Full Paper

Administration of *Bifidobacterium pseudolongum* suppresses the increase of colonic serotonin and alleviates symptoms in dextran sodium sulfate-induced colitis in mice

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Previous studies suggested that altered gut serotonin (5-HT) signaling is implicated in the pathophysiology of inflammatory bowel disease (IBD). Indeed, 5-HT administration reportedly exacerbated the severity of murine dextran sodium sulfate (DSS)-induced colitis that mimics human IBD. Our recent study suggested that *Bifidobacterium pseudolongum*, one of the most predominant bifidobacterial species in various mammals, reduces the colonic 5-HT content in mice. The present study thus tested whether the administration of *B. pseudolongum* prevents DSS-induced colitis in mice. Colitis was induced by administering 3% DSS in drinking water in female BALB/c mice, and *B. pseudolongum* (10^9 CFU/day) or 5-aminosalicylic acid (5-ASA, 200 mg/kg body weight) was intragastrically administered once daily throughout the experimental period. *B. pseudolongum* administration reduced body weight loss, diarrhea, fecal bleeding, colon shortening, spleen enlargement, and colon tissue damage and increased colonic mRNA levels of cytokine genes (*Il1b*, *Il6*, *Il10*, and *Tnf*) almost to an extent similar to 5-ASA administration in DSS-treated mice. *B. pseudolongum* administration also reduced the increase of colonic 5-HT content, whereas it did not alter the colonic mRNA levels of genes that encode the 5-HT synthesizing enzyme, 5-HT reuptake transporter, 5-HT metabolizing enzyme, and tight junction-associated proteins. We propose that *B. pseudolongum* is as beneficial against murine DSS-induced colitis as the widely used anti-inflammatory agent 5-ASA. However, further studies are needed to clarify the causal relationship between the reduced colonic 5-HT content and reduced severity of DSS-induced colitis caused by *B. pseudolongum* administration.

Key words: erotonin, *Bifidobacterium pseudolongum*, dextran sodium sulfate (DSS)-induced colitis, inflammation

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT), a bioactive monoamine, is synthesized in serotonergic neurons of the central nervous system and enterochromaffin (EC) cells in the gut epithelium, and more than 90% of total body 5-HT is synthesized in the latter cells [1–3]. Synthesis of 5-HT in EC cells relies on the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1), which metabolizes the 5-HT precursor, L-tryptophan. Upon stimulation by chemical and mechanical stimuli in the gut lumen and neural and endocrine inputs, EC cells release 5-HT from their basal surfaces into the interstitial space of the lamina propria [1–3]. The released 5-HT participates in various functions in the gut, which include peristalsis, secretion, and vasodilation, by acting on a diverse range of receptors expressed on smooth muscle, enteric neurons, and epithelial cells [1–3]. For instance,

5-HT-induced gut segmentation serves to mix the small intestinal contents and increase their exposure to digestive enzymes during digestion [1]. In addition, intestinal 5-HT modulates pancreatic enzyme secretion, which is the mechanism by which the gut communicates with the pancreas to fulfill its exocrine enzyme needs, depending on the contents in the gut [1]. 5-HT signaling in the gut is terminated by the removal of 5-HT from the interstitial space through the serotonin reuptake transporter (SERT) expressed in the epithelial cells. 5-HT is then converted to 5-hydroxyindoleacetic acid by a monoamine oxidase-A (MAO-A) in the epithelial cells [1–3]. Otherwise, extracellular 5-HT is transported to platelets to circulate.

Inflammatory bowel disease (IBD) comprising ulcerative colitis (UC) and Crohn's disease (CD) is a chronic relapsing inflammatory condition of the gastrointestinal tract. Currently, the prevalence of IBD is increasing globally [4]. Although its etiology

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is not well understood, the pathogenesis of IBD is thought to involve the complex interaction between genetic predisposition, inappropriate immune response, gut dysbiosis, a weakened gut barrier, and environmental factors [5–7]. Previous studies have suggested that altered 5-HT signaling in the gut is implicated in the pathophysiology of IBD. Regarding the 5-HT content in the gut tissues, Magro *et al.* [8] and Coates *et al.* [9] showed a reduction in the colonic mucosa of UC and CD patients, whereas El-Salhy *et al.* [10] reported contrasting data. As Coates *et al.* [11] mentioned in their review, this difference may be associated with disease severity. However, most animal studies have shown significant increases in colonic 5-HT content in experimental colitis. For instance, Oshima *et al.* [12] and Matsumoto *et al.* [13] reported that the 5-HT content in the colonic mucosa was increased in dextran sodium sulfate (DSS)-induced colitis in rats and mice, respectively. DSS-induced colitis has been widely used as a chemically induced gut inflammation model that morphologically and symptomatically mimics human UC [14]. Previous studies suggested that an increased colonic 5-HT content contributes to the aggravation of DSS-induced colitis. For instance, Chen *et al.* [15] showed that the severity of DSS-induced colitis was exacerbated by enema administration of 5-HT in mice. Salaga *et al.* [16] showed that chronic intraperitoneal administration of 5-HT exacerbated DSS-induced colitis in mice. In contrast, Ghia *et al.* [17] reported that a deficiency of the *Tph1* gene that encodes TPH1 reduced the severity of DSS-induced colitis in mice. Therefore, decreasing the colonic 5-HT content may prevent DSS-induced colitis.

Recent studies have highlighted the involvement of gut microbiota in the gut serotonergic system [18, 19]. Their effect has been demonstrated in germ-free mice displaying a lower level of 5-HT content, lower rate of 5-HT synthesis, and lower density of 5-HT-producing EC cells in the colon. More specifically, in terms of bacterial species, a recent study of ours suggested that *Bifidobacterium pseudolongum*, one of the most predominant bifidobacterial species in various mammals [20], reduces the colonic 5-HT content in mice [21]. We observed that the consumption of 1-kestose, an indigestible oligosaccharide, increased cecal *B. pseudolongum* populations and decreased the colonic 5-HT content in mice [21]. In addition, oral administration of *B. pseudolongum*, isolated from mouse feces in our laboratory [22], reduced the colonic 5-HT content [21]. These findings suggest that increased populations of *B. pseudolongum* at least partly mediate the decrease in 5-HT content induced by dietary 1-kestose. Considering the possibility that reducing the colonic 5-HT content might prevent DSS-induced colitis, it is possible that *B. pseudolongum* could prevent DSS-induced colitis. The present study thus tested this idea.

MATERIALS AND METHODS

Animals and diets

All study protocols were pre-approved by the Animal Use Committee of Hokkaido University (approval no. 19-0017), and all mice were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Female BALB/c mice (age, 6 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled ($23 \pm 2°C$) room under a 12-hr light/dark cycle. They were allowed free access to a commercial AIN-93G pellet diet (D10012G, Research Diets, New Brunswick, NJ, USA) and water. Mice were acclimatized for 1 week before the experiment.

Experimental design

Mice were randomly allocated to four groups (n=6 in each group), fed the AIN-93G pellet diet *ad libitum*, and intragastrically administered 200 μL of phosphate-buffered saline (PBS), PBS containing 1×10^9 CFU *B. pseudolongum,* or PBS supplemented with 5-aminosalicylic acid (5-ASA, 200 mg/kg body weight, LKT Labs, Minneapolis, MN, USA) once daily throughout the experimental period. The preparation of *B. pseudolongum* is described below. One week after starting the treatments, mice were allowed free access to deionized water (DW) or DW supplemented with 3% (w/v) DSS (molecular weight 36,000–50,000, MP Biomedicals, Illkirch, France) as drinking water. Thus, mice in the four groups received PBS/DW, PBS/DSS, *B. pseudolongum*/DSS, or 5-ASA/DSS and were referred to as the NON, CON, BIF, and ASA groups, respectively. The start date of DSS administration was referred to as day (d) 0. The disease severity of DSS-induced colitis was assessed daily, as described below. On d 5, DSS was omitted from the drinking water; subsequently, mice received normal water for 2 d. On d 7, mice were anesthetized by inhalation of sevoflurane and killed by cervical dislocation. DSS treatment was performed according to a previous study [22] that investigated the role of 5-HT signaling in DSS-induced colitis. Following a laparotomy, the colon and spleen were excised, and the colon length and spleen weight were measured. The luminal contents of the colon were flushed with ice-cold PBS. The colon tissue was then opened longitudinally, and a 1-cm section was excised and subjected to immunohistochemistry as described below. The mucosa of the remainder of the colon tissue was scraped with a glass slide, immediately plunged into liquid nitrogen, and then stored at −80°C for 5-HT measurement and RNA isolation as described below. The cecal contents were isolated, weighed, and then subjected to the quantification of *B. pseudolongum,* as described below.

Preparation of B. pseudolongum

B. pseudolongum isolated previously from the feces of BALB/c mice [23] was routinely cultured in MRS broth supplemented with 0.05% (w/v) L-cysteine HCl using an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 72 hr [24]. The bacterial culture was washed with sterile PBS before intragastric administration. Meanwhile, viable cell counts were determined by applying serial dilutions of the bacterial culture supplemented with anaerobic phosphate buffer, which was composed of 2% (w/v) buffered peptone water, 0.05% (w/v) L-cysteine HCl, 0.1% (w/v) Tween 80, and 0.1% (w/v) BL agar (Nissui Pharmaceutical, Tokyo, Japan), onto MRS agar plates and by counting colonies formed after incubation at 37°C for 72 hr under anaerobic conditions [25].

Colitis severity assessment

To assess the severity of DSS-induced colitis, stool consistency, fecal bleeding, and body weight were monitored throughout the experimental period. The disease activity index (DAI) was determined by combining the scores of stool consistency (0, normal; 1 and 2, loose stool; 3 and 4, diarrhea), gross fecal bleeding (0, negative; 1, +; 2, ++; 3, +++; 4, ++++), and body

weight loss (0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4: >20%) according to Cooper *et al.* [26]. In addition, colonic tissue damage was histologically scored based on a published scoring system [27]. The tissue samples were fixed in a fixating agent (Ufix, Sakura Finetek Japan, Tokyo, Japan) for 3 hr at room temperature. The samples were then dehydrated through a graded ethanol series, embedded in paraffin, and sliced at a thickness of 5 μm on a microtome (REM-710, Yamato Koki, Asaka, Japan). After deparaffinization and rehydration, tissue sections were stained with hematoxylin and eosin (H&E), followed by microscopic histological assessment in a blind fashion. The histological score ranged from 0 to 12, representing the sum of scores from 0 to 3 each for loss of epithelium, crypt damage, depletion of goblet cells, and infiltration of inflammatory cells [27].

Measurement of 5-HT

Colonic mucosa samples were homogenized in a cell lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 1% (w/v) Triton X-100, 0.15 M NaCl, 0.1% (w/v) sodium dodecyl sulfate (SDS), and 1% (w/v) sodium deoxycholate as described previously [27]. After centrifugation, the concentration of 5-HT in the supernatants was determined using a Serotonin ELISA Kit (Cat# KA2518, Abnova, Taipei, Taiwan) according to the manufacturer's instructions. The 5-HT concentration was normalized to the protein concentration in the supernatants, as determined by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Indianapolis, IN, USA) according to the manufacturer's instructions.

Quantification of bifidobacteria in the cecal contents

DNA was isolated from whole cecal contents in each mouse using a QIAamp DNA Stool Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA samples served as a template for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of the 16S rRNA gene fragment to estimate the numbers of total bifidobacteria and *B. pseudolongum* as previously described [23]. RT-qPCR was performed using GeneAce SYBR qPCR Mix α No ROX (Nippon Gene, Toyama, Japan) with a Thermal Cycler Dice TP800 (Takara Bio, Otsu, Japan). The reaction conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec and 60°C for 60 sec. A melting curve analysis was performed after amplification to assess the specificity of RT-qPCR. The sequences of primers used for RT-qPCR are described in Table 1. For RT-qPCR standards, subcloned 16S rRNA gene fragments were prepared from *Bifidobacterium animalis* (JCM 1190ᵀ) and *B. pseudolongum* subsp. *pseudolongum* (JCM1264) for total bifidobacteria and *B. pseudolongum*, respectively, as previously described [23]. RT-qPCR was carried out in duplicate.

Analysis of mRNA expression

Messenger RNA expression was analyzed as previously described [28, 29]. Total RNA was extracted from colonic mucosa samples using a ReliaPrep RNA Tissue Miniprep System (Promega Japan, Tokyo, Japan) according to the manufacturer's instructions. The RNA concentration was monitored with a spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific) by measuring the absorbance at 260 nm (A260). An A260 of 1.0 was considered 40 μg/mL of the extracted RNA. The ratio of A260 to absorbance at 280 nm was measured, and the samples with a balance of 1.8 to 2.0 were used for reverse transcription. Total RNA (1 μg) was reverse transcribed to generate first-strand cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The reaction was run in a thermal cycler (Life Eco, Bioer Technology, Hangzhou, China) with a thermal profile of 37°C for 15 min followed by 5 min at 95°C, and the obtained cDNA was stored at −20°C for subsequent PCR reactions. Murine interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, MUC2, TPH1, SERT, MAO-A, ZO-1, occludin, claudin-1, claudin-3, and β-actin are encoded by *Il1b, Il6, Il10, Tnf, Muc2, Tph1*, *Slc6a4, Maoa, Tjp1, Ocln, Cldn1, Cldn3*, and *Actb* genes, respectively. To compare the mRNA levels of these genes between the groups, RT-qPCR was performed using GeneAce SYBR qPCR Mix α No ROX with a TP800 Thermal Cycler Dice Real Time System. The thermal profile was adjusted to denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 30 sec and annealing and extension at 60°C for 60 sec. A melting curve analysis was performed after amplification to assess the specificity of RT-qPCR. The data was calculated through the $2^{-\Delta\Delta Ct}$ method with the geometric mean of the endogenous reference gene, i.e., *Actb*. Relative mRNA expression levels for each sample were normalized to that of *Actb*. RT-qPCR was carried out in duplicate. The sequences of primers used for RT-qPCR are described in Table 2.

Statistical analyses

Data are presented as the median ± 95% confidence interval and compared using Welch's ANOVA followed by Dunnett's T3 multiple comparisons post hoc test or two-way repeated measures ANOVA followed by Dunnett's T3 multiple comparisons post hoc test. Data were analyzed using GraphPad Prism software for Macintosh (version 8, GraphPad Software, San Diego, CA, USA). P values of <0.05 were considered statistically significant.

RESULTS

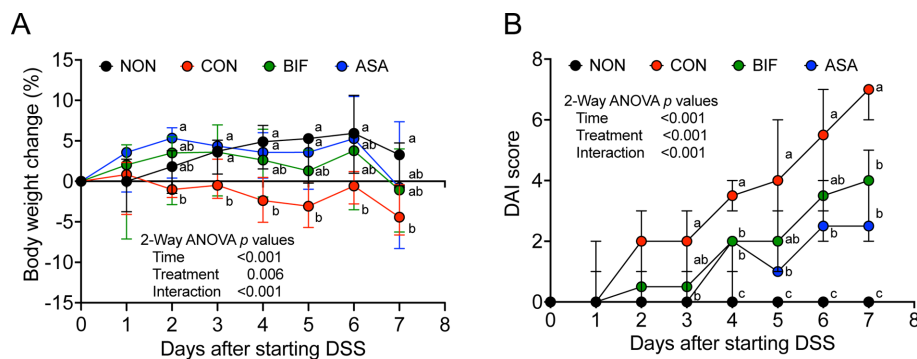### Administration of B. pseudolongum *alleviated DSS-induced colitis*

After starting DSS administration, daily body weight changes in the CON group tended to decrease (Fig. 1A). On d 2 to 7, body weight change was significantly lower in the CON group than in the NON group. The body weight change in the BIF and ASA groups did not differ from that in the NON group. The DAI scores in the CON, BIF, and ASA groups, but not in the NON group, continued to increase during the experimental period (Fig. 1B). The score was significantly higher in the CON group than in the

Table 1. Primer sequences for bacterial 16S rRNA gene

Taxon	Forward	Reverse
Genus *Bifidobacterium*	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC
B. pseudolongum	CCCTTTTTCCGGGTCCTGT	ATCCGAACTGAGACCGGTT

Table 2. Primer sequences for murine genes

Gene	Forward	Reverse
Il1b	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il6	GAGGATACCACTCCCAACAGACC	GAGGATACCACTCCCAACAGACC
Il10	CACAGCAGCTTTGAGGATGA	ATGGGGGACTCTTGGTTAGG
Tnf	CA TCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Muc2	GCTGACGAGTGGTTGGTGAATG	GATGAGGTGGCAGACAGGAGAC
Tph1	AACAAAGACCATTCCTCCGAAAG	TGTAACAGGCTCACATGATTCTC
Slc6a4	TGGGCGCTCTACTACCTCAT	ATGTTGTCCTGGGCGAAGTA
Maoa	CCGCTCCTTTTCCATGCCTCTCAA	CCCTTGTACCGCCCCTTGACTGAA
Tjp1	CCACCGGAGTCTGCCATTACACG	GGGTGGGCTCCTCCAGGCTGACATTAG
Ocln	CTCCCATCCGAGTTTCAGGT	GCTGTCGCCTAAGGAAAGAG
Cldn1	GGCTTCTCTGGGATGGATCG	CTTTGCGAAACGCAGGACAT
Cldn3	GACCGTACCGTCACCACTAC	CAGCCTAGCAAGCAGACTGT
Actb	CTGGGACGATATGGAGAAGA	AGAGGCATACAGGGACAACA

Fig. 1. Effect of intragastric administration of *Bifidobacterium pseudolongum* on the changes in body weight (chart A) and disease activity (DAI) score (chart B) after starting dextran sodium sulfate (DSS)-administration in BALB/c mice. Mice in the NON group received drinking water without DSS, and mice in the CON, BIF, and ASA groups received DSS-supplemented water and were administered PBS, PBS supplemented with live *B. pseudolongum*, or 5-aminosalicylic acid daily, respectively. Data are presented as the median ± 95% confidence interval (*n*=6 in each group). Data without a common letter at a time point differ as estimated using Dunnett's T3 multiple comparisons post hoc test following two-way repeated measures ANOVA (p<0.05). NON: PBS/DW; CON: PBS/DSS; BIF: *B. pseudolongum*/DSS; ASA: 5-ASA/DSS.

NON group on d 3 to 7, and the scores in the BIF and ASA groups were intermediate between those in the NON and CON groups. The score was significantly lower in the BIF group than in the CON group on d 4 and 7 and was significantly lower in the ASA group than in the CON group on d 4 to 7.

Colon length was significantly shorter in the CON group than in the NON group. The lengths in the BIF and ASA groups were significantly longer than that in the CON group and did not differ from that in the NON group (Fig. 2A). Spleen weight was significantly higher in the CON group than in the NON group. The weights in the BIF and ASA groups were intermediate between those the NON and CON groups (Fig. 2B). Figure 2D shows representative light photomicrographs of colonic sections stained with H&E. A severe case in the CON group showed epithelial loss, crypt loss, and marked inflammatory cell infiltration. Although the epithelium was maintained in a mild case in the CON group, mild crypt loss, goblet cell depletion, and inflammatory cell infiltration were still observed. In the BIF and ASA groups, the epithelium, crypts, and goblet cells were maintained, whereas mild infiltration of inflammatory cells was observed. The dissociation between mucosal and muscular layers was due to technical problems we encountered. The total

histological score was significantly higher in the CON and BIF groups than in the NON group and significantly lower in the ASA group than in the CON group (Fig. 2C).
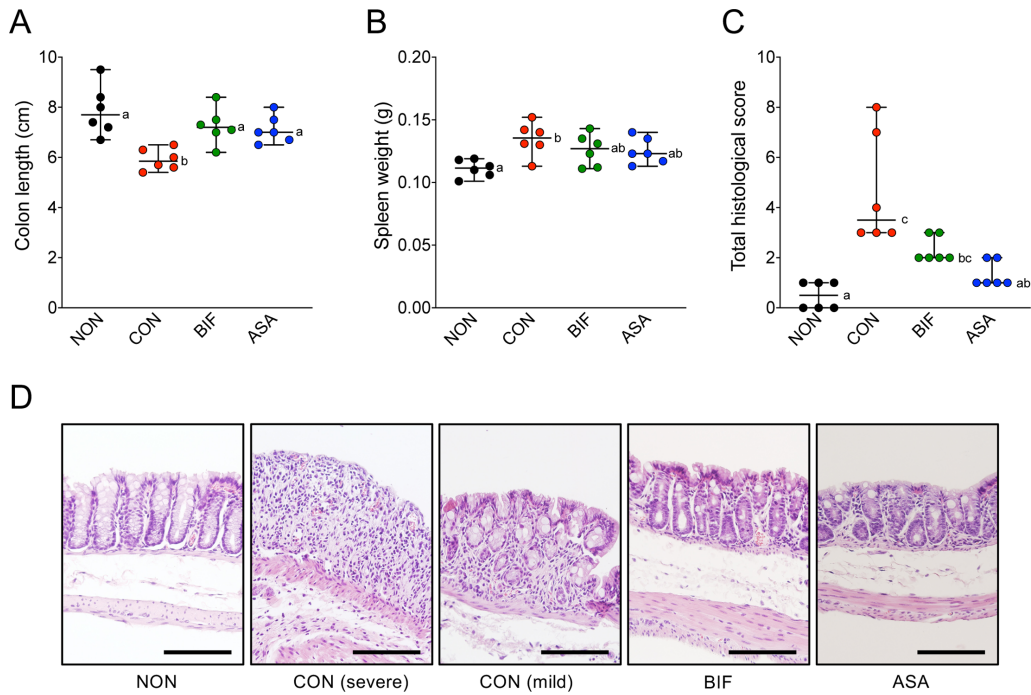
Administration of B. pseudolongum *suppressed the increased expression of inflammatory cytokine genes in DSS-induced colitis*

The mRNA levels of the *Il1b*, *Il6, Il10,* and *Tnf* genes (Fig. 3A, 3B, 3C, and 3D, respectively) were significantly higher in the CON group than in the NON group and significantly lower in the BIF and ASA groups than in the CON group. The mRNA levels of these genes in the BIF and ASA groups did not differ from those in the NON group. The mRNA level of the *Muc2* gene was significantly lower in the CON, BIF, and ASA groups than in the NON group (Fig. 3E).
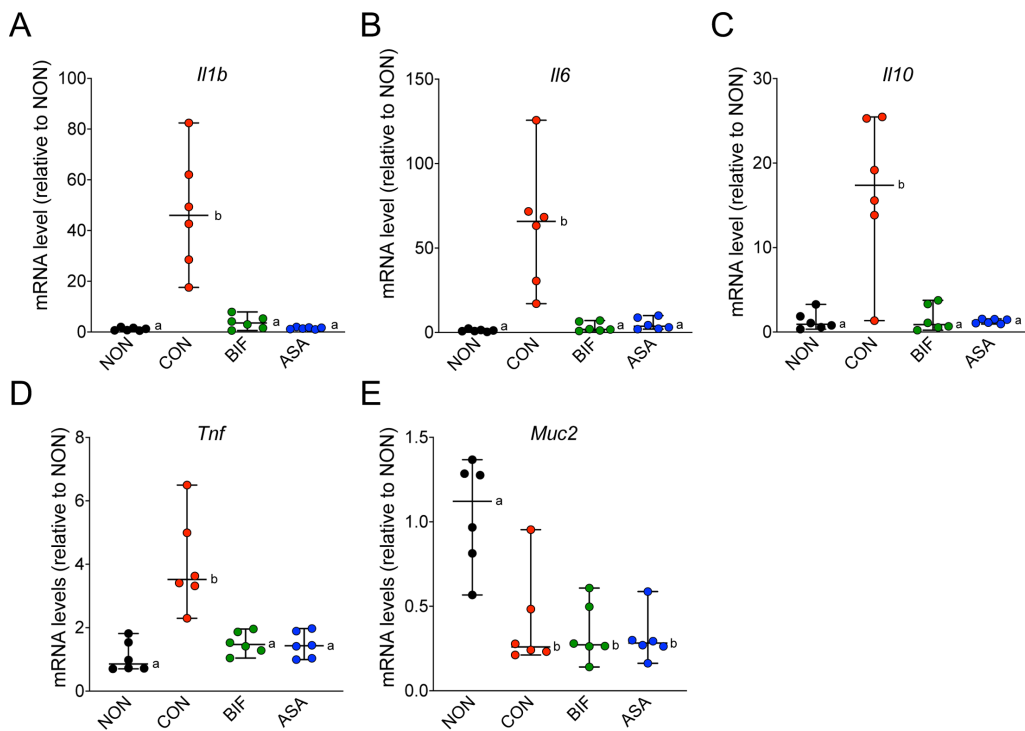
Administration of B. pseudolongum *restored the diminished population of bifidobacteria in DSS-induced colitis*

The numbers of total bifidobacteria and *B. pseudolongum* in the cecal contents were estimated by RT-qPCR of the 16S rRNA gene fragments (Fig. 4A and 4B, respectively). No bifidobacteria were detected in the CON and ASA groups. In contrast, no

Fig. 2. Effect of intragastric administration of *Bifidobacterium pseudolongum* on colon length (chart A), spleen weight (chart B), and total histological score (chart C) and representative light photomicrographs of colonic sections (chart D) in BALB/c mice treated with dextran sodium sulfate (DSS). Mice in the NON group received drinking water without DSS, and mice in the CON, BIF, and ASA groups received DSS-supplemented water and were administered PBS, PBS supplemented with live *B. pseudolongum*, or 5-aminosalicylic acid daily, respectively. Data are presented as the median ± 95% confidence interval (*n*=6 in each group). Data without a common letter differ as estimated using Dunnett's T3 multiple comparisons post hoc test following Welch's ANOVA (p<0.05). In chart D, the bar represents 100 μm. NON: PBS/DW; CON: PBS/DSS; BIF: *B. pseudolongum*/DSS; ASA: 5-ASA/DSS.

Fig. 3. Effect of intragastric administration of *Bifidobacterium pseudolongum* on the mRNA levels of the *Il1b*, *Il6*, *Il10*, *Tnf*, and *Muc2* genes (charts A, B, C, D, and E, respectively) in the colon of BALB/c mice treated with dextran sodium sulfate (DSS). Mice in the NON group received drinking water without DSS, and mice in the CON, BIF, and ASA groups received DSS-supplemented water and were administered PBS, PBS supplemented with live *B. pseudolongum*, or 5-aminosalicylic acid daily, respectively. Data are presented as the median ± 95% confidence interval (*n*=6 in each group). Data without a common letter differ as estimated using Dunnett's T3 multiple comparisons post hoc test following Welch's ANOVA (p<0.05). NON: PBS/DW; CON: PBS/DSS; BIF: *B. pseudolongum*/DSS; ASA: 5-ASA/DSS.

difference was observed in the numbers of total bifidobacteria and *B. pseudolongum* between the NON and BIF groups.

Administration of B. pseudolongum *suppressed the increased 5-HT in DSS-induced colitis*

The colonic 5-HT content was significantly higher in the CON group than in the NON group (Fig. 5A). The colonic 5-HT contents in the BIF and ASA groups were intermediate between the NON and CON groups but did not differ significantly from those two groups. The mRNA level of the *Tph1* gene that encodes TPH1, the rate-limiting enzyme for 5-HT synthesis, was significantly lower in the BIF and ASA groups and tended to be lower in the CON group, as compared with the NON group (Fig. 5B). No significant difference was observed in the *Tph1* mRNA levels among the CON, BIF, and ASA groups. The mRNA levels of the *Slc6a4* and *Maoa* genes (Fig. 5C and 5D, respectively), which encode the 5-HT reuptake transporter SERT and 5-HT-metabolizing enzyme MAO-A, were the same in comparisons among the NON, CON, BIF, and ASA groups.
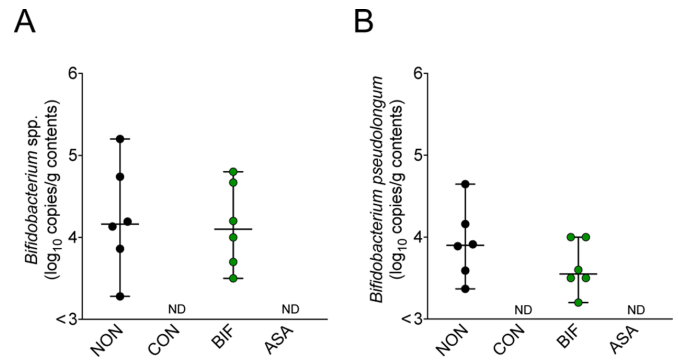
Administration of B. pseudolongum *failed to restore the reduced expression of tight junction (TJ)-associated protein genes in DSS-induced colitis*

The mRNA levels of the *Tjp1* and *Cldn3* genes (Fig. 6A and 6D, respectively) were significantly lower in the CON group than in the NON group, and the mRNA levels of the *Ocln* and *Cldn1* genes (Fig. 6B and 6C, respectively) tended to be lower in the CON group than in the NON group. The mRNA levels of these genes in the BIF and ASA groups did not differ from those in the NON group.

DISCUSSION

Because previous studies suggested that an increased colonic 5-HT content contributes to the aggravation of DSS-induced colitis [15–17], decreasing the colonic 5-HT content may prevent DSS-induced colitis. Therefore, the present study tested whether the administration of *B. pseudolongum,* which has been shown to reduce the colonic 5-HT content [21], prevented DSS-induced colitis in mice. Body weight loss, diarrhea, fecal bleeding, colon shortening, and spleen enlargement are common phenomena in DSS-induced colitis [26, 30, 31]. In the present study, intragastric administration of *B. pseudolongum* reduced these symptoms in the DSS-treated mice. In addition, DSS administration resulted in tissue damage, including epithelial loss, crypt loss, and inflammatory cell infiltration in the colon, and this damage was partly reduced by *B. pseudolongum* administration. Furthermore, we observed significant increases in the mRNA levels of cytokine genes, i.e., *Il1b*, *Il6*, *Il10*, and *Tnf*, in the colon of DSS-treated mice. These increases were almost completely prevented by *B. pseudolongum* administration. These results suggest that *B. pseudolongum* administration prevented DSS-induced colitis, as evidenced by the reduced tissue damage and inflammation in the colonic mucosa and systemic symptoms.

Like previous studies [12, 13], we observed that DSS administration significantly increased the colonic 5-HT content. In addition, we found that intragastric administration of *B. pseudolongum* suppressed the DSS-induced increase of colonic 5-HT content. These results suggest that preventing DSS-induced colitis by *B. pseudolongum* administration is associated with a

Fig. 4. Effect of intragastric administration of *Bifidobacterium pseudolongum* on total bifidobacteria and *B. pseudolongum* (charts A and B, respectively) in the cecal contents of BALB/c mice treated with dextran sodium sulfate (DSS). Mice in the NON group received drinking water without DSS, and mice in the CON, BIF, and ASA groups received DSS-supplemented water and were administered PBS, PBS supplemented with live *B. pseudolongum*, or 5-aminosalicylic acid daily, respectively. Data are presented as the median ± 95% confidence interval (*n*=6 in each group). ND: not detected. NON: PBS/DW; CON: PBS/DSS; BIF: *B. pseudolongum*/DSS; ASA: 5-ASA/DSS.
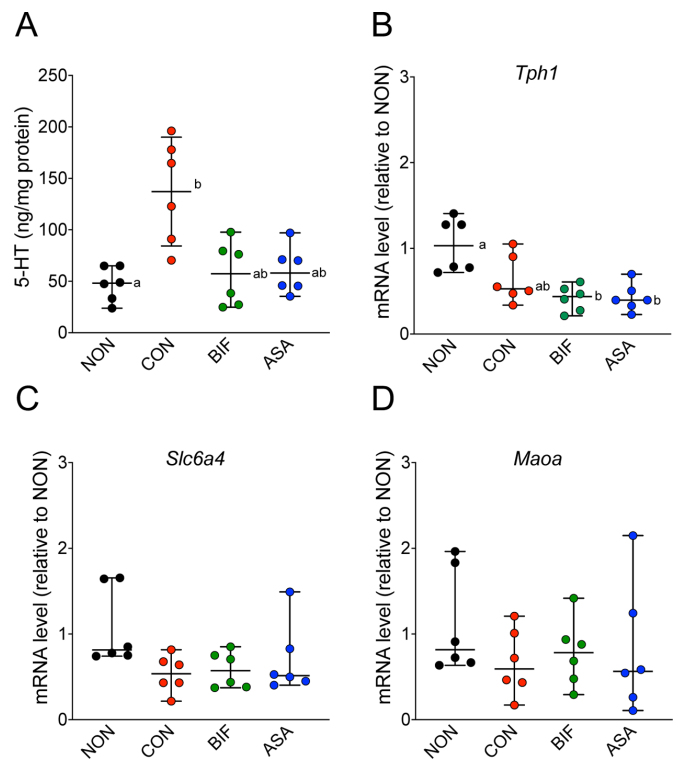
Fig. 5. Effect of intragastric administration of *Bifidobacterium pseudolongum* on serotonin (5-HT) content (chart A) and mRNA levels of the *Tph1*, *Slc6a4*, and *Maoa* genes (charts B, C, and D, respectively) in the colon of BALB/c mice treated with dextran sodium sulfate (DSS). Mice in group NON received drinking water without DSS, and mice in the CON, BIF, and ASA groups received DSS-supplemented water and were administered PBS, PBS supplemented with live *B. pseudolongum*, or 5-aminosalicylic acid daily, respectively. Data are presented as the median ± 95% confidence interval (*n*=6 in each group). Data without a common letter differ as estimated using Dunnett's T3 multiple comparisons *post hoc* test following Welch's ANOVA (p<0.05). NON: PBS/DW; CON: PBS/DSS; BIF: *B. pseudolongum*/DSS; ASA: 5-ASA/DSS.
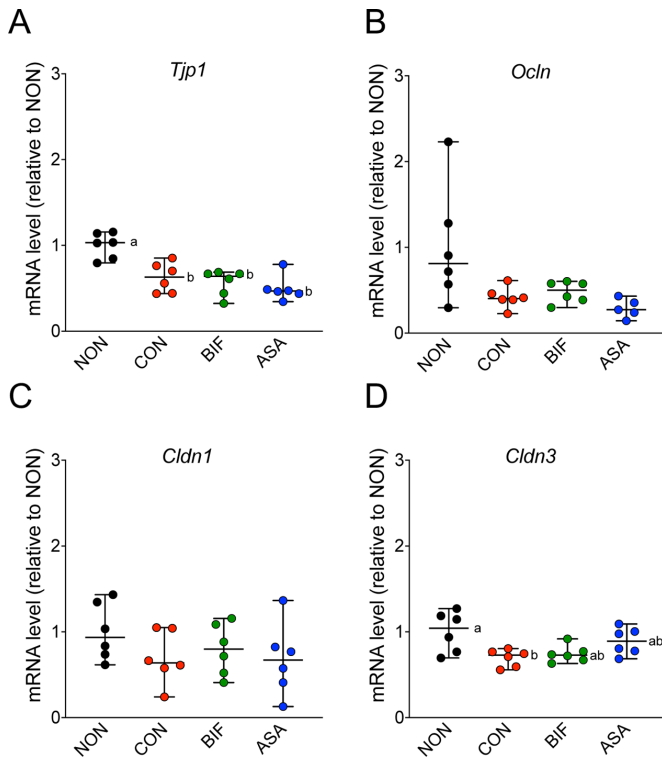
Fig. 6. Effect of intragastric administration of *Bifidobacterium pseudolongum* on the mRNA levels of the *Tjp1*, *Ocln*, *Cldn1*, and *Cldn3* genes (charts A, B, C, and D, respectively) in the colon of BALB/c mice treated with dextran sodium sulfate (DSS). Mice in the NON group received drinking water without DSS, and mice in the CON, BIF, and ASA groups received DSS-supplemented water and were administered PBS, PBS supplemented with live *B. pseudolongum*, or 5-aminosalicylic acid daily, respectively. Data are presented as the median ± 95% confidence interval (*n*=6 in each group). Data without a common letter differ as estimated using Dunnett's T3 multiple comparisons post hoc test following Welch's ANOVA (p<0.05). NON: PBS/DW; CON: PBS/DSS; BIF: *B. pseudolongum*/DSS; ASA: 5-ASA/DSS.

reduced colonic 5-HT content. Ghia *et al.* [17] reported that a deficiency of the *Tph1* gene that encodes TPH1, the rate-limiting enzyme for 5-HT synthesis, reduced the severity of DSS-induced colitis in mice. In addition, Utsumi *et al.* [32] showed that administration of 5-HT3 receptor antagonists, i.e., ramosetron and ondansetron, reduced the severity of DSS-induced colitis in mice. These studies suggested that lowering 5-HT signaling could prevent DSS-induced colitis. The fact that 5-HT activates immune cells to produce pro-inflammatory mediators [33] also supports this idea. However, the causal relationship between the reduced colonic 5-HT content and reduced severity of DSS-induced colitis caused by *B. pseudolongum* administration in the present study remains to be investigated. Further studies are required to elucidate this issue. For instance, it would be informative to test whether 5-HT administration abrogates the reducing effect of *B. pseudolongum* administration on DSS-induced colitis.

The 5-HT content in colon tissue should be regulated by the 5-HT synthesis in the EC cells and 5-HT removal by the surrounding epithelial cells. Indeed, Wang *et al.* [34] reported that a DSS-induced increase of colonic 5-HT content was coupled with the upregulation of the *Tph1* gene that encodes the 5-HT-

synthesizing enzyme and the downregulation of the *Slc6a4* gene that encodes the 5-HT reuptake transporter in mice. In the present study, however, DSS administration tended to reduce the mRNA level of the *Tph1* gene and produced no significant changes in the mRNA expression of the *Slc6a4* and *Maoa* genes. We also observed that the administration of *B. pseudolongum* in addition to DSS significantly lowered the mRNA level of the *Tph1* gene as compared with mice without DSS administration. Considering that DSS damages the gut epithelium, it would not be surprising if DSS administration reduced the mRNA expression of the *Tph1*, *Slc6a4*, and *Maoa* genes in colonic epithelial cells. Indeed, we observed that DSS administration reduced the mRNA level of the *Muc2* gene that encodes a mucin glycoprotein, MUC2, expressed in the epithelial goblet cells. Nevertheless, further studies are needed to elucidate the mechanism by which the administration of DSS and *B. pseudolongum* alters the colonic 5-HT content and whether reduced expression of the *Tph1* gene contributes to the reduction of colonic 5-HT content caused by *B. pseudolongum* administration in DSS-treated mice.

The present study employed 5-ASA, used as the standard therapy for UC [35], as a positive control for preventing DSS-induced colitis. As expected, the intragastric administration of 5-ASA reduced body weight loss, diarrhea, fecal bleeding, colon shortening, and spleen enlargement in DSS-treated mice. In addition, DSS-induced colonic tissue damage assessed by the total histological score was significantly reduced by 5-ASA administration. Furthermore, 5-ASA administration almost completely prevented the upregulation of cytokine genes in the colon of DSS-treated mice. Because 5-ASA administration under the same conditions that lead to protection from DSS-induced colitis suppressed the increase of colonic 5-HT content almost to an extent similar to *B. pseudolongum* administration, it might be possible that part of the anti-inflammatory actions of 5-ASA is due to the reduction of 5-HT signaling. However, the present study presented no evidence to support this hypothesis, and it will have to be investigated in future studies.

DSS-induced colitis reportedly showed disruption of the TJ barrier in the colonic epithelium [36]. In the present study, gene expression of ZO-1 and claudin-3, key components of the TJ, was reduced by DSS treatment, which was consistent with previous studies [37–39]. In addition, in line with previous studies [38, 39], the present study showed reduced expression of *Muc2*, which encodes mucin 2, a key component of the mucus barrier in the colon. However, both *B. pseudolongum* administration and 5-ASA treatment failed to restore the expression of these genes. These results suggest that the reduction of DSS-induced colitis caused by *B. pseudolongum* and 5-ASA is not achieved by restoration of TJ- and mucus-barrier functions. Considering that inflammatory cytokines could reduce the expression of TJ-associated proteins [40], DSS-increased expression of the *Il1b*, *Il6*, and *Tnf* genes might be involved in the reduced expression of genes encoding TJ-associated proteins in the present study. In addition, 5-HT has been suggested to induce TJ-barrier dysfunction by reducing the expression of occludin and claudin-1 in the small and large intestines of mice with stress-induced diarrhea [41]. An *in vitro* study using Caco-2 cells, a human intestinal epithelial cell line, also showed that 5-HT supplementation reduced the expression of occludin [42]. Thus, an additional possibility is that the DSS-reduced expression of genes encoding TJ-associated proteins might be mediated by an increased colonic 5-HT content.

Conversely, whether TJ- and mucus-barrier dysfunctions increase the colonic 5-HT content remains to be elucidated.

Previous studies showed that many bacterial strains, primarily *Bifidobacterium* spp. and *Lactobacillus* spp., reduced the severity of DSS-induced colitis in mice [43]. Different mechanisms underlying this beneficial action of bacterial strains have also been proposed, including the promotion of epithelial cell proliferation, the improvement of gut barrier function, the induction of regulatory T cells, and the inhibition of toll-like receptor 4-linked NF-κB activation [43]. Whether these mechanisms are involved in *B. pseudolongum* reducing the severity of DSS-induced colitis warrants further investigations.

In conclusion, in agreement with the prediction that reducing the colonic 5-HT content would be useful against DSS-induced colitis, the present findings suggest that *B. pseudolongum,* which has been shown to reduce colonic 5-HT content [21], is as beneficial against murine DSS-induced colitis as the widely used anti-inflammatory agent 5-ASA. However, further studies are needed to clarify the causal relationship between the reduced colonic 5-HT content and reduced severity of DSS-induced colitis caused by *B. pseudolongum* administration.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ACKNOWLEDGMENTS

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