B. subtilis* had an outstanding ability to prevent DSS-induced colitis.

4,4'-Diaponeurosporene is also a kind of carotenoids. Our previous study showed that the carotenoid 4,4'-diaponeurosporene modulated hydrogen peroxide-induced oxidative stress in mouse dendritic cells (23), indicating its antioxidant activity. Moreover, it has been reported that the addition of *B. subtilis* to chicken diets can enhance the activity of antioxidant enzymes to scavenge free radicals (41,42). And present study shows that *B.s-Dia* had a more positive effect on DSS-induced colitis, as compared with *B. subtilis*. This result implied the potential beneficial effect of 4,4'-diaponeurosporene on colitis, possibly because of its antioxidant activity. Consequently, Bs-Dia has potential application value for the prevention and treatment of IBD and colitis in clinic.

In brief, the results of the present preliminary study show that Bs-Dia can effectively improve the DSS-induced intestinal mucosal epithelial injury, bleeding, crypts gland rupture and submucosal thickening and other pathological manifestations, greatly alleviate DSS-induced colitis. However, we should note that the mechanism underlies the protection of *B. subtilis* or carotenoids might be complex, and more efforts are needed to illustrate it. Our future studies will aim to investigate the specific factors (intrinsic regulatory mechanisms and epithelial tight junction protein) that *B.s-Dia* can alleviate colitis, and the dose-dependent effects of *B.s-Dia* pre-treatment on colitis.

Figure 4. Influences of B.s-Dia on histological damage induced by DSS. (A) The colon of mice from different groups was fixed in 4% paraformaldehyde, embedded in paraffin and sliced into sections of 5 µm thickness, and followed by hematoxylin and eosin staining. bleeding (black arrow), cell detachment (red arrow), thickened submucosal layer (double sided arrow). (B) Colitis was assessed with tissue sections and was scored in a blinded experimental set-up as described in ‘Materials and methods’. Histological scores were analyzed by the Mann-Whitney U-test (*P<0.05, **P<0.01). n=5. Scale bar, 200 nm. DSS, dextran sodium sulfate; B.s, Bacillus subtilis; B.s-Dia, 4,4'-diaponeurosporene-producing Bacillus subtilis.
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