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Glycosaminoglycan Degradation-Inhibitory Lactic Acid Bacteria Ameliorate 2,4,6-Trinitrobenzenesulfonic Acid-Induced Colitis in Mice

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To evaluate the effects of lactic acid bacteria (LAB) in inflammatory bowel diseases (IBD), we measured the inhibitory effect of several LAB isolated from intestinal microflora and commercial probiotics against the glycosaminoglycan (GAG) degradation by intestinal bacteria. *Bifidobacterium longum* HY8004 and *Lactobacillus plantarum* AK8-4 exhibited the most potent inhibition. These LAB inhibited colon shortening and myeloperoxidase production in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced experimental colitic mice. These LAB also blocked the expression of the proinflammatory cytokines, IL-1β and TNF-α, as well as of COX-2, in the colon. LAB also blocked activation of the transcription factor, NF-κB, and expression of TLR-4 induced by TNBS. In addition, LAB reduced the TNBS-induced bacterial degradation activities of chondroitin sulfate and hyaluronic acid. These findings suggest that GAG degradation-inhibitory LAB may improve colitis by inhibiting inflammatory cytokine expression *via* TLR-4-linked NF-κB activation and by inhibiting intestinal bacterial GAG degradation.

Keywords: Lactic acid bacteria, inflammatory bowel disease, glycosaminoglycan, TLR-4, intestinal bacteria

The pathogenic mechanism of inflammatory bowel diseases (IBD), ulcerative colitis, and Crohn's disease involve dysregulation of the intestinal immune response to intestinal antigens, such as intestinal microflora [4, 5]. IBD occurs most frequently in the terminal ileum and colon, where many intestinal microbes reside [6, 11]. IBD does not develop significantly or progress in germ-free animals [6, 11], suggesting that intestinal microflora play an important role in initiating and perpetuating colonic inflammation.

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Normal intestinal microflora consist of an estimated 400 bacterial species and reach their highest concentrations in the terminal ileum and colon [23, 37]. Intestinal microflora produce toxic sources, such as gram-negative bacterial endotoxins, and harmful enzymes, such as β-glucuronidase and tryptophanase, which produce cytotoxic or carcinogenic agents [12, 17, 37]. Cytotoxin and endotoxins are potent stimuli of innate immune responses, induce proinflammatory cytokines in colonic epithelial cells, and cause IBD [5]. Belmiro *et al.* [3] reported increased biosynthesis of extracellular matrix glycosaminoglycans (GAGs) in the intestinal mucosa of IBD patients. These glycosaminoglycans induce the GAG-degrading enzymes, chondroitinase and heparinase, in intestinal bacteria [18, 35].

Lactic acid bacteria (LAB) are gram-positive, non-spore-forming, non-respiring cocci or rods that ferment carbohydrates and produce lactic acid as the main product [39]. The common LAB genera in fermented foods, such as cheese, yogurt, and *kimchi*, are *Enterococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp., and *Streptococcus* sp. Although the genus *Bifidobacterium* is unrelated to these LAB phylogenetically, *Bifidobacteria* are considered LAB because they maintain health by residing in the gastrointestinal tract of humans and animals [19, 39]. LAB are safe microorganisms [15] that improve disturbances of indigenous microflora [9, 31], ameliorate the development of beneficial microflora [14], have anticolitic effects [9, 22, 27, 30], and induce nonspecific activation of the host immune system [10]. LAB inhibit proinflammatory cytokine expression, productivity of intestinal bacterial GAG-degrading enzymes, and β-glucuronidase in dextran sulfate sodium-induced colitic animals [27].

Therefore, in the present study, we tested the ability of LAB to inhibit GAG degradation and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), tetramethyl benzidine, Griess reagent, TNBS, hexadecyl trimethyl ammonium bromide, *p*-nitrophenyl-β-D-glucuronide, tryptophan, chondroitin sulfate, hyaluronic acid, tetramethyl benzidine, and radio-immunoprecipitation assay (RIPA) lysis buffer were purchased from Sigma Co. (St. Louis, MO, U.S.A.). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). ELISA kits were from Pierce Biotechnology, Inc. (Rockford, IL, U.S.A.). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, U.S.A.).

Bacterial Strains and Growth Conditions

Fourteen LAB were used in this study: *Lactobacillus suntoryeus* 7J3-8, *Lactobacillus plantarum* AK8-4, *Lactobacillus paracasei* A3-4, *L. paracasei* J2-1, *Lactobacillus* sp. EJ-I3, *Lactobacillus* sp. MD19-1, *Lactobacillus* sp. K4-7, *Lactobacillus* sp. K5-8, *Bifidobacterium longum* KJ-6, *B. longum* HY8004, *B. longum* B10J4-1, *Bifidobacterium* sp. KJ-8, *Bifidobacterium* sp. APB11-1, and *Bifidobacterium* sp. APB6-2 isolated by Korea Yakult Co. (Seoul, Korea).

These bacteria were grown to an optical density of between 0.3 and 0.4 at 600 nm (early stationary phase) in tryptic soy broth for 24 h (37°C), harvested by centrifugation (10,000 ×*g* for 30 min) and washed with PBS, and then orally administered to mice as a resuspension in 0.2 M $NaHCO_3$ buffer containing 1% glucose and 2×10^{10} CFU per kilogram of body weight [21, 27].

Inhibitory Activity Assay of LAB Against GAG Degradation of *Bacteroides stercoris*

Probiotic bacteria were cultured as described above. *Bacteroides stercoris* HJ-15 (KCTC 12437), which potently degrades GAG, was anaerobically cultured in tryptic soy broth. The previously cultured *B. stercoris* HJ-15 (1×10^8 CFU) and each LAB (1×10^8 CFU) were simultaneously inoculated into 100 ml of tryptic soy broth and grown anaerobically at 37°C for 72 h. The cultured bacteria were collected by the centrifugation (10,000 ×*g*, 30 min), washed with PBS twice, and then disrupted by an Ultrasonicator (Eyela Co., Tokyo, Japan) and centrifuged at 10,000 ×*g* for 30 min. The GAG degradation activity of the supernatant was measured.

Animals

Male ICR mice (24–28 g) were purchased from Jung-Ang Lab Animal, Inc. (Seoul, Korea). All animals were housed in wire cages at 20–22°C and 50%±10% humidity, fed standard laboratory chow (Samyang, Seoul, Korea), and allowed water *ad libitum*. All procedures relating to the animals and their care conformed to the national and international guidelines outlined in *Principles of Laboratory Animals Care* (NIH Publication No. 85-23 revised 1985, and Kyung Hee University 2006).

Preparation of Experimental Colitic Mice

Male ICR mice were randomly divided into 7 groups of normal and TNBS-induced colitic groups treated with and without LAB or sulfasalazine. TNBS-induced colitis was induced by administration of 2.5% (w/v) TNBS solution (100 μl) in 50% ethanol into the colon of lightly anesthetized mice *via* a thin round-tip needle equipped with a 1-ml syringe [2, 30]. The tip of the needle was inserted such that the tip was 3.5–4 cm proximal to the anal verge. To distribute the agents within the entire colon and cecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >95% of the mice retained the TNBS enema. Any animal that quickly excreted the TNBS-ethanol solution was omitted from the remainder of the study. The LAB (50 mg/kg or 100 mg/kg of *Lactobacillus* AK8-4 or 50 mg/kg or 100 mg/kg of *Bifidobacterium* HY8004) were orally administered once a day, beginning 3 days before TNBS treatment until the day before sacrifice. The mice were anesthetized with ether and sacrificed on the 3rd day after TNBS administration. The colon was quickly removed, opened longitudinally, gently cleared of stool, and used for further study.

Assay of Myeloperoxidase (MPO) Activity in Colonic Mucosa

Colons were homogenized in a solution containing 0.5% hexadecyl trimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0) and then centrifuged for 30 min at 20,000 ×*g* and 4°C. An aliquot (50 μl) of the supernatant was added to a reaction mixture of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2 and incubated at 37°C. The absorbance was then obtained at 650 nm over time. MPO activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37°C and expressed in unit/mg protein [28]. The protein content was assayed by the Bradford method [8].

Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoblot

For the ELISA of IL-1β and IL-6, colons were homogenized in 1 ml of ice-cold lysis buffer (radio-immunoprecipitation assay, RIPA) containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (15,000 ×*g*, 4°C) for 15 min, and the supernatant was transferred to 96-well ELISA plates. IL-1β and IL-6 concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

For the immunoblot of pp65 (phospho-NF-κB), p65 (NF-κB), COX-2, and β-actin, the colon tissue was carefully homogenized to obtain viable single cells, which were resuspended in 1 ml of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, the supernatant was used for the immunoblot assay. The protein from collected cells was subjected to electrophoresis on an 8–10% sodium dodecyl sulfate-polyacrylamide gel, and then transferred to nitrocellulose membrane. Levels of pp65, p65, COX-2, and β-actin were assayed as previously described [27, 36]. Immunodetection was performed using an enhanced chemiluminescence detection kit.

Preparation of Fecal Bacterial Suspension

Fresh mouse stools (0.5 g) from each group were separately collected in sterilized plastic cups, carefully suspended in 20 volumes of saline in a cooled tube, and centrifuged at 250 ×*g* for 5 min. The supernatant was recentrifuged at 10,000 ×*g* for 20 min. The resulting pellets were used as the sources for the fecal enzyme assays. All procedures were performed at 4°C.

Assay of Tryptophanase Activity

The reaction mixture (0.5 ml), which contained 0.2 ml of complete buffer mixture (2.75 mg of pyrophosphate, 19.6 mg of disodium EDTA dehydrate, and 10 mg of BSA in 100 ml of 0.05 M potassium

phosphate buffer, pH 7.5), 0.2 ml of 20 mM tryptophan, and 0.1 ml of the fecal suspension, was incubated at 374°C for 1 h. The reaction was terminated by the addition of 2 ml of color reagent (14.7 g of *p*-dimethylaminobenzaldehyde, 52 ml of sulfuric acid, and 948 ml of 95% ethanol) and centrifuged at 3,000 ×*g* for 10 min. The enzyme activity was measured by monitoring the absorbance at 550 nm.

Assay of β-Glucuronidase Activity

The reaction mixture (2.0 ml), which consisted of 0.04 ml of 2 mM *p*-nitrophenyl-β-D-glucuronide, 0.76 ml of 0.1 M phosphate buffer (pH 7.0), and 0.2 ml of fecal suspension, was incubated for 30 min at 37°C, and the reaction was terminated by the addition of 1 ml of 0.5 M NaOH. The mixture was then centrifuged at 3,000 ×*g* for 10 min and the absorbance was measured at 405 nm.

Assay of Chondroitin Sulfate and Hyaluronic Acid Degradation Activities

Reaction mixtures containing 0.2 ml of chondroitin sulfate A (or hyaluronic acid) (1.0 mg/ml) and 0.6 ml of the fecal suspension were incubated at 37°C for 1 h and then centrifuged at 3,000 ×*g* at 4°C. The supernatant (500 μl), 0.1 ml of 0.4 M NaOH, and 0.1 ml of 0.4 M potassium borate were boiled for 5 min, and then cooled to room temperature; 3 ml of 67 mM *p*-dimethylaminobenzaldehyde was then added. The mixture was incubated at 37°C for 20 min and the absorbance measured at 585 nm.

Statistical Analysis

All data are expressed as the mean±standard deviation. Statistical significance was analyzed using one-way ANOVA followed by a Student-Newman-Keuls test.

Results

Inhibitory Effect of LAB Against GAG Degradation by *Bacteroides stercoris*

We first tested the ability of LAB to inhibit GAG degradation by *Bacteroides stercoris*, a bacterium that produces GAG degradation enzymes in human intestine, in co-cultivation experiments (Table 1). Most LAB inhibited GAG degradation. *Bifidobacterium* HY8004 showed the highest inhibitory effect among Bifidobacteria and *Lactobacillus* AK8-4 exhibited the greatest potency among Lactobacilli.

Antiinflammatory Effect of LAB in Experimental Colitic Mice

We then tested the inhibitory effects of *Bifidobacterium* HY8004 and *Lactobacillus* AK8-4 on TNBS-induced colitis in mice (Fig. 1). TNBS treatment caused body weight loss and severe inflammation, manifested by shortened and erythematous colons on the 3rd day following intrarectal administration of TNBS. LAB treatment inhibited the colon shortening and severe inflammation induced by TNBS. *Bifidobacterium* HY8004 and *Lactobacillus* AK8-4 at 100 mg/kg inhibited the colon shortening by 54% and 43%, respectively. TNBS treatment also induced MPO activity, an inflammatory marker, in colon epithelial cells. These LAB inhibited MPO activity by 82% and 67%, respectively. Their inhibitory effects are comparable to that of sulfasalazine, a commercial agent used for IBD.

TNBS treatment induced the expression of the proinflammatory cytokines, IL-1β and TNF-α, but did not affect β-actin levels (Fig. 2). *Bifidobacterium* HY8004 and *Lactobacillus* AK8-4 at 100 mg/kg inhibited IL-1β and TNF-α expression by 78% and 74% and by 84% and 68%, respectively. TNBS also increased the activation of NF-κB, which regulates the expression of IL-1β, TNF-α [15], and TLR-4, a potential mediator of LPS signaling [24]. Oral administration of LAB inhibited the activation of NF-κB and TLR-4 expressions.

LAB Inhibit Harmful Enzyme Production by Intestinal Microflora in Experimental Colitic Mice

TNBS treatment increased GAG (chondroitin sulfate and hyaluronic acid) degradation, as well as tryptophanase and β-glucuronidase activities (Fig. 3). LAB treatment significantly reduced the degradation activities of chondroitin sulfate and hyaluronic acid, as well as the activities of tryptophanase and β-glucuronidase ($p<0.05$). The inhibitory effects of these LAB are comparable to those of sulfasalazine.

Discussion

IBD is a severe form of intestinal inflammation. Although our understanding of the pathogenesis is incomplete, IBD

Table 1. Inhibitory effect of lactic acid bacteria against chondroitin sulfate degradation of *Bacteroides stercoris* HJ-15.

Strains	Inhibition[a] (%)	Strains	Inhibition (%)
B. longum KJ-6	93±3	*L. paracasei* J2-1	93±2
B. longum B10J4-1	89±4	*L. plantarum* AK8-4	94±5
B. longum HY8004	99±2	*L. paracasei* A3-4	93±3
Bifidobacterium sp. KJ-8	95±3	*Lactobacillus* sp. EJ-I3	83±1
Bifidobacterium sp. APB11-1	94±2	*Lactobacillus* sp. MD19-1	93±5
Bifidobacterium sp. APB6-2	92±4	*Lactobacillus* sp. K4-7	36±5
L. sontoryeus 7J3-8	93±3	*Lactobacillus* sp. K5-8	84±3

[a]Inhibition (%) was calculated as 100×[(the absorbance at 530 nm of control group treated with *Bacteroides* HJ-15 alone − that of the blank treated with vehicle alone) − (that of the group treated with *Bacteroides* HJ-15 and test LAB − that of the blank treated with vehicle alone)] / (the absorbance at 530 nm of control group treated with *Bacteroides* HJ-15 alone − that of the blank treated wih vehicle alone).

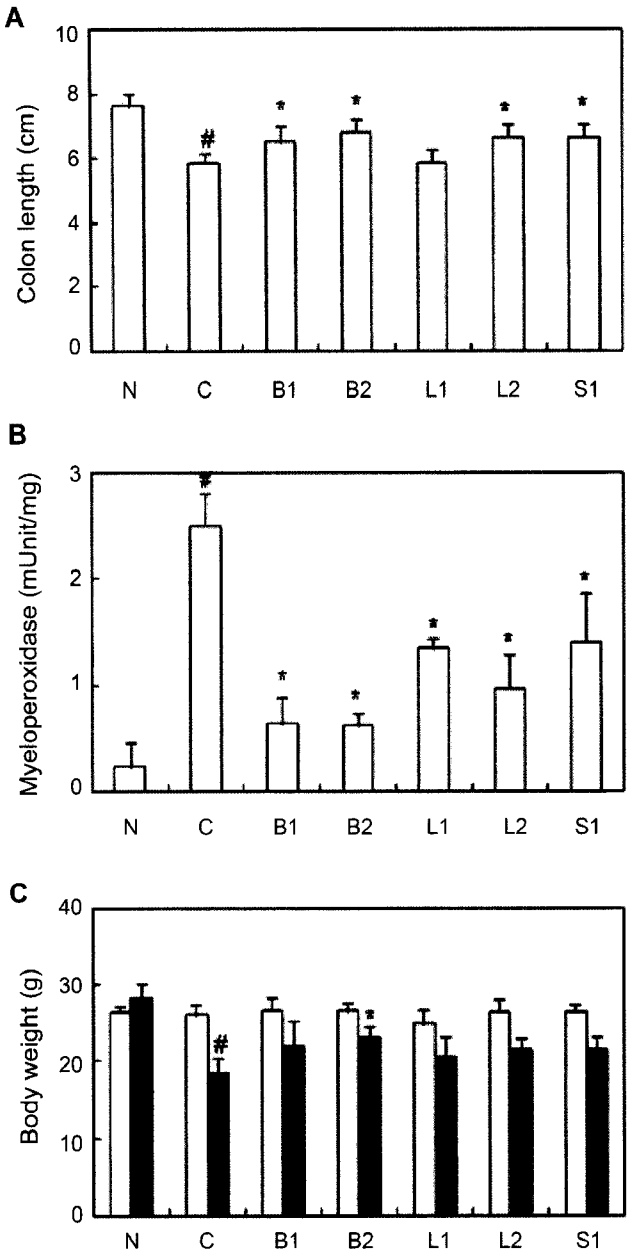

Fig. 1. Effects of LAB on colon length (**A**), colonic myeloperoxidase activity (**B**), and body weight (**C**) in TNBS-induced colitic mice. TNBS, except in the normal group (N, normal group treated vehicle alone), was intrarectally administered in the control (C), LAB, and sulfasalazine groups. LAB (B1, 50 mg/kg *Bifidobacterium longum* HY8004, and TNBS; B2, 100 mg/kg *Bifidobacterium* HY8004, and TNBS; L1, 50 mg/kg of *Lactobacillus plantarum* AK8-4, and TNBS; L2, 100 mg/kg of *Lactobacillus* AK8-4, and TNBS; S1, 50 mg/kg sulfasalazine, and TNBS), except in the normal and control groups, were orally administered beginning 3 days prior to TNBS treatment. White and black bars indicate body weights before and after TNBS and/or LAB, respectively. Enzyme activity values are the mean±SD (n=10). #Significantly different vs. normal group (p<0.05). *Significantly different vs. control group (p<0.05).

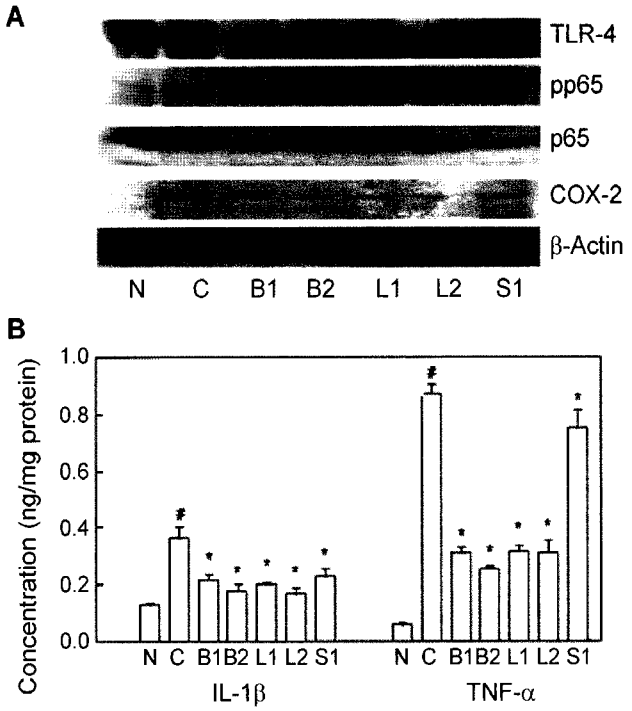

Fig. 2. Effect of LAB on the protein expression of TLR-4 and COX-2 and the activation of the transcription factor NF-κB (**A**) and inflammatory cytokines (**B**) in TNBS-induced colitis in mice. The test agents (N, normal group treated with vehicle alone; C, TNBS-treated control group; B1, 50 mg/kg *Bifidobacterium longum* HY8004, and TNBS; B2, 100 mg/kg *Bifidobacterium* HY8004, and TNBS; L1, 50 mg/kg *Lactobacillus plantarum* AK8-4, and TNBS; L2, 100 mg/kg *Lactobacillus* AK8-4, and TNBS) were orally administered beginning 3 days prior to TNBS treatment. The mice were anesthetized and killed following treatment. Colon epithelial cells were collected; TLR-4 and COX-2 expression and NF-κB activation were measured by immunoblot analysis, and IL-1β and TNF-α were measured by ELISA. All values are the mean±S.D. (n=5). #Significantly different vs. normal group (p<0.05). *Significantly different vs. control group (p<0.05).

involves complex mucosal immune responses to antigens of resident enteric bacteria. The numbers and species of enteric bacteria between normal and colitic mice are not significantly different [25, 29]. However, mucosal damage, such as that observed in IBD, may be caused by toxic compounds, such as endotoxin produced by resident enteric bacteria [11, 24, 33]. The extracellular matrix GAGs of the intestinal mucosa may also be disturbed in IBD [3]. GAG degradation increases in the intestinal contents of dextran sulfate sodium (DSS)-induced colitic mice [27]. In the present study, we tested the ability of LAB to inhibit colitis in TNBS-treated mice. TNBS induced colon shortening, and epithelial MPO activity in TNBS-treated mice, in addition to inducing the GAG-degrading enzymes, β-glucuronidase and tryptophanase, of intestinal bacteria, which may promote development of colitis.

LAB inhibited colon shortening and intestinal epithelial myeloperoxidase activity in TNBS-stimulated mice. Of the LAB tested, *Bifidobacterium* HY8004 and *Lactobacillus* AK8-4 most potently inhibited GAG degradation and also inhibited the induction of IL-1β, TNF-α, and COX-2 [1, 2, 24, 38]. LAB inhibited the activation of the transcription factor, NF-κB, which is induced by TNBS and in colitic patients [10], and reduced the expression of TLR-4, a

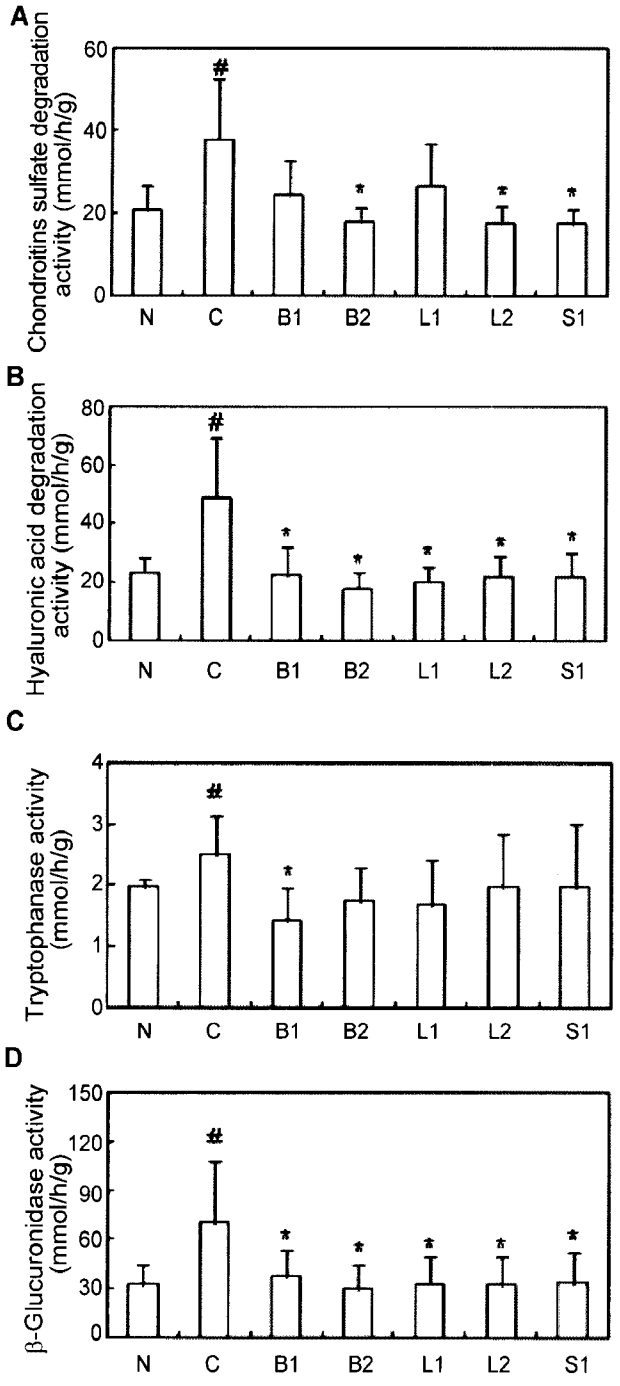

Fig. 3. The effects of LAB in fecal chondroitin degradation (**A**), hyaluronidase degradation (**B**), and tryptophanase (**C**), and β-glucuronidase (**D**) activities in TNBS-induced colitis mice.
Test agents (N, normal group treated with vehicle alone; C, TNBS-treated control group; B1, 50 mg/kg *Bifidobacterium longum* HY8004, and TNBS; B2, 100 mg/kg *Bifidobacterium* HY8004, and TNBS; L1, 50 mg/kg *Lactobacillus plantarum* AK8-4, and TNBS; L2, 100 mg/kg *Lactobacillus* AK8-4, and TNBS) were orally administered beginining 3 days prior to TNBS treatment. #Significantly different vs. normal group ($p<0.05$). *Significantly different vs. control group ($p<0.05$). Enzyme activities are indicated as the mean±SD (n=5).

mediator of LPS signaling [16]. LAB may also improve colitis by inhibiting the biosynthesis of the proinflammatory cytokines, IL-1β and TNF-α.

The probiotics, bifidobacteria and lactobacilli, suppress the growth of pathogens by releasing antimicrobial factors and compete with microbial pathogens for the limited number of receptors on epithelial cells [26, 34]. Therefore, probiotics may be an alternative and safe treatment for IBD. VSL#3, a mixture of LAB (*B. longum*, *B. infantis*, *B. breve*, *L. acidophilus*, *L. casei*, *L. delbrueckii* sp. *bulgaricus*, *L. plantarum*, and *Streptococcus salivarius* sp. *thermophilus*), increased the expression of the antiinflammatory cytokine IL-10 [7]. *Lactobacillus casei* also inhibited the expression of inflammatory cytokines in DSS-induced colitic mice [13, 31]. *E. coli* Nissle 1917 improved DSS-induced mouse colitis *via* TLR-2- and TLR-4-dependent pathways, although it activated TLR-4-linked NF-κB activation [9, 20]. The LAB screened here also inhibited the GAG degradation induced by TNBS, as previously reported [27], as well as TLR-4-linked NF-κB activation.

These findings suggest that LAB may inhibit intestinal bacterial GAG degradation in the intestine and may improve colitis by inhibiting inflammatory cytokine expression *via* TLR-4-linked NF-κB activation and by preventing GAG degradation by intestinal microflora.

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