Lactobacillus fermentum species ameliorate dextran sulfate sodium-induced colitis by regulating the immune response and altering gut microbiota

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ABSTRACT

We evaluated immunometabolic functions of novel Lactobacillus fermentum strains (KBL374 and KBL375) isolated from feces of healthy Koreans. The levels of inflammatory cytokines, such as interleukin (IL)-2, interferon-γ, IL-4, IL-13, and IL-17A, were decreased, and that of the anti-inflammatory cytokine IL-10 was increased, in human peripheral blood mononuclear cells (PBMCs) treated with the L. fermentum KBL374 or KBL375 strain. When these strains were orally administered to mice with dextran sulfate sodium (DSS)-induced colitis, both L. fermentum KBL374 and KBL375 showed beneficial effects on body weight, disease activity index score, colon length, cecal weight, and histological scores. Furthermore, both L. fermentum KBL374 and KBL375 modulated the innate immune response by improving gut barrier function and reducing leukocyte infiltration. Consistent with the PBMC data, both L. fermentum KBL374- and KBL375-treated DSS mice demonstrated decreased Th1-, Th2-, and Th17-related cytokine levels and increased IL-10 in the colon compared with the DSS control mice. Administration of L. fermentum KBL374 or KBL375 to mice increased the CD4+CD25+Foxp3+Treg cell population in mesenteric lymph nodes. Additionally, L. fermentum KBL374 or KBL375 administration reshaped and increased the diversity of the gut microbiota. In particular, L. fermentum KBL375 increased the abundance of beneficial microorganisms, such as Lactobacillus spp. and Akkermansia spp. Both L. fermentum KBL374 and KBL375 may alleviate inflammatory diseases, such as inflammatory bowel disease, in the gut by regulating immune responses and altering the composition of gut microbiota.

Introduction

Inflammatory bowel diseases (IBDs), such as Crohn’s disease (CD) and ulcerative colitis (UC), are characterized clinically by chronic inflammation in the intestine. IBDs affect people worldwide, with increasing incidence rate particularly where Westernization has progressed significantly. The etiology of IBD is unclear; however, accumulating data suggest that a genetic predisposition or susceptibility factors, defective mucosal barrier function, immune stimulation, and altered microbial composition and function in the enteric environment contribute to the initiation and the recurrence of these diseases. Dextran sulfate sodium (DSS)-induced colitis is one of the most commonly used in vivo animal models, in which animals present increased permeability of the mucus layer, recruitment of leukocytes, such as neutrophils, monocytes, and macrophages, and secretion of cytokines leading to epithelial damage. Cytokines play a key role as modulators of T cell differentiation into T helper 1 (Th1), Th2, Th17 and regulatory T (Treg) cells, and imbalances among cytokine levels lead to IBD. Activation of a number of genes associated with inflammatory cytokine expression is important in immune responses, such as leukocyte infiltration, when DSS-induced colitis occurs. Th1, Th2, and Th17 cells are involved in the pathogenesis of DSS-induced colitis, and Treg cells are reportedly crucial in immune regulation via IL-10 production. The microbiota is a dense and complex ecosystem that is becoming increasingly recognized as an essential component of host immune regulation. Intestinal bacteria actively interact with the host...
immune system, and dysregulation of these interactions can lead to inflammation-related disorders. Microbial dysbiosis, a gut microbiota imbalance, leads to alterations in the intestinal epithelial mucosa and promotes inflammation by modulating cytokine activity. UC has a significant association with reduced gut microbiota diversity and loss of key commensal species necessary to maintain immune homeostasis.

Live biotherapeutic products (LBPs) contain whole live microorganisms with intended therapeutic or preventive effects in humans. Lactobacillus spp. have been developed as LBPs. Many studies have demonstrated that LBPs, such as probiotics, contribute to modulating intestinal inflammation, which in turn may alleviate disease. For example, previous studies have demonstrated that LBPs enhance intestinal barrier function of the epithelium by reducing paracellular permeability, preserving the expression of tight junction proteins, and ameliorating clinical symptoms of DSS-induced colitis. LBPs promote the recovery of intestinal microbiota homeostasis by modulating the properties of the mucus layer and immune system.

The effects of the LBP Lactobacillus fermentum on the host immune response in relation to Th1-, Th2- and Th17-type cytokines and the anti-inflammatory cytokine IL-10, as well as the detailed changes in the gut microbiome and gut microbial metabolites, have not been well studied. We evaluated the biological effects of L. fermentum strains KBL374 and KBL375 isolated from healthy Korean feces because of the vast differences in the biological functions of each strain even within the same species. Therefore, we investigated how the probiotic candidates L. fermentum strains KBL374 and KBL375 improve the gut ecosystem in a murine IBD model, in terms of modulation of the inflammatory cytokines associated with innate and adaptive immune responses and alterations in microbiota diversity and composition that alleviate DSS-induced colitis symptoms.

Material & methods

1. Preparation of L. fermentum

We initially screened over 700 isolates from healthy Korean adult and infant feces and identified L. fermentum KBL374 and KBL375 in fecal samples from healthy adults. We identified the strains to the species level by 16S ribosomal RNA gene sequencing (Macrogen, Inc., Seoul, Republic of Korea) and the EzBioCloud Bacteria Identification Service (ChunLab, Inc., Seoul, Republic of Korea; http://www.Ezbiocloud.net). Both strains showed outstanding resistance to bile salts and low pH.

The L. fermentum KBL374 and KBL375 strains and an Escherichia coli strain, as a positive control, from fecal samples of healthy adults were cultured in Lactobacilli MRS Agar (BD Difco, Sparks, MD, USA) supplemented with 0.05% L-cysteine-hydrochloride at 37°C under anaerobic conditions using the Anaeropack (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) for 24 h. The bacterial concentration was determined by measuring the optical density at 600 nm. The cells were harvested by centrifugation (1,200 × g), washed twice with 1× sterile phosphate-buffered saline (PBS), re-suspended in PBS containing 20% glycerol (final concentration: 5 × 10^8 colony forming units [CFU]/mL), and stored at −80°C.

2. Immunomodulatory effects of Lactobacillus strains on peripheral blood mononuclear cells (PBMCs)

PBMCs from healthy donors (ZenBio, Inc., Research Triangle Park, NC, USA) were cultured in RPMI 1640 medium (Gibco, Paisley, UK) containing 1% penicillin/streptomycin (Gibco), 1% gentamycin (Gibco), and 10% fetal bovine serum (Gibco). The viable cultured PBMCs were evaluated by trypan blue staining and counted using the CKX31 inverted microscope (Olympus Corp., Tokyo, Japan).

For in vitro PBMC assay, Lactobacillus strains including L. fermentum KBL374 and KBL375 were cultured from bacteria stocks and were harvested by centrifugation (1,200 × g) and washed twice with PBS. A total of 2 × 10^5 PBMCs were incubated either with 1 µg/mL anti-CD3 monoclonal antibody (OKT3; Thermo Fisher Scientific, Inc., Waltham, MA, USA) alone or in combination with the E. coli and each tested strain in 96-well plates (SPL Life Sciences Co., Ltd., Pocheon-si, Republic of Korea) which were counted using a bacteria counting kit (Molecular Probes, Eugene, OR, USA) and flow cytometry (BD Accuri C6 Plus flow cytometer; BD Biosciences,
San Jose, CA, USA) for 50:1 bacteria:PBMC ratio at 37°C as described previously. After incubation for 72 h, the supernatant was collected to measure the concentrations of the cytokines interleukin (IL)-2, interferon (IFN)-γ, IL-4, IL-17A, and IL-10 using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. The concentration of IL-13 was measured using the IL-13 Human ELISA Kit (BMS231-3; Thermo Fisher Scientific) according to the manufacturer’s instructions. Lactate dehydrogenase cytotoxicity assay in PBMCs with L. fermentum was performed and did not show any significant toxic effects in PBMCs (data not shown).

3. Experimental animal model of colitis

Seven- to eight-week-old female C57BL/6N mice (Central Lab Animals Inc., Seoul, Republic of Korea) were separated into groups of eight mice per experimental condition. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-170831–1 and SNU-160602–9-8). Colitis was induced by adding 2% (w/v) DSS (molecular mass 36,000–50,000 Da; MP Biomedical, LLC., Santa Ana, CA, USA) to the drinking water for 7 days. L. fermentum KBL374 or KBL375 (1 × 10^9 CFU each) were also administered simultaneously with DSS treatment to 8 days. Briefly, we cultured bacteria in Lactobacilli MRS broth for 24 h supplemented with 0.05% L-cysteine-hydrochloride at 37°C under anaerobic conditions using an Anaeropack (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) from stocks. The cells were harvested by centrifugation (1,200 × g) and washed twice with PBS before administration to mice. On day 9, the mice were sacrificed, and the colon, stool, cecum, and mesenteric lymph nodes were isolated for further analyses. The change in body weight was measured, and the percentage weight change relative to the initial weight prior to DSS treatment on day 0 was calculated. The changes of disease activity index (DAI) were measured using the following criteria: (1) weight loss (%), (2) stool consistency and (3) blood in feces as previously described (Table S1). The distal colon samples were fixed in 10% formaldehyde and stained with hematoxylin and eosin. To identify the occurrence of colitis, the stained tissues were analyzed using a panoramic viewer (3DHISTECH, Ltd., Budapest, Hungary). Histological scores were categorized into four groups: (1) loss of epithelium, (2) crypt damage, (3) depletion of goblet cells and (4) inflammatory cell infiltration (Table S2).

4. Western blot

The colon samples were weighed and homogenized in 1× RIPA buffer (Thermo Fisher Scientific) with the Halt protease inhibitor cocktail (Thermo Fisher Scientific) using the MM 400 Mixer Mill homogenizer (Retsch, GmbH, Haan, Germany). The homogenate was centrifuged at 4°C for 10 min at 15,000 × g, and the supernatant was collected. Protein concentration was measured with BCA protein assay kit (Pierce, Rokford, IL, USA) according to the manufacturer’s instructions. Briefly, samples (20 µg/lane) were run on a 4% to 15% gradient gel (Bio-Rad Laboratories, Hercules, CA, USA) at a constant voltage of 100 for 1 h against high molecular weight standards (Bio-Rad Laboratories). Separated proteins were transferred to nitrocellulose membrane (GE Healthcare Life science, Freiburg, BW, Germany). The membrane was blocked with 5% skimmed milk for 1 h and incubated with a rabbit polyclonal antibody ZO-1 (40–2200; Thermo Fisher Scientific), ZO-2 (#2847; Cell Signaling Technology, Danvers, MA, USA), Claudin-2 (#48120; Cell Signaling Technology, Danvers, MA, USA), Claudin-3 (SC-517546; Santa Cruz Biotechnology Inc., Dallas, TX, USA) or Claudin-4 (36–4800; Thermo Fisher Scientific), with a mouse monoclonal antibody Occludin (33–1500; Thermo Fisher Scientific), E-cadherin (#3195, Cell Signaling Technology, Danvers, MA, USA) or β-actin (A2228; Sigma Aldrich, St. Louis, MO, USA) for loading control overnight at 4°C and incubated with horseradish-peroxidase-conjugated anti-rabbit (31460; Thermo Fisher Scientific) or mouse IgG (31430; Thermo Fisher Scientific) for 1 h. Bands were visualized using enhanced chemiluminescence reagent (Bio-Rad Laboratories, Hercules, CA, USA) in G:BOX (Syngen, Bengaluru, India) and band intensity was quantified by Gene Tools analysis software (Syngen) and band intensities were normalized by the results of β-actin.
5. Myeloperoxidase (MPO) measurement

Colon tissue samples were homogenized as described above. The homogenates were centrifuged at 15,000 x g for 10 min at 4°C, and the MPO concentration in the supernatant was measured using an ELISA kit (Hycult Biotech. Inc., Plymouth Meeting, PA, USA) according to the manufacturer’s instructions.

6. Cytokine levels in colon samples

The supernatant from colon tissue samples was collected as described above. The concentration of IL-1β, Monocyte chemoattractant protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 1 (CXCL1) and cytokine IL-13 were measured using the Murine IL-1β, J E/MCP-1(CCL2), CXCL1 Mini ABTS ELISA Development Kit (#900-M47; #900-M126, #900-M127; PeproTech, Rocky Hill, NJ, USA) and Mouse IL-13 DuoSet ELISA (#DY413-05; R&D Systems. Inc., Minneapolis, MN, USA) respectively, according to the manufacturer’s instructions. IFN-γ, IL-6, tumor necrosis factor (TNF), IL-17A, IL-10 and IL-4 levels were measured in the supernatant using the BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences).

7. Immunological assays and flow cytometry analysis

Mesenteric lymph nodes (MLNs) were collected from the mice. The tissues were carefully crushed and filtered through a cell strainer (100 μm pore diameter). The cells were isolated, counted and subjected to FcγR blocking. The surfaces of the cells were stained for 30 min at 4°C using Fixable Viability Stain 510 (FVS510; BD Bioscience) for live cells and CD3+ fluorescein isothiocyanate (145-2C11; BD Bioscience), CD4+ Percep-Cyanine 5.5 (RM4-5; BD Bioscience) and CD25+ phycoerythrin (PC61; BD Bioscience) for cell surface staining. The cells were permeabilized in fixation/permeabilization buffer (eBioscience, San Diego, CA, USA) and subjected to intracellular Foxp3 staining using the Alexa Fluor 647 anti-Foxp3 antibody (MF23; BD Bioscience). IgG isotypes were used as a control for all fluorescence-activated cell sorting experiments.

The CD4+CD25+Foxp3+Treg population was analyzed using the BD FACSVersa™ Flow Cytometer (BD Bioscience).

8. Taxonomic and functional analyses of the gut microbiota

Total genomic DNA was extracted from cecal contents using the QIAamp Fast DNA Stool Mini Kit (Qiagen). The V4-V5 region of the 16S ribosomal RNA gene was amplified using the barcoded primers 515F and 926R. The PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the MiSeq platform (Illumina Inc., San Diego, CA, USA). Raw FASTQ files were demultiplexed and quality filtered, and the data were processed using Quantitative Insights into Microbial Ecology 1.8.0 software (QIIME development team; http://qiime.org/). The sequences were clustered into operational taxonomic units of at least 97% identity, and the relative abundances of the microbial taxa (genus to kingdom) were generated from nonrarefied operational taxonomic unit tables. Species richness (alpha diversity) was measured using the Chao1 index. Beta diversity was calculated using the UniFrac distance between samples and visualized in three-dimensional plots based on a weighted principal coordinate analysis. A linear discriminant analysis effect size (LEfSe) analysis (http://huttenhower. org/galaxy) was performed to identify significantly different phylotypes among the experimental groups. In addition, a phylogenetic investigation of communities was performed by reconstructing the unobserved states to identify functional genes in the sampled microbial community based on data in the KEGG pathway database (http://www.genome.jp/kegg/pathway.html).

9. Metabolite analysis using ultra-performance liquid chromatography (UPLC)

Cecal content (30 mg) was immersed in 600 μL liquid chromatography-grade methanol and vortexed for 5 min to investigate the effects of the L. fermentum strains on the total amino acid content in the DSS model. The samples were centrifuged at 15,000 x g for 5 min. The extracts (10 μL) were transferred to an autosampler vial and derivatized by adding 70 μL AccQ•Tag™ Ultra Borate Buffer.
Amino acids were separated on the Acquity UPLC (Waters Corp.) equipped with an HSS T3 column (2.1 mm × 10 mm, 1.7 μm) heated to 45°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was as follows: 0–0.5 min, 4% B; 2.5 min, 10% B; 5 min, 18% B; 5.1 min, 95% B; and 6.2–7.5 min, 4% B. The flow rate was 0.6 mL/min.

The amino acids were analyzed using the SYNAPT G2-Si mass spectrometer (Waters Corp.) equipped with an ESI probe. The mass acquisition mode was time-of-flight–multiple reaction monitoring mode with ESI positive ionization mode. The capillary voltage was set to 1.5 kV. The sampling cone ranged from 20 to 60 V depending on the amino acids. The desolvation gas and cone gas flow rate were 600 and 50 L/h, respectively. The desolvation temperature was 250°C. The identity of the amino acids was confirmed by alignment to the AA-S-18 analytical standards mixture (Sigma-Aldrich, St. Louis, MO, USA) and the individual standards T0254 (L-tryptophan), G3126 (L-glutamine), and A08884 (L-asparagine) (Sigma-Aldrich). Data acquisition and quantitation were carried out using MassLynx software 4.1 (Waters Corp.).

10. Statistical analysis

Data are expressed as the mean ± standard error of the mean. GraphPad Prism 5.04 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to visualize and analyze all data using the Mann–Whitney U-test. A P value < 0.05 was considered significant.

Results

1. Lactobacillus fermentum spp. differentially induce cytokine production in PBMCs

IL-2, IFN-γ, IL-4, IL-13, and IL-10 production was increased significantly in PBMCs stimulated by *E. coli* compared with those stimulated with PBS and CD3 only (Figure S1a-d and f). IL-17A production tended to increase in PBMCs stimulated by *E. coli* compared with CD3 only (Figure S1e). Compared with *E. coli*, *L. fermentum* KBL374 and KBL375 significantly decreased the production of Th1-type (IL-2 and IFN-γ), Th2-type (IL-4 and IL-13), and Th17-type (IL-17A) cytokines and IL-10 (Figure S1a–f). In addition, compared with CD3 only, *L. fermentum* KBL374 and KBL375 significantly decreased the production of Th1-type (IFN-γ), Th2-type (IL-4 and IL-13), and Th17-type (IL-17A) cytokines but increased that of IL-10 (Figure S1b–f). However, *L. fermentum* KBL374 and KBL375 had no significant effect on IL-2 production (Figure S1a). The production of Th1-, Th2-, and Th17-type cytokines decreased and that of IL-10 increased in the presence of the two *L. fermentum* strains compared with CD3-only stimulation.

2. *L. fermentum* KBL374 and KBL375 alleviate the symptoms of DSS-induced colitis in vivo

The effects of *L. fermentum* were further investigated in an in vivo murine DSS-induced colitis model. The weight of mice treated with DSS + PBS decreased sharply on day 5 and decreased to 83.6% of the control by day 9 (Figure 1a). The weight of mice treated with DSS + *L. fermentum* KBL374 or DSS + *L. fermentum* KBL375 showed a similar change as that of the mice treated with DSS + PBS, with decreases observed after 5 days. The weight change was significant between the PBS-treated and *L. fermentum* KBL375-treated DSS mice after day 5. The weights of the *L. fermentum* KBL374- and *L. fermentum* KBL375-treated DSS mice decreased to 88.1% and 89.7% of the control on day 9, respectively. DSS + PBS had an average DAI score of 9.0 while administration of *L. fermentum* KBL374 and *L. fermentum* KBL375 significantly reduced an average DAI score of 6.0 and 6.4 on day 9, respectively (Figure 1b).

The *L. fermentum*-treated DSS mice had significantly longer colons and increased cecal weights than those of the PBS-treated DSS mice (Figure 1c,d). Increased epithelial structural damage and inflammation occurred in the colonic mucosa of PBS-treated DSS mice compared with water + PBS-treated mice (Figure 1e). However, the *L. fermentum* KBL374- and *L. fermentum* KBL375-treated DSS mice exhibited fewer damaged crypts and a thickened epithelial...
layer. The histology scores of mice treated with either *L. fermentum* strain indicated significantly less loss of goblet cells, less distortion of crypts, and decreased leukocyte infiltration in the colonic mucosa compared with PBS treatment (Figure 1f).

3. Administration of *L. fermentum* KBL374 and KBL375 increases tight junction-related protein levels in the colon

*L. fermentum* KBL375 significantly increased E-cadherin or Claudin-3 levels in inflamed colonic tissue of DSS mice compared with PBS treatment (Figure 2a,b). In contrast, *L. fermentum* KBL374-treated DSS mice showed no significant difference in tight junction protein compared with PBS-treated DSS mice (Figure 2a,b).

4. *L. fermentum* KBL374 and KBL375 reduce inflammation markers in the colon

After DSS treatment, the PBS-treated DSS mice showed significantly higher MPO concentrations than those in the water + PBS-treated mice (Figure 3a). MPO concentrations were significantly lower in *L. fermentum* KBL375-treated DSS mice and slightly lower (but not significantly) in *L. fermentum* KBL374-treated DSS mice compared with PBS-treated DSS mice. Pro-inflammatory cytokine IL-1β and the chemokines CCL2 and CXCL1 were significantly induced by DSS treatment compared with those in the water + PBS-treated mice (Figure 3b–d). Moreover, *L. fermentum* KBL374 and KBL375 significantly decreased IL-1β, CCL2 and CXCL1 compared with PBS-treated DSS mice (Figure 3b–d).

5. *L. fermentum* KBL374 and KBL375 downregulate the protein levels of Th1, Th2, and Th17 cytokines in the colon

We measured the protein levels of Th1-, Th2-, and Th17-type cytokines in the colon after *L. fermentum* treatment. *L. fermentum* KBL374 significantly suppressed the protein levels of Th1-type (IFN-γ), Th2-type (IL-13) and Th17-type (TNF) cytokines compared with PBS in DSS mice (Figure 4a,c and g). *L. fermentum* KBL375 significantly decreased the protein levels of Th1-type (IFN-γ), Th2-type (IL-4 and IL-13) and Th17-type (IL-6, TNF and IL-17A) cytokines compared with PBS (Figure 4a–d and f,g). The anti-inflammatory cytokine IL-10 was increased significantly in the PBS-treated DSS...
mice compared with the water + PBS-treated mice (Figure 4e). *L. fermentum* KBL374 tended to increase, whereas *L. fermentum* KBL375 significantly increased, IL-10 concentrations compared with PBS treatment in DSS mice (Figure 4e).

6. **L. fermentum KBL374 and KBL375 increase the proportion of Treg cells in MLNs**

We investigated the CD4+CD25+Foxp3+Treg population in MLNs in DSS-induced colitis to confirm the potent immunomodulatory effects of the *L. fermentum* KBL374 and KBL375 strains in MLNs. Oral administration of *L. fermentum* KBL374 and KBL375 significantly increased the proportion of the CD4+CD25+Foxp3+Treg population compared with PBS treatment in DSS mice (Figure 5a,b).

7. **L. fermentum KBL374 and KBL375 administration significantly changes the community structure of the gut microbiota**

The microbial population was significantly decreased in PBS-treated DSS mice compared with water + PBS-
treated mice but increased in *L. fermentum* KBL374- and *L. fermentum* KBL375-treated DSS mice compared with PBS-treated DSS mice (Figure 6a,b). The bacterial communities in mice within the same groups had a greater tendency to cluster together, but there were differences in the gut microbiota patterns among the four groups (Figure 6c). In particular, principal coordinates analysis showed that the gut...
microbiota structure was different between the PBS-treated DSS mice and water + PBS-treated mice. DSS treatment caused a disturbance in the gut microbiota, which was less severe when the mice were administered *Lactobacillus fermentum* KBL374 or KBL375. The prominent microbiota at the genus level in the water + PBS-treated mice were *S24-7_unclassified* (42%) followed by *Clostridiales_unclassified* (10.7%) and *Oscillospira* (4.8%). The relative abundance of *S24-7_unclassified* (6%) was lower, whereas that of *Bacteroides* (31.5%) was markedly higher, in the PBS-treated DSS mice compared with the water + PBS-treated mice. The

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**Figure 6.** Taxonomic changes in the microbiota of the mouse cecum induced by *Lactobacillus fermentum* KBL374 and KBL375. A microbiome analysis was conducted using cecal samples collected from 7–16 mice per group. (a) A rarefaction plot measured using the Chao1 diversity index. (b) The Chao1 diversity index at 5,500 sequences per sample. (c) Principal coordinates analysis of the cecal microbiota structure measured by weighted UniFrac distance. (d) Average relative abundances of taxa at the genus level. (e) Significantly different taxa among the water + PBS (Green), DSS + PBS (Orange), DSS + KBL374 (Red), and DSS + KBL375 (Blue) treatment groups, as measured by LEfSe analysis (threshold > 2.0). (f) Comparison of the relative abundances of significantly different microbial taxa at the genus level. The statistical analysis was performed using the Mann–Whitney *U*-test, with comparison with the DSS + PBS-treated mice. Error bars represent standard errors. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.
relative abundances of S24-7_unclassified were lower in *L. fermentum* KBL374- and *L. fermentum* KBL375-treated DSS mice compared with the water + PBS-treated mice, but higher compared with the PBS-treated DSS mice. In contrast, the proportions of *Bacteroides* were greater in the *L. fermentum* KBL374- and *L. fermentum* KBL375-treated DSS mice compared with the water + PBS-treated mice but lower compared with the PBS-treated DSS mice, despite the DSS treatment (Figure 6d).

8. Administration of *L. fermentum* KBL374 or KBL375 alters the cecal microbiota composition at the genus level

The four groups had abundant taxa at the genus level according to the LEfSe algorithm (Figure 6e). The relative abundance of *Lactobacillus* in the water + PBS-treated mice was significantly higher compared with the PBS-treated DSS mice. *Lactobacillus* abundance was significantly higher in the *L. fermentum* KBL375-treated compared with the PBS-treated DSS mice. The PBS-treated DSS mice contained significantly higher proportions of *Bacteroides* and *Mucisprillium* compared with the other three groups. However, the abundances of these two genera were significantly lower in the *L. fermentum* KBL374- and KBL375-treated compared with the PBS-treated DSS mice. Compared with PBS-treated DSS mice, the relative abundance of *Akkermansia* was significantly lower in water + PBS-treated mice but significantly higher in *L. fermentum* KBL374- and *L. fermentum* KBL375-treated DSS mice (Figure 6f).

9. KBL374 and KBL375 change amino acid metabolic functions

The KEGG pathway analysis revealed differentially expressed genes in the cecum (Figure 7a). Significant changes in amino acid biosynthesis...
and metabolism pathways were observed in the water + PBS-treated mice. However, DSS treatment led to changes in a number of pathways, including those involving transporters, bacterial motility proteins, and several others. *L. fermentum* KBL374 treatment altered genes involved in carbohydrate metabolism (e.g., galactose) and amino acid metabolism (e.g., alanine, aspartate, glutamate, glycine and tryptophan). Energy metabolism and amino acid metabolism (glycine, serine, threonine, D-glutamine and D-glutamate) pathways were upregulated in *L. fermentum* KBL375-treated DSS mice. Levels of amino acids such as glutamine, glutamic acid, and glycine were significantly lower in the cecum of the *L. fermentum* KBL374- and *L. fermentum* KBL375-treated compared with the PBS-treated DSS mice (Figure 7b).

**Discussion**

We demonstrated that *L. fermentum* strains KBL374 and KBL375 modulated the immune response and improved the symptoms of IBD via the gut microbiota and their metabolites based on *in vitro* and *in vivo* models. In this study, we report the capability of two *L. fermentum* strains to modulate the concentrations of different cytokines. Significant reductions in Th1-type (IL-2 and IFN-γ), Th2-type (IL-4 and IL-13) and Th17-type (IL-17A) cytokine concentrations were observed in PBMCs stimulated with *L. fermentum* KBL374 or KBL375 after T cell stimulation. In addition, stimulation with *L. fermentum* KBL374 or KBL375 significantly increased IL-10 secretion, which is often associated with anti-inflammatory effects. The ratio of IL-10 to IFN-γ was increased in *L. fermentum* KBL374 and KBL375 compared with *E. coli* stimulated with CD3, as reported previously (data not shown). The capability of *L. fermentum* to alter PBMC cytokine levels (increased IL-10 and decreased Th1-, Th2- and Th17-related cytokines) *in vitro* indicates that applying specific *L. fermentum* strains *in vivo* will have a protective function against IBD.

The DSS-induced murine colitis model is a useful tool to examine the clinical efficacy and possible working mechanism of probiotics in IBD development. Mice treated with 2% DSS that have no mortality rate for 5–7 days showed extensive bodily harm, including severe depletion of crypts and slow regeneration of colonic epithelium, which were associated with clinical features such as weight loss, loose stools/diarrhea and rectal bleeding. In our experiment, oral administration of either *L. fermentum* KBL374 or KBL375 significantly prevented weight loss and DAI score and decreases in the length and weight of the cecum compared with the DSS control group. In addition, *L. fermentum* KBL374 and KBL375 treatment recovered mucous and crypt structures by reducing mucosa-associated lymphoid tissue, as shown in the histological analysis. There could be multiple reasons for the improvements in the colitis model. First, our study confirmed that *L. fermentum* KBL375 increases E-cadherin and Claudin-3 levels, which are important transmembrane and intracellular tight junction proteins. Alterations in tight junction protein expression and distribution have been considered key factors in the onset of colonic inflammation and UC.

Furthermore, the *L. fermentum* KBL374 and KBL375 treatments reduced the innate immune response, which agrees with a previous study. Changes in MPO levels reflect the degree of neutrophil infiltration and tissue damage in the colon. TNF and CCL2, which are a key pro-inflammatory cytokine and chemokine, respectively, are primarily produced by monocytes and macrophages. Moreover, TNF-α is an activator of nuclear factor-κB, which further promotes the secretion of TNF and upregulates other pro-inflammatory cytokines, such as IL-1β and IL-6. CXCL1 is a chemokine with dual roles in recruiting and activating neutrophils. Overall, the colons of the *L. fermentum* KBL374- and KBL375-treated DSS mice contained reduced levels of these inflammatory markers (Figure 3).

Penetration of bacteria into the mucosal layer leads to activation of resident innate immune cells, which in turn leads to an adaptive immune response involving T cells. Indeed, it has been demonstrated that antigen-specific T cells develop during the acute stage of DSS-induced colitis. A previous study postulated that it is essential to target both Th1 and Th17 cells to treat CD, a major form of IBD. In addition, transient depletion of Treg cells increases the severity of DSS-induced colitis. In fact, protein
levels of Th1-, Th2- and Th17-type cytokines were decreased and IL-10 was increased after *L. fermentum* KBL375 treatment. Oral administration of *L. fermentum* KBL375 successfully altered the mucosal immune response to modulate the levels of cytokines.

*L. fermentum* KBL375 significantly upregulated the level of anti-inflammatory cytokine IL-10 in the colons of mice (Figure 4), similar to the in vitro PBMC model. IL-10 inhibits the expression of other inflammatory cytokines (i.e., IL-1, IL-6 and IL-12) and chemokines produced by activated monocytes or macrophages. Moreover, administering either *L. fermentum* KBL374 or KBL375 increased the CD4+CD25+Foxp3+Treg cell population in MLNs (Figure 5). CD11+dendritic cells (DCs) are converted into regulatory DCs, which express IL-10 and convert CD4+Foxp3–T cells into CD4+Foxp3 +Treg cells. Treg cells in MLNs migrate to the inflamed region to suppress disease progression. These findings are consistent with a previous study reporting that Treg cells are restored in MLNs after inoculation with *L. fermentum*. The role of the gut microbiota in controlling the host immune response has received increasing attention. The immune system influences the composition of the gut microbiota. A number of studies have demonstrated that the gut microbiota plays a critical role in the attenuation or development of IBD, and that inflammation via IBD can drive the loss of microbiota diversity, leading to a distinct microbial community composition. Our results showed that mice treated with DSS had an altered composition and reduced diversity of the gut microbiota (Figure 6a–c). However, *L. fermentum* KBL374 and KBL375 partly restored the gut microbiota in terms of diversity and composition, in line with a previous study. We evaluated the composition of the gut microbiota at the genus level. Decreased *Bacteroides* and increased in S24-7_unclassified abundances were observed in the *L. fermentum* KBL374- and KBL375-treated DSS groups compared with the PBS-treated DSS group (Figure 6d,f), suggesting that these strains ameliorate colitis and reshape gut microbiota to promote recovery. Consistent with our results, previous studies have reported that the relative abundance of *Bacteroides* is higher, and that of S24-7 is lower, in DSS-treated groups. *Bacteroides* is a possible indicator of disease onset in the mouse model of DSS-induced colitis, and its abundance decreases upon recovery of the mice. In contrast, the abundance of S24-7 decreased at the onset of colitis. Increased abundance of *Lactobacillus* was observed in the *L. fermentum* KBL375-treated DSS group and of *Akkermansia* in both the *L. fermentum* KBL374- and KBL375-treated DSS groups, compared with the DSS control group. *Lactobacillus* stimulates DCs to produce regulatory IL-10, and *Akkermansia* reduces inflammation via anti-inflammatory activities such as upregulation of Foxp3+Treg cells in adipose tissue. In addition, *Akkermansia* stimulates IL-10 production and plays an immunological role in homeostasis of the gut mucosa and barrier function. Oral administration of *L. fermentum* KBL374 or KBL375 may contribute to restoring *Lactobacillus* and *Akkermansia* populations, which would increase IL-10 levels and the integrity of the mucus layer, thereby alleviating DSS-induced colitis. Previous studies demonstrated that administration of probiotics for short period could affect the alteration of the abundance and composition of specific gut microbiota. The effect of probiotics on the composition of gut microbiota could be faster than host physiology. The administration of metabolically active microorganisms could change the composition of other microorganisms. Previous study indicated that different diet could rapidly change the composition of gut microbiota within 24 h. Additionally, our study coincided with the results of previous study reporting that *L. acidophilus* increased the relative abundance of *Akkermansia*. Each of microorganisms in the gut was highly metabolically dependent each other and mutually networked based on their metabolic pathways and products. The exact mechanism of these changes should be experimentally evaluated in future.

Changes in the composition of the gut microbiota lead to metabolite changes that impact IBD pathogenesis. We found that both *L. fermentum* KBL374 and KBL375 were involved in the metabolism of amino acids such as glutamine, glutamic acid and glycine, indicating that the two strains regulate metabolism related to these amino acids in the gut. The specific functions of each microbial taxa in the gut microbiota need further evaluation.

Consumption of LBPs may ameliorate IBD by manipulating the intestinal microbiota. The diverse effects of LBPs have been demonstrated,
and the effects of probiotics on cytokine regulation have been widely studied. However, the capabilities of these immunomodulatory bacteria are largely strain dependent. We suggest that both *L. fermentum* KBL374 and KBL375 may guide therapeutic strategies in manipulating the microbiota to control IBD. Our novel *L. fermentum* strains were evaluated for various antibiotics resistance. Additionally, anti-bacterial effects against pathogens could be further evaluated in future.

In conclusion, novel strains of *L. fermentum* isolated from Korean feces were identified, and these strains affected immune cytokine expression and microbiome and metabolic functions, leading to differential efficacy in the amelioration of DSS-induced colitis. *L. fermentum* KBL374 and KBL375 are promising novel probiotic candidates to treat IBD.

**Disclosure of potential conflicts of interest**

The authors declare that there is no conflict of interest.

**Funding**

This work was supported by the Technology development Program (S2518735) funded by the Ministry of SMEs and Startups (MSS, Korea).

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