보건학석사 학위논문

Combine effects of *Lactobacillus gasseri* KBL697 and infliximab against DSS-induced colitis by regulating immune response and altering gut microbiota in mice

락토바실러스 가세리 균주와 인플릭시맵 병행처리의 염증성 장 질환 완화 효과

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한 대 희

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Abstract

Combine effects of *Lactobacillus gasseri* KBL697 and infliximab against DSS-induced colitis by regulating immune response and altering gut microbiota in mice

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We hypothesized that *Lactobacillus gasseri* (*L. gasseri*) KBL697 and infliximab (IFX) have combine effects to prevent dextran sodium sulfate (DSS)-induced colitis induced by inflammatory cytokines and gut microbiota disruption. We first evaluated immunomodulation effect of *L. gasseri* KBL697. In bone-merrowed-derived macrophage cells

(BMDMs), *L. gasseri* KBL697 has effects of decreasing inflammatory cytokine interleukin (IL)-1β and increasing anti-inflammatory cytokine IL-10. Also, we found that *L. gasseri* KBL697 has combine effect of barrier protection with IFX in Caco-2 cell. Furthermore, we demonstrated that combination of IFX and *L. gasseri* KBL697 has beneficial effects than single treatment on DSS-induced colitis. Combination treatment significantly prevented weight loss than single treatment after DSS treatment cessation. Also, colon length, cecum weight and histological scores confirmed that combination treatment shows beneficial effects in DSS-induced colitis. Our results showed that combination treatment reduces inflammatory cytokine levels and also increases anti-inflammatory cytokine levels than single treatment. Furthermore, combination treatment has effect of increasing CD4+CD25+Foxp3+ T cell population than single treatment in mesenteric lymph nodes (MLN). Taxonomic and functional analyses of gut microbiota showed significantly higher cecum bacterial diversities and abundances including genus *Akkermansia*, *Prevotella* and *Ruminococcus* in DSS+Combine-treated mice. Combination treatment also showed effects of increasing beneficial metabolite such as purine, glycine, serine and threonine metabolism as well as acetate and

butyrate in the cecum. Through these results, we demonstrate that combination treatment has effects of alleviating colitis symptoms than single treatment by modulating the immune response and altering gut microbiota.

Key words: *Lactobacillus gasseri*, infliximab, colitis, cytokine, metabolome, microbiome

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List of Abbreviations

Bone merrowed derived (BMDM)

Transepithelial electrical resistance (TEER)

Dextran sulfate sodium (DSS)

Disease activity index (DAI)

Mesenteric lymph nodes (MLN)

Inflammatory Bowel Disease (IBD)

Phosphate buffer solution (PBS)

Man, Rogosa and Sharpe (MRS)

Enzyme-linked immunosorbent assay (ELISA)

Optical density (OD)

Room temperature (RT)

Fetal bovine serum (FBS)

Radioimmunoprecipitation assay (RIPA)

Quantitative Insights into Microbial Ecology (QIIME)

Operational taxonomic units (OTUs)

Principle coordinate analysis (PCoA)

Linear discriminant analysis (LDA)

Linear discriminant analysis effect size (LEfSe)

Standard error of the mean (SEM)

Gas chromatography (GC)

Zonula occludens (ZO)

Fluorescence-activated cell sorting (FACS)

Tight junction (TJ)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

I. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that affects the gastrointestinal tract.[1] In spite of various pharmaceutical treatment for IBD,[2] current therapeutic agents are only moderately effective. These aspects emphasize the need for other therapeutic options. Although the pathogenesis of IBD is not completely elucidated, it is currently widely accepted that IBD involves disruption of the homeostasis between the mucosal immune system and gut microbiota.[3]

The gut microbiota composition is essential for maintaining the structural integrity of the gut mucosal barrier and immunomodulation. Moreover, the microbiota helps the host to distinguish commensal organisms from pathogens to induce the adequate responses.[4] Also, this particular bacterial composition can synthesize short chain fatty acids (SCFAs) which play the key role for adjusting the function of the host metabolism and regulating immune response.[5] However, when the composition of the microbiota is impaired, this symbiotic relationship does not function properly and trigger an inappropriate immune response. This alteration could ultimately lead to inflammatory conditions such as IBD.[6]

Different mediators, tumor necrosis factor (TNF)-α is a pivotal pro-inflammatory cytokine implicated in the immune-pathogenesis of IBD, leading to the activation of immune cells.[7] New insights in the immunopathology of IBD have allowed the introduction of biologics, among which IFX represents the first example in IBD. Infliximab (IFX) is a chimeric mouse–human monoclonal antibody composed by constant regions of human immunoglobulin (Ig) G1k coupled to the variable regions of a high-affinity that are able to neutralize human TNF-α. Therefore, IFX has the effect of alleviating IBD symptoms by neutralizing TNF-α.[8]

Probiotics are useful for the prevention and treatment of a variety of acute and chronic human diseases.[9,10] While some attention has been given to the potential mechanisms by which probiotics improve health by modulating the intestinal microbiota, direct interactions between probiotics and the intestinal epithelium and immune system have also been shown.[9,10] Moreover, *Lactobacillus gasseri* (*L. gasseri*) strains can alleviate IBD via modulation of gut microbiota, and affecting host metabolic pathways.[11,12]

Therefore, we hypothesized that *L. gasseri* KBL697 isolated from the feces of healthy donors would enhance the effects of IFX on IBD

by regulating immune responses and gut microbiota. After the treatment of IFX and *L. gasseri* KBL697, we assessed the ameliorations of IBD symptoms. Then, we evaluated if *L. gasseri* KBL697 improve effect of IFX on IBD, 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.

II. Materials and Methods

1. Bacterial strains preparation

L. paracasei KBL382 and *L. gasseri* KBL697 were isolated from fecal samples of healthy Korean adult. *Escherichia coli* (E. coli) 0157:H7 strain used as a positive control. These bacterial strains were gifts from Kobiolabs Inc (Seoul, Korea) and received in lyophilized condition (*L. paracasei* KBL382: 3 x 10^{11} CFU/g, *L. gasseri* KBL697: 2 x 10^{10} CFU/g). All experiments were performed by diluting lyophilized bacteria in phosphate buffer solution (PBS).

2. The measurement of transepithelial electrical resistance in Caco-2 *in vitro* model

To identify the effect of IFX and *L. gasseri* KBL697, Caco-2 cells from the American Type Culture Collection (ATCC) were cultured in Minimum Essential Medium (MEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; GenDEPOT, Barker, TX), 1% nonessential amino acids, 1% HEPES, 1% sodium bicarbonate solution, 50 μg/mL of gentamicin and 10 U/mL of penicillin-streptomycin at 37°C in a 5% CO2 environment. For *in vitro* Trans Epithelial Electrical Resistance (TEER) assay, the cells were seeded onto Transwell inserts (Corning Inc., Corning, NY) at 3 x 10^5 cells/mL density, cultured for 7 days. Before bacterial treatment, cells were starved with FBS-free and antibiotic-free medium to synchronize the cell cycle. Cells were treated with bacteria at multiplicity of infection (MOI) of 100 or in combination with 20 μg of IFX (Remicade®, Centocor B.V.). The integrity of the differentiated cells was measured as the TEER value using an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) at 0 h and 24 h after treatment.

3. The measurement of immunomodulatory effects in BMDM *in vitro* model

To measure the immunomodulatory effects of *L. gasseri* KBL697, Rodent bone-marrow-derived macrophages (BMDM), obtained as described previously,[13] were cultured in DMEM medium (Gibco, Paisley, UK) containing 1% penicillin/streptomycin (Gibco), 1% gentamycin (Gibco), and 10% fetal bovine serum (Gibco). To confirm cell viability, cultured BMDM were stained with trypan blue and counted using a CKX31 inverted microscope (Olympus Corp., Tokyo, Japan). BMDM at a density of approximately 1×10^5 cells were incubated with 4×10^6 CFU bacterial strains in the presence of 500 ng/mL LPS in 96-well plates at 37°C for 24 h. Then the supernatant was collected to measure the concentrations of the cytokines TNF, interleukin (IL)-6, IL-1β and IL-10 using the Murine TNF, IL-6, IL-1β Mini ABTS ELISA Development Kit (#900-M54, #900-M54, #900-M47; PeproTech, RockyHill, NJ, USA) and IL-10 mouse ELISA kit (#88-7105-22; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

4. Experimental colitis *in vivo*

The overall experimental procedure is illustrated in Supplementary figure 1A. Female 7-week-old C57BL/6N mice were purchased from Central Lab Animals Incorporated (Seoul, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University (Case Number: SNU-190528-1). Five groups of mice (n=8) were treated with (1) Water+PBS, (2) DSS+PBS, (3) DSS+IFX, (4) DSS+KBL697, (5) DSS+Combine. To induce colitis, all groups except Water+PBS group were fed the water containing 2% (w/v) DSS (molecular mass 36,000–50,000 Da; MP Biomedical, LLC., Santa Ana, CA, USA) from day 0 to day 7. Then, the Water+PBS, DSS+PBS and DSS+IFX groups were orally administered 200 μL of PBS while DSS+KBL697 and DSS+Combine groups were orally administered *L. gasseri* KBL697 (2×10^9 CFU) suspended in 200 μL of PBS from day 0 to day 8. On day 3, the Water+PBS, DSS+PBS and DSS+KBL697 groups were intravenously injected of 200 μL of PBS while DSS+IFX and DSS+Combine groups were intravenously injected IFX (5mg/kg) suspended in 200 μL of PBS. The changes of disease activity index (DAI) were measured every day using the following criteria: (1) weight loss (%), (2) stool consistency

and (3) blood in feces as previously described (Table 1).[11] On day 9, the mice were sacrificed for collection of the colon, stool, cecum and mesenteric lymph nodes (MLN).

5. Histological analysis

To identify the occurrence of colitis, the distal colon samples were stained with hematoxylin and eosin as previously described.[14] 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 as previously described with some modification (Table 2).[15]

Table 1. Scoring system to calculate DAI

Score	Weight loss	Consistency	Bleeding
0	None	Normal	None
1	1-5%	Normal	None
2	5-10%	Loose	Slight bleeding
3	10-15%	Loose	Slight bleeding
4	>15%	Diarrhea	Gross bleeding

Table 2. Histological score of colitis induced by DSS

Histological feature	Score	Description
Loss of epithelium	0	None
	1	0-5% loss of epithelium
	2	5-10% loss of epithelium
	3	Over 10% loss of epithelium
Crypt damage	0	None
	1	0-10% loss of crypt
	2	10-20% loss of crypt
	3	Over 20% loss of crypt
Depletion of goblet cells	0	None
	1	Mild
	2	Moderate
	3	Severe
Infiltration of cells	0	None
	1	Mild
	2	Moderate
	3	Severe

6. Real-time PCR

To measure the mRNA expression of various cytokines or chemokines in the colon tissue, total RNA was extracted from each distal colonic tissue using an easy-spin total RNA Extraction Kit (Intron, Seoul, Korea) following the manufacturer's protocol. All RNA samples were quantified with the Nano drop® ND 1000 (Thermo Scientific). RNA was reverse transcribed and amplified using High-Capacity RNA-to-cDNA kit (Thermo Scientific). The resulting cDNA was amplified using Power SYBR Green PCR Master Mix (Thermo Scientific) and specific primers for each gene: zonula occludens (ZO)-1, ZO-2, claudin4, mucin (MUC)-2 and Transforming growth factor (TGF)-β (Table 3). The reaction for 40 cycles with denatured at 95°C for 5 min followed by 95°C for 5 s and 60°C for 10 s were performed using a Step-One-Plus Real-Time PCR System (Applied Biosystems, Forster, CA USA). The expression levels of target mRNA were normalized to Hypoxanthine guanine Phospho-Ribosyl-Transferase (HPRT). Results were expressed as relative expression ratios to the Water group. Also, miRNA was extracted from the distal colonic tissue using miRNeasy mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All miRNA samples were quantified with the

Nano drop® ND 1000 (Thermo Scientific) and 500 ng of miRNA was reverse transcribed and amplified using the miScript II RT kit (Qiagen). The resulting cDNA (2 ng) was amplified using QuantiTects SYBR Green PCR Master Mix (Qiagen), miScript Universal Primer (Qiagen) and miScript Primer Assay (Qiagen): miR-155 (cat: MS00001701), miR-150 (cat: MS00001673), miR-223 (cat: MS00001960), miR-146 (cat: MS00001638), miR-107 (cat: MS00023961). The reaction for 40 cycles with denatured at 95°C for 15 min followed by 94°C for 15 s and 55°C for 30 s and 70°C for 30 s were performed using a Real-Time PCR System (Applied Biosystems). The expression levels of target miRNA were normalized to Small nuclear RNA, C/D box 95 (SNORD95) (Qiagen, cat: 218300). Results were expressed as relative expression ratios to the Water+PBS-treated mice.[16]

Table 3. PCR primers

Gene	Sequence (5'→3')	Reference
HPRT	Fw: 5′-TTA TGG ACA GGA CTG AAA GAC-3′	31
	Rv: 5′-GCT TTA ATG TAA TCC AGC AGG T-3′	31
ZO-1	Fw: 5′-ACC CGA AAC TGA TGC TGT GGA TAG-3′	30
	Rv: 5′-AAA TGG CCG GGC AGA ACT TGT GTA-3′	30
ZO-2	Fw: 5′-CTA GAC CCC CAG AGC CCC AGA AA-3′	30
	Rv: 5′-TCG CAG GAG TCC ACG CAT ACA AG-3′	30
Claudin4	Fw: 5′-ACT TTT TGT GGT CAC CGA CT-3′	30
	Rv: 5′-GCG AGC ATC GAG TCG TAC AT-3′	30
MUC2	Fw: 5′-TCG TCT ATT TCC TTG CCC TG-3′	31
	Rv: 5′-ATT ACC TGC CGA AAC CTC CT-3′	31

Fw: Represents sequences of a forward primer

Rv: Represents sequences of a reverse prime

7. Myeloperoxidase (MPO) measurement

To measure the protein level of MPO in the colon tissue, 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. The homogenates were centrifuged at 15,000 × 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.

8. Measurement of cytokines in protein level

To measure the protein level of various cytokines or chemokines in the colon tissue, the supernatant from colon tissue samples was collected as described above. The expression of Interferon (IFN)-γ, IL-2, IL-17A, IL-6, TNF and IL-10 were measured using the BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The concentration of IL-1β, C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 1 (CXCL1) were measured using the Murine IL-1β, CCL2, CXCL1 Mini ABTS ELISA Development Kit (#900-M47; #900-M126, #900-M127; PeproTech) according to the manufacturer's instructions.

9. Flow cytometry analysis

To measure the regulatory T cell (Treg) population in the colon tissue, MLN from mice was carefully smashed and filtered through a cell strainer with a 100 μm pore diameter. The cells were stained with Fixable Viability Stain 510 (FVS510; BD Horizon) for live cells, CD3+ FITC (BD bioscience), CD4+ Percep-cy5.5 (BD bioscience) and CD25+ PE (BD Bioscience) for cell surface staining and were permeabilized with fixation/permeabilization buffer (ebiosciecnce) and were stained Foxp3 (Alexa Flow 647) for intracellular staining. The CD4+CD25+Foxp3+ T cells population was analyzed using the BD FACSVerse™ Flow Cytometer (BD Bioscience).

10. Gut microbiota and predicted community metagenomic analysis

All sequenced data and detailed methods were illustrated in the previous study.[17] Briefly, total bacterial DNA was extracted from cecal contents using the QIAmp Fast DNA Stool Mini Kit (Qiagen). The V4-V5 region of 16S rRNA was amplified by MID-PCR reaction with the reference primers (515F and 806R).[18] After purification, the PCR amplicons were quantified, and aliquots were mixed in equal amounts. The final library was sequenced using Illumina Miseq platform (Illumina Inc., San Diego, CA, USA).[19] The processing of the raw reads were conducted following the Quantitative Insights into Microbial Ecology (QIIME) 1.8.0 pipeline.[19] 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 (α-diversity) was measured using the Phylogenetic Diversity (PD) whole tree index. β-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, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was applied to generate predicted functional metagenomes based on the acquired operational taxonomic units (OTU) table.[20] The relative abundance of predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was calculated after excluding non-microbial functional pathways.

11. Short-chain fatty acids measurement

To quantify the total short-chain fatty acids (SCFAs) content, cecum samples were analyzed using a gas chromatography (GC) flame ionization detector (FID) system as previously described.[21] Briefly, cecum samples immersed in 1 mL of distilled water (DW) and vortexed for 1 min. The supernatant was obtained, and 10% volume of sulfuric acid was added for stability. After centrifuged at 13,000 rpm for 5 min, 400 μL of supernatant was acquired and 40 μL of internal standard (1% 2-methylpentanoic acid) was added. Then, 400 μL of ethyl ether was added and vortex for 1 min. After centrifuged at 13,000 rpm for 5 min, the organic layer was transferred into vial (Agilent Technologies, Santa Clara, CA, USA) and measured by gas chromatography. Nitrogen was used as the carrier gas. The oven temperature was set at 170 °C. FID and injection port were set to 225 °C. The retention times and peak areas of the standard mix were used as references for the unknown samples.[21]

12. 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 one-way ANOVA followed by Tukey's test. A P value < 0.05 was considered significant.

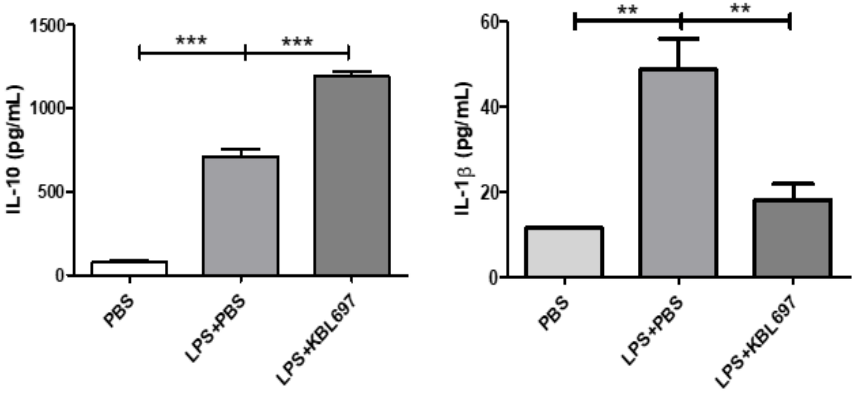
III. Results

1. *Lactobacillus gasseri* KBL697 has effects of immunomodulation in BMDMs

To assess the immunomodulatory capacity of the *L. gasseri* KBL697, BMDMs were stimulated with a combination of LPS and *L. gasseri* KBL697, and the cytokine levels were analyzed. *L. gasseri* KBL697 significantly decreased IL-6 and increased IL-10 compared with those stimulated with PBS or LPS only (Figure 1A).

2. Combination treatment has combine effect of improve tight junction in Caco-2 monolayer

We measured the intestinal barrier enhancement effect of *L. gasseri* KBL697 and IFX. Combination of *L. gasseri* KBL697 and IFX significantly increased relative TEER change than *L. gasseri* KBL697. However, *L. paracasei* KBL382, known to be effective against colitis,[6] had no significant effect. Also, there was no effect of combination of *L. paracasei* KBL382 and IFX (Figure 1B).


IL-10 (pg/mL)
1500
1000
500
0
***
***
PBS
LPS+PBS
LPS+KBL697
IL-1β (pg/mL)
60
40
20
0
**
**
PBS
LPS+PBS
LPS+KBL697

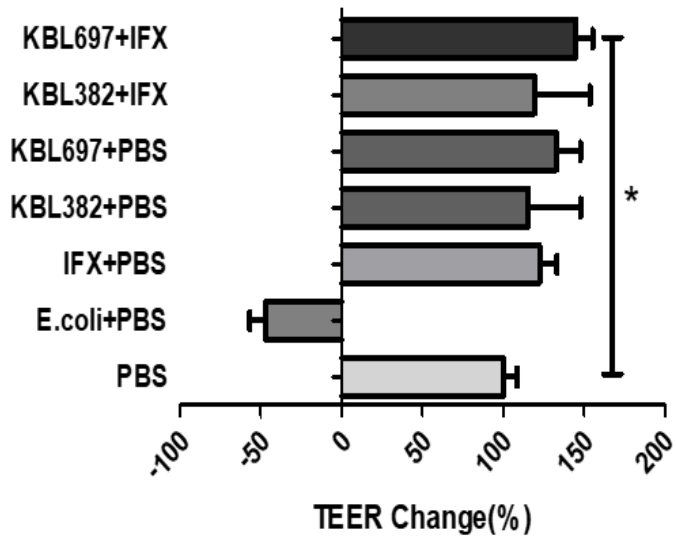

KBL697+IFX
KBL382+IFX
KBL697+PBS
KBL382+PBS
IFX+PBS
E.coli+PBS
PBS
*
-100
-50
0
50
100
150
200
TEER Change(%)

Figure 1. Effects of *Lactobaciilus gasseri* KBL697 and Infliximab (IFX) on relative transepithelial electrical resistance (TEER) change and cytokines level in the *in vitro* model

Cytokines level was measured in the bone marrow derived macrophage. (A) Interleukin (IL)-10 and IL-1β were quantified by enzyme-linked immunosorbent assay (ELISA). In Caco-2 transwell system, (B) relative TEER change was measured after *Lactobaciilus gasseri* KBL697 and IFX treatment for 24 h. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Combination treatment has therapeutic effect of DSS-induced colitis *in vivo*

To study the combine effect of the *L. gasseri* KBL697 and IFX *in vivo*, the murine DSS-induced colitis model was used. The weight of DSS+PBS-treated mice decreased rapidly on day 5 and decreased by 77.28% on day 9 (Figure 2A). The weight of DSS+*L. gasseri* KBL697-treated or DSS+IFX-treated mice was increased after day 8 compare to DSS+PBS-treated mice but there were not significant. On the other hand, the weight of the DSS+Combine-treated mice was significantly increased to 85.78% compare to DSS+PBS-treated mice on day 9. Also, DSS+PBS-treated mice had an average DAI score of 8.3 while treatment of *L. gasseri* KBL697 or IFX significantly reduced an average DAI score of 6.3 or 6.7 on day 9, respectively. Interestingly, combination treatment reduced the DAI score by 5.2 and significantly more than KBL697 or IFX treatment (Figure 2B). DSS treatment decreased colon length, cecum weight and increased histology score. Combination of *L. gasseri* KBL697 and IFX led to significant reduction of DSS-induced colon shortening, decreased cecum weight, colon epithelial damage and cellular infiltration as compare to mice with DSS treatment alone (Figure C and D). In particular, histology score of

DSS+Combine-treated mice was significantly decreased than DSS+*L. gasseri* KBL697- or DSS+IFX-treated mice. These data indicate that combination treatment has effects on the alleviation of colitis symptoms than single treatment.

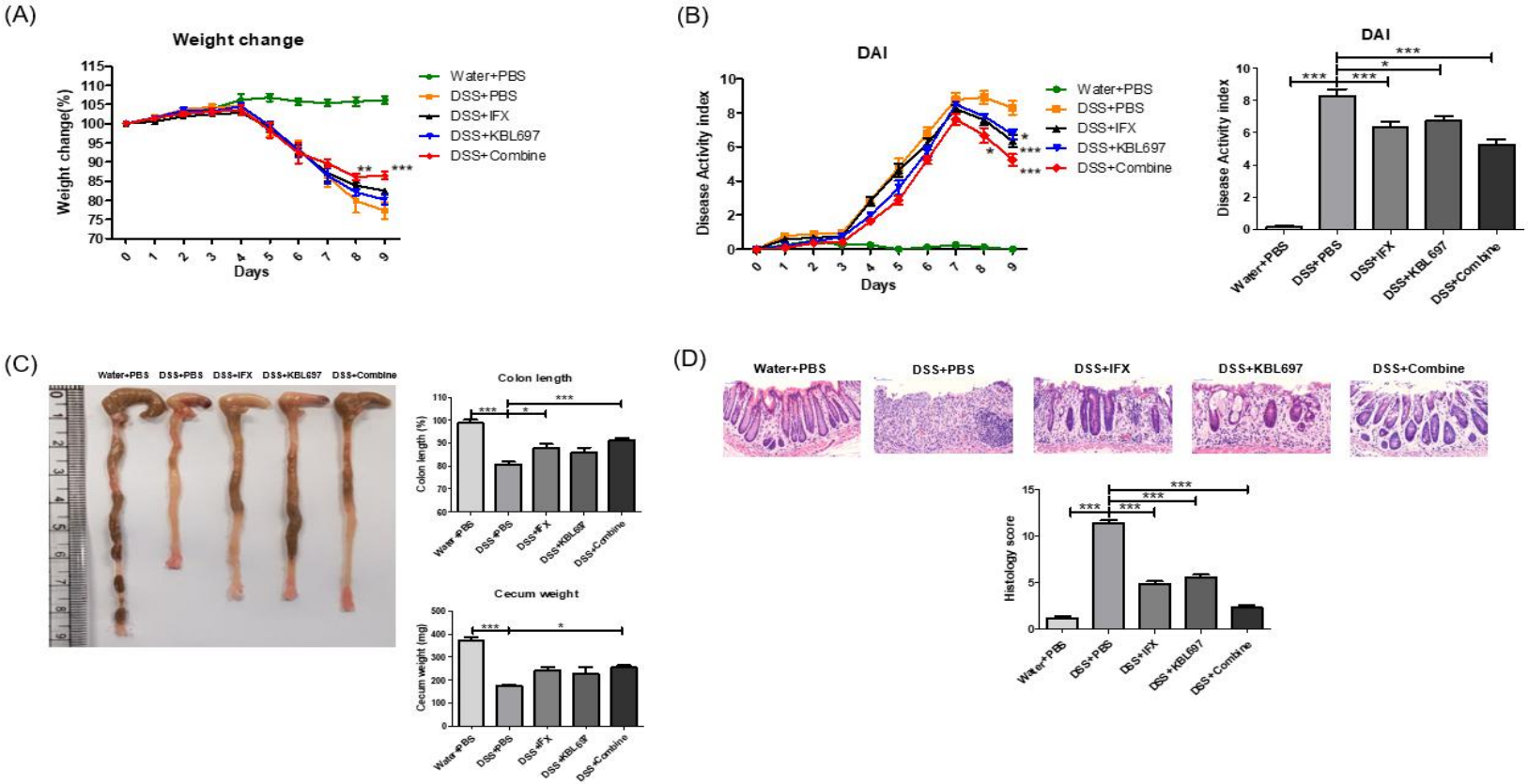

(A)
Weight change
Weight change(%)
Days
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(B)
DAI
Disease Activity index
Days
DAI
(C)
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
Colon length
Colon length (%)
Cecum weight
Cecum weight (mg)
(D)
Histology score

Figure 2. Combination treatment alleviated the symptoms of inflammatory bowel disease (IBD) in an *in vivo* dextran sodium sulfate (DSS)-induced colitis model

(A, B) The body weight and disease activity index (DAI) score were evaluated in DSS-induced colitis mice over the 9 day experimental period. (C) Relative colon length and cecum weight were measured after sacrifice on day 9. (D) The distal colonic tissue was stained with hematoxylin and eosin (H&E) to examine degree of inflammation and the histology score was measured. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4. Combination treatment downregulates miRNA level of inflammation-related gene in colon

To investigate the combine effects of *L. gasseri* KBL697 and IFX on the expression of miRNAs, we measured the miRNA level of miR-146, miR-150, miR-150 and miR-223. The expression level of miR-146, miR-150, miR-150 and miR-223 were significantly decreased in the DSS+Combine-treated mice compare to DSS+PBS-treated mice and tended to decrease more than single treatment. (Figure 3A-D).

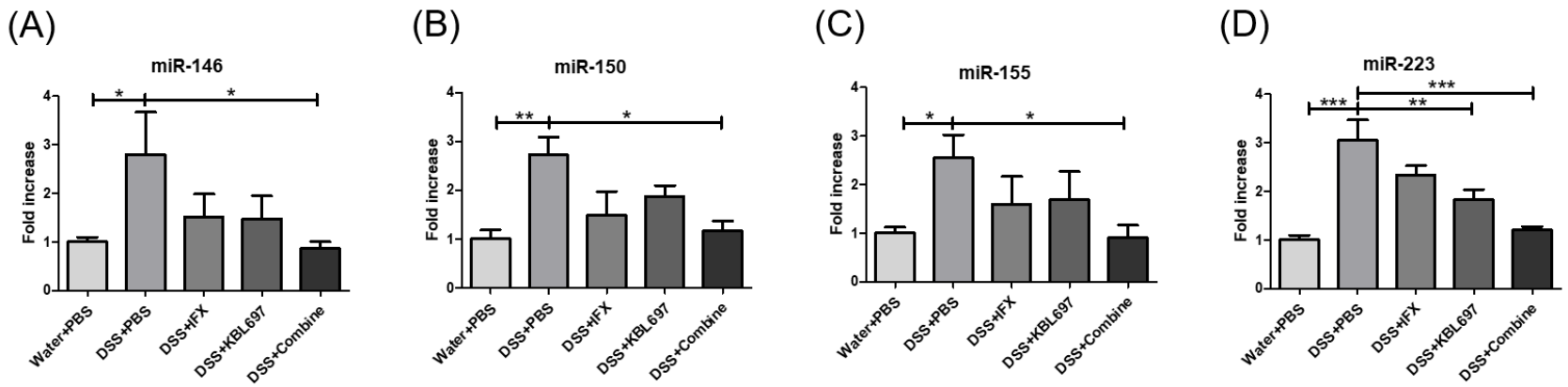


Figure 3. Effects of combination in DSS-induced colitis on miRNAs involved in the pathogenesis of intestinal inflammation

Expressions of (A) miR-146, (B) miR-150, (C) miR-155 and (D) miR-223 in the colonic tissue were quantified by real-time PCR. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

5. Combination treatment increases mRNA level of tight junction-related gene in colon

As the combination treatment was able to enhance intestinal barrier *in vitro*, we investigated if combination treatment induced similar chnages *in vivo* during colitis. DSS-induced colitis, on its own, significantly decrased the mRNA expression of ZO-1, ZO-2, claudin4 and MUC2. Compared to DSS+PBS-treated mice, ZO-1, ZO-2, claudin4 and MUC2 tends to increase slightly in the DSS+*L. gasseri* KBL697- or DSS+IFX-treated mice while there are significantly increase in the DSS+Combine-treated mice (Figure 4A-D).

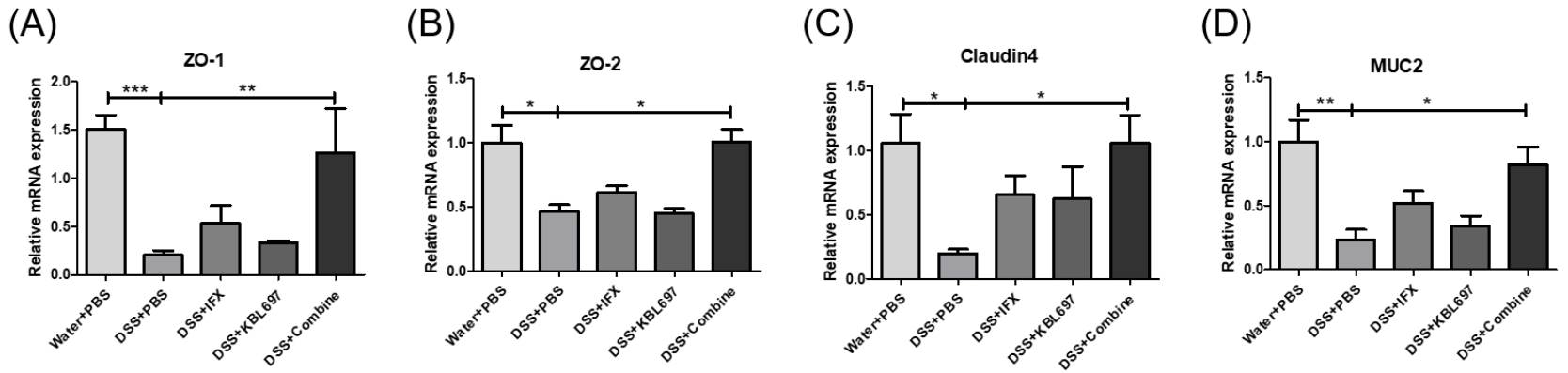


Figure 4. Increase of tight junction-related mRNA levels in the colon by combination

(A) ZO-1, (B) ZO-2, (C) claudin4 and (D) MUC2. Intestinal mRNA expression levels of tight junction-related genes in the colonic tissue were calculated with real-time PCR. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

6. Combination treatment reduces leukocyte recruitment in colon

After DSS treatment, DSS+PBS-treated mice showed significantly increase of chemokine CCL-2, CXCL1 and cytokine IL-1β and infiltration marker MPO compare to Water+PBS-treated mice. However, DSS+*L. gasseri* KBL697- or DSS+IFX-treated mice showed slighly reduce of these chemokines, cytokine and MPO than DSS+PBS-treated mice in the colonic tissue. Also, DSS+Combine-treated mice tended to decrease more than DSS+*L. gasseri* KBL697- or DSS+IFX-treated mice (Figure 5A-D).

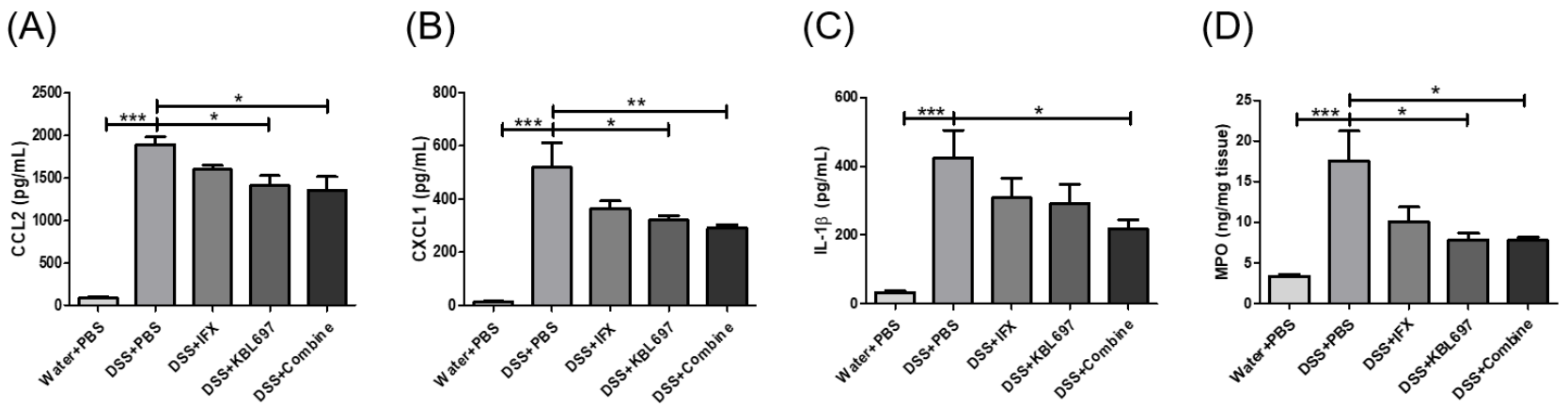


Figure 5. Regulation of innate immune response in the colonic tissue by combination.

(A) chemokine (C-C motif) ligand 2 (CCL2), (B) chemokine (C-X-C motif) ligand 1 (CXCL1), (C) IL-1β and (D) Myeloperoxidase (MPO) levels were measured by an ELISA. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

7. Combination treatment regulates protein level of cytokines in colon

Due to DSS-induced inflammation, DSS+PBS-treated mice showed significantly increase of pro-inflammatory cytokines such as TNF and inflammatory cytokines such as Th1-(IFN-γ and IL-2) and Th17-(IL-6 and IL-17A) while anti-inflammatory cytokine IL-10 was increased compare to Water+PBS-treated mice (Figure 6A-F). However, combination treatment reduced pro-inflammatory cytokines and inflammatory cytokines, but significantly increased anti-inflammatory cytokine than DSS+PBS-, DSS+*L. gasseri* KBL697- and DSS+IFX-treated mice in the colonic tissue. (Figure 6A-F). These data indicate that combination treatment has effect on the immunomodulation than single treatment.

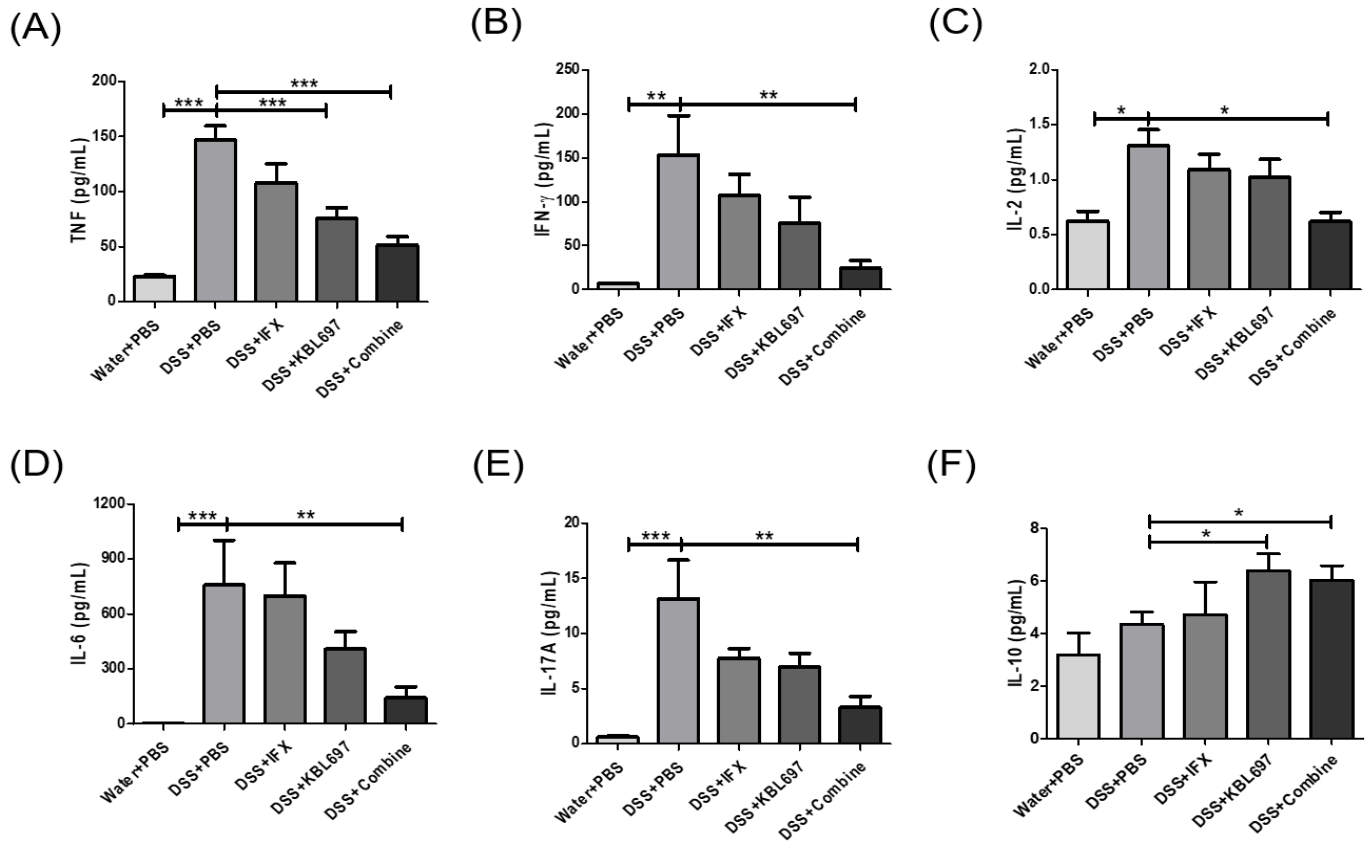

(A)
TNF (pg/mL)
200
150
100
50
0
***
***
***
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(B)
IFN-γ (pg/mL)
250
200
150
100
50
0
**
**
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(C)
IL-2 (pg/mL)
2.0
1.5
1.0
0.5
0.0
*
*
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(D)
IL-6 (pg/mL)
1200
900
600
300
0
***
**
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(E)
IL-17A (pg/mL)
20
15
10
5
0
***
**
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(F)
IL-10 (pg/mL)
8
6
4
2
0
*
*
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine

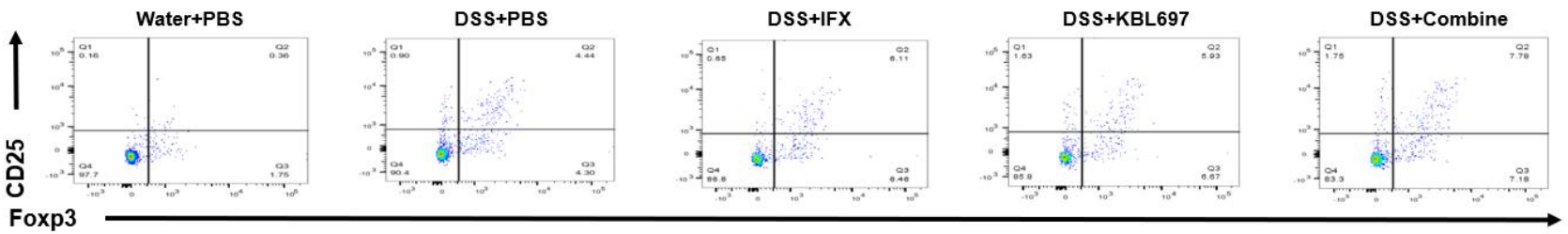
Figure 6. Downregulation of Th1 and Th17 cytokine levels in the colonic tissue by combination.

Concentrations of the Th17-type cytokines (A) tumor necrosis factor (TNF), (D) IL-6, (E) IL-17A, the Th1-type cytokine (B) IFN-γ, (C) IL-2 and the anti-inflammatory cytokine (F) IL-10 were measured by the BD Cytometric Bead Array Mouse Th1/ Th2/Th17 Cytokine Kit and ELISA kit. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

8. Combination treatment increases regulatory T cells population in MLN

In order to assess combine effect of combination, we investigate CD4+CD25+Foxp3+ T cell population in MLN and they were measured by fluorescence-activated cell sorting (FACS) in the DSS-induced colitis. After combination treatment, CD4+CD25+Foxp3+ T cells population were significantly increased than single treatment (Figure 7). This observation indicates that combination treatment has effect of inducing generation of CD4+CD25+Foxp3+ T cells population than single treatment in MLN with potentiated the suppressor function.

(A)


Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
CD25
Foxp3

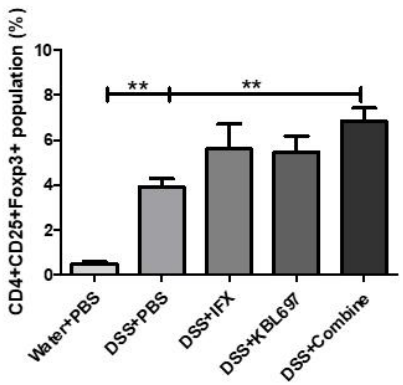

CD4+CD25+Foxp3+ population (%)
10
8
6
4
2
0
**
**
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine

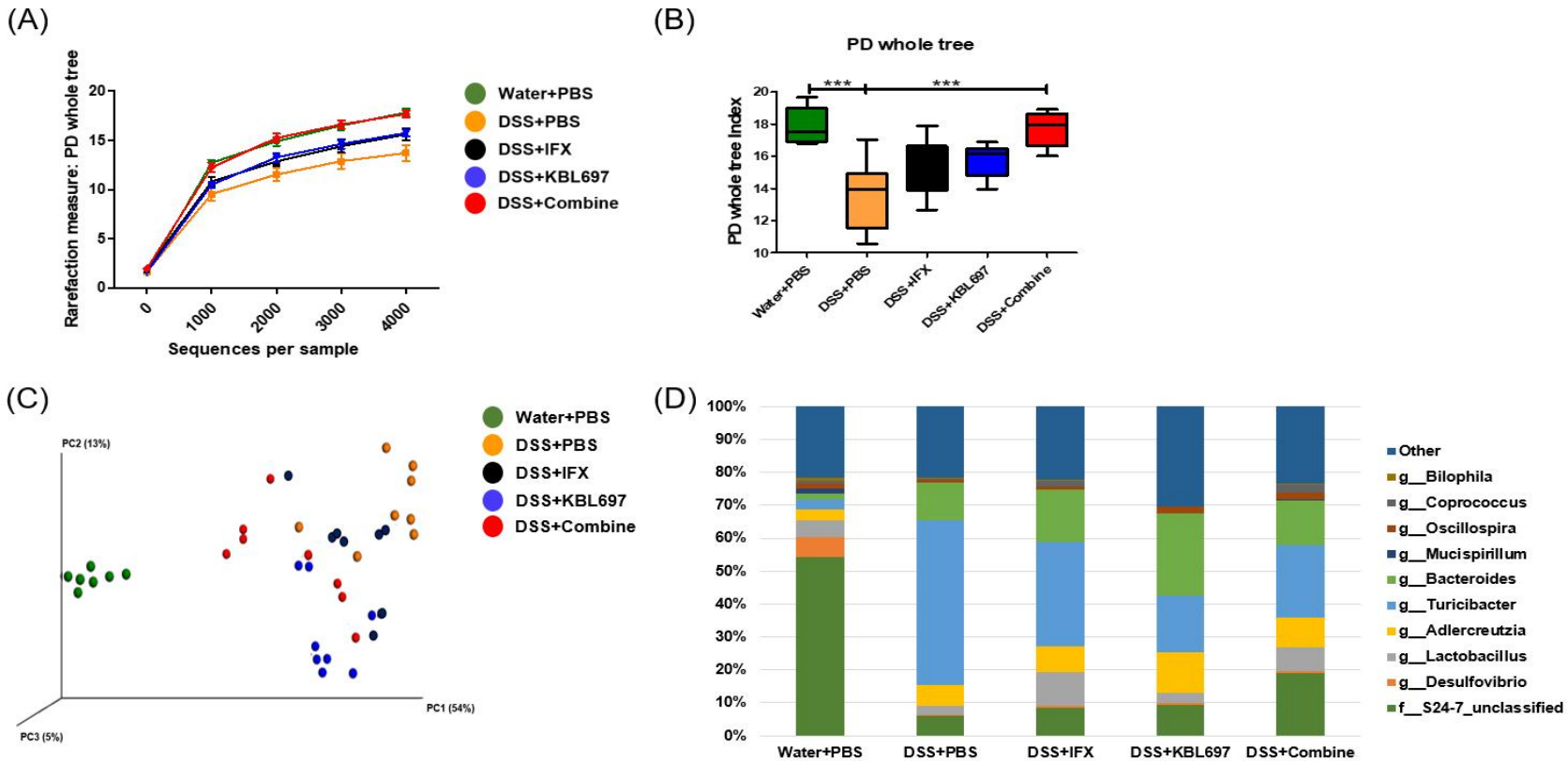
Figure 7. CD4+CD25+Foxp3+T cell population is increased in MLN from combination treatment.

By using MLN cells, surface CD3, CD4 and CD25, and intracellular Foxp3 staining were performed and then measured by FACS. (A) The numbers of CD4+CD25+Foxp3 T cells were calculated based on percentage of total MLN cell counts. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. **, $P < 0.01$.

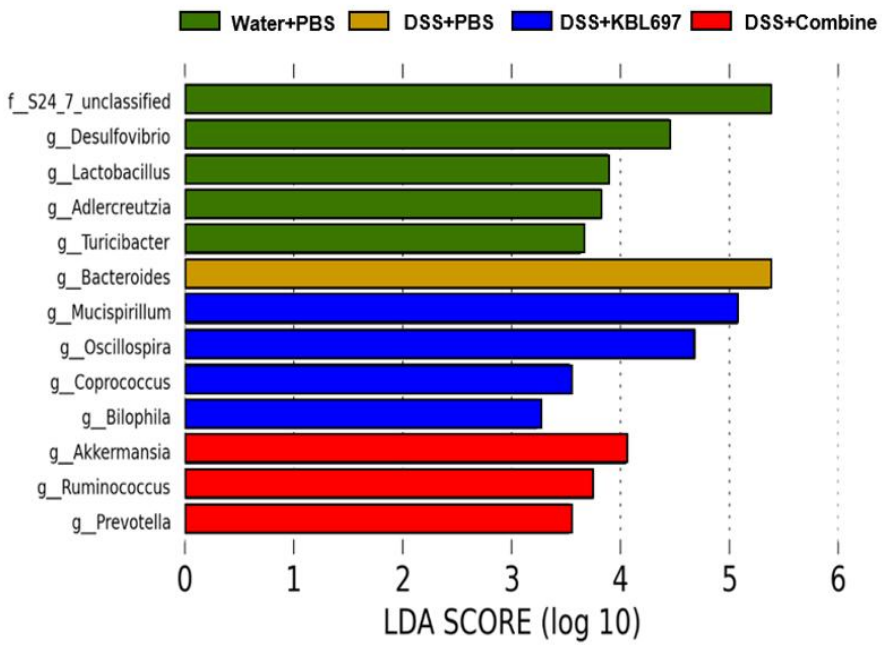
9. Combination treatment significantly changes the community structure of the gut microbiota

As the symptoms of DSS-induced colitis worsen, the microbial population was significantly decreased compare to Water+PBS-treated mice but increased in DSS+*L. gasseri* KBL697- or DSS+IFX-treated mice. In particular, the DSS+Combine-treated mice had more increased microbial population than DSS+*L. gasseri* KBL697- and DSS+IFX-treated mice (Figure 8A and 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 five groups (Figure 8C). In particular, the Principal coordinate analysis (PCoA) plots demonstrate that the microbiota in mice were significantly changed by DSS treatment, which was less severe when the mice were treated *L. gasseri* KBL697 or IFX. Also, the DSS+Combine-treated mice was less changed than DSS+*L. gasseri* KBL697- and DSS+IFX-treated mice. At genus level, remarkable microbiota in the Water+PBS-treated mice was *f_S24-7_unclassified* (54%) followed by *Desulfovibrio* (6%). Strikingly in the DSS+PBS-treated mice, the relative abundance of *f_S24-7_unclassified* (5.9%) was decreased and *Turicibacter* (49.7%) was rapidly increased

compared to the Water+PBS-treated mice. Interestingly, DSS+Combine-treated mice was increased *f_S24-7_unclassified* and decreased *Turicibacter* compared to the DSS+PBS-treated mice (Figure 8D). Next, we observed that five groups had abundant taxa at the genus level using the LEfSe algorithm (Figure 8E). In the DSS+PBS-treated mice, *Akkermansia*, *Ruminococcus* and *Prevotella* were slightly decreased compare to Water+PBS-treated mice, being significantly reversed after combination treatment. Relative abundance of *Bacteroides* was significantly increased in the DSS+PBS-treated mice compare to Water+PBS-treated mice while *L. gasseri* KBL697 or IFX treatment were suppressed compare to DSS+PBS-treated mice (Figure 8F).


(A)
Rarefaction measure: PD whole tree
Sequences per sample
0
1000
2000
3000
4000
0
5
10
15
20
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(B)
PD whole tree
PD whole tree Index
***
***
10
12
14
16
18
20
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(C)
PC2 (13%)
PC1 (54%)
PC3 (5%)
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
(D)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
Other
g__Bilophila
g__Coprococcus
g__Oscillospira
g__Mucispirillum
g__Bacteroides
g__Turicibacter
g__Adlercreutzia
g__Lactobacillus
g__Desulfovibrio
f__S24-7_unclassified

(E)


Water+PBS
DSS+PBS
DSS+KBL697
DSS+Combine
f__S24_7_unclassified
g__Desulfovibrio
g__Lactobacillus
g__Adlercreutzia
g__Turicibacter
g__Bacteroides
g__Mucispirillum
g__Oscillospira
g__Coprococcus
g__Bilophila
g__Akkermansia
g__Ruminococcus
g__Prevotella
0
1
2
3
4
5
6
LDA SCORE (log 10)

(F)

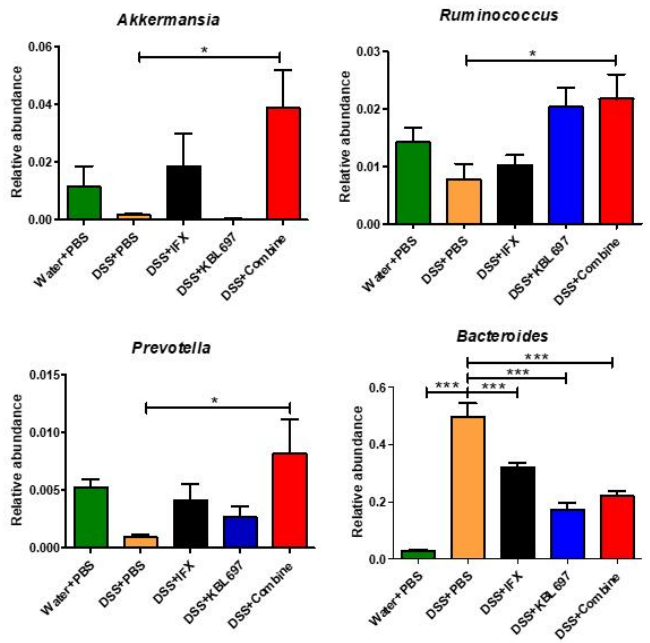

Akkermansia
Ruminococcus
Prevotella
Bacteroides
Relative abundance
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
*
***

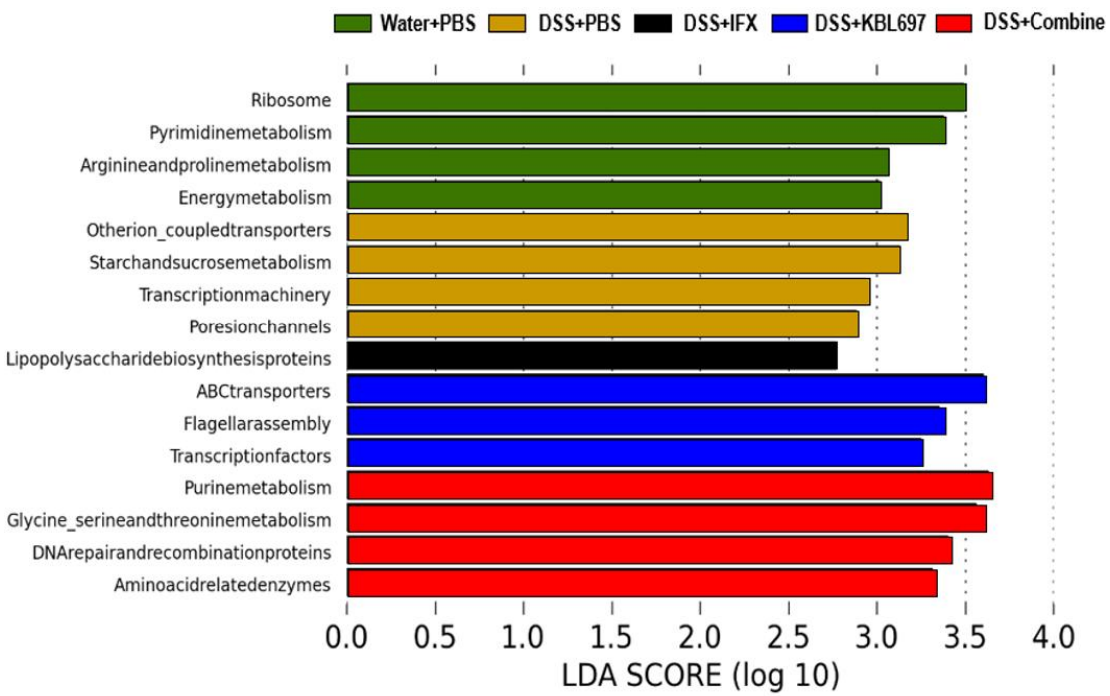
Figure 8. Effects of combination treatment on cecal microbiota in mice.

(A) A rarefaction plot measured using the PD whole tree diversity index. (B) The PD whole tree diversity index at 4,000 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 + KBL697 (Blue), and DSS + Combine (Red) treatment groups, as measured by LEfSe analysis (threshold > 2.5). (F) Comparison of the relative abundances of significantly different microbial taxa at the genus level. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; ***, $P < 0.001$.

10. Combination treatment changes in metabolic functions and SCFA levels

As the altering microbiota change metabolic function, we investigated metabolic pathway using PICRUSt which predicted KEGG orthology annotation. KEGG pathways showed differentially expressed genes. In Water+PBS-treated mice, significant changes in amino acid biosynthesis and energy metabolism were observed. However, DSS treatment led to a number of pathways including transporters and several other pathways. IFX treatment shows genes encoding lipopolysaccharide biosynthesis proteins. *L. gasseri* KBL697 treatment led to ABC transporter and flagellar assembly. Combination treatment led to increase several amino acid metabolism such as purine, glycine, serine and threonine (Figure 9A). Also, we examine SCFAs (Acetate, butyrate and propionate) to examine combine effect of the *L. gasseri* KBL697 and IFX. Compare to DSS+PBS-treated mice, acetate was significantly increased in the DSS+Combine-treated mice. Furthermore, compare to DSS+PBS-treated mice, butyrate was significantly increased in the DSS+Combine-treated mice while there were no significant in the DSS+*L. gasseri* KBL697- and DSS+IFX-treated mice (Figure 9B).

(A)


Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
Ribosome
Pyrimidinemetabolism
Arginineandprolinemetabolism
Energymetabolism
Otherion_coupledtransporters
Starchandsucrosemetabolism
Transcriptionmachinery
Poresionchannels
Lipopolysaccharidebiosynthesisproteins
ABCtransporters
Flagellarassembly
Transcriptionfactors
Purinemetabolism
Glycine_serineandthreoninemetabolism
DNArepairandrecombinationproteins
Aminoacidrelatedenzymes
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
LDA SCORE (log 10)

(B)

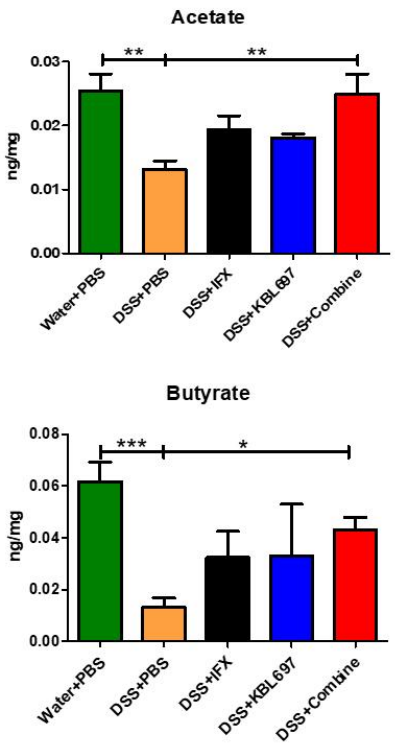

Acetate
**
**
0.03
0.02
0.01
0.00
ng/mg
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine
Butyrate
***
*
0.08
0.06
0.04
0.02
0.00
ng/mg
Water+PBS
DSS+PBS
DSS+IFX
DSS+KBL697
DSS+Combine

Figure 9. Functional alterations related to cecum microbiota by combination in mice.

(A) Profiles were suggested using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) and LEfSe analyses with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (threshold >2.5). (B) Analysis of short chain fatty acid (SCFA) levels by gas chromatography. The statistical analysis was performed using the one-way ANOVA followed by Tukey's test. The error bars represent standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IV. Discussion

We found that combination of IFX and *L. gasseri* KBL697 treatment had combine effects of immunomodulation based on *in vitro* and *in vivo* model. In *in vitro* BMDM model, *L. gasseri* KBL697 significantly reduced pro-inflammatory cytokine IL-1β and increased anti-inflammatory cytokine IL-10 compare to LPS-treated cell (Figure 1A). IL-1β plays key roles in acute and chronic inflammatory and IL-10 is correlated with various anti-inflammatory effects.[22] Through these results, we confirm that *L. gasseri* KBL697 has immunosuppressive effects.

IFX has been used repeatedly in mouse preclinical models with associated claims that anti-inflammatory effects due to neutralizing TNF-α.[8] In *in vitro* Caco-2 model, combination of IFX and *L. gasseri* KBL697 treatment increases TEER value than single treatment. It means that this combination has combine effect of increase TJ in Caco-2 monolayer and can be used to strength the gut barrier function.[23] Therefore, we expect that combination of IFX and *L. gasseri* KBL697 treatment has therapeutic effects in *in vivo* DSS-induced colitis mice by increasing TJ and immunomodulation.

Mice treated with 2% DSS for 5-7 days showed severe damage with crypt depletion and slow recovery of colonic epithelium associated with clinical features such as weight loss, diarrhea, and rectal bleeding.[24] In our experiment, combination treatment had protective effects on the clinical features such as weight loss and DAI score compare to single treatment (Figure 2). Also, the crypt organization and mucosal architecture of combination treatment group was significantly better structured than single treatment group (Figure 2D). Through these results, we found that combination treatment has a better therapeutic effects for colitis than single treatment. There may be several reasons why combination treatment is more effective than single treatment in relieving colitis symptoms.

First, our study confirmed miRNAs based on previous studies about DSS-induced colitis model.[25–27] The impact on colonic miRNA is associated with several biological process including cell differentiation, proliferation and immune system. Therefore, miRNA can be indicator of inflammation and TJ integrity.[28,29] For example, miR-146 has been reported that it promotes the Th17 and severe symptoms of colitis.[26] The expression of miR-150 and miR-155 is increased in inflammatory conditions and promote the inflammation through increasing immune

cells, including macrophages, as well as Th1 and Th17 cells.[25] Similarly, miR-223 expression is also increased in inflammatory condition involved in the modulation of the inflammasome complex, which is closely related to IL-1β production in immune cells.[27] In our study, combination treatment decreased these miRNAs than single treatment (Figure 3). Through these results we found that combination treatment successfully altered the mucosal immune response than single treatment by modulating the miRNAs level.

Additionally, TJ proteins is important factor for epithelial barrier function. For example, ZO-1 and ZO-2 proteins are intracellular TJ proteins which are play a role in linking the cell cytoskeleton to the transmembrane TJ proteins.[30] Claudin4 is transmembrane epithelial proteins which are important in intestinal barrier function.[30] Also, MUC2 is one of the components of mucus layer to maintain the protective capacities during DSS-induced colitis.[31] In the present study, DSS-induced colitis was also associated with a reduction in the expression of ZO-1, ZO-2, claudin4 and MUC2. However, combination treatment significantly increaed the expression of TJ proteins and MUC2 than single treatment (Figure 4). This effect could preserve the mucus-secreting layer and facilitate the restoration of the epithelial

barrier function and integrity, thus improving intestinal permeability and promoting colonic recovery, in consistency with previously described for IFX and probiotics.[10,32]

Furthermore, the combination treatment reduced the innate immune response. Changes in MPO levels reflect the degree of neutrophil infiltration and tissue damage in the colon.[10] IL-1β and CCL2, which are a key pro-inflammatory cytokine and chemokine, respectively, are primarily produced by monocytes and macrophages, driving intestinal inflammation.[11] CXCL1 is a chemokine with dual roles in recruiting and activating neutrophils.[10] Overall, combination treatment reduced levels of these inflammatory markers than single treatment (Figure 5).

Previous studies demonstrated that activation of resident innate immune cells leads to an adaptive immune response.[32] Indeed, it has been demonstrated that antigen-specific T cells develop during the acute stage of DSS-induced colitis.[33] Also, a previous study postulated that increased Th1 and Th17 cells and decreased Treg cells exacerbate the severity of DSS-induced colitis.[34,35] In our study, protein levels of Th1-, Th17-type cytokines were decreased and IL-10 was increased after combination treatment. Also, combination treatment made more

significant changes than single treatment (Figure 6). Combination treatment successfully regulated the activation of adaptive immune response than single treatment by modulating the levels of T cell-related cytokines.

In addition, combination treatment significantly increased CD4+CD25+Foxp3+ T cell populations than single treatment in the MLN of DSS-treated mice (Figure 8). Activated Treg cells produce inhibitory cytokines such as IL-10 that may help regulate Th17 cells to acquire a protective rather as a pathogenic phenotype, and it is increased in inflamed region to suppress disease progression.[35] Collectively, our data suggest that combination treatment significantly increase Treg cell to relieve DSS-induced colitis symptoms, while single treatment has slightly effect of Treg cell upregulation.

To further understand why this combine effects occur, we analyzed gut microbiota. In the previous studies, gut microbiota has a key role in the progression of IBD. Many studies have demonstrated that altering microbiota diversity and composition can drive inflammation in IBD patients.[36] Our results showed that DSS treatment changed the composition and decreased the diversity of gut microbiota while the combination treatment restored the composition and

decreased the diversity of gut microbiota than single treatment (Figure 9A-C). We next observed change of microbiota at genus level. Our results showed that DSS+IFX- and DSS+*L. gasseri* KBL697-treated mice were decreased *Turicibacter* abundance and increased *S24-7* compared to DSS+PBS-treated mice (Figure 9D). *Turicibacter* have been identified as a possible indicator of disease onset in the mouse model of colitis and decreased after beginning recovery.[37] Conversely, the abundance of *S24-7* is decreased from the onset of colitis.[10,11] Compare to DSS+PBS-treated mice, DSS+Combine-treated mice was increased *Akkermansia*, *Ruminococcus* and *Prevotella* abundance than DSS+IFX- and DSS+*L. gasseri* KBL697-treated mice (Figure 9E-F). *Akkermansia*, *Prevotella* and *Ruminococcus* reduced inflammation by anti-inflammatory activity, inducing Foxp3 regulatory T cells (Tregs) and increasing IL-10 for homeostasis of the gut mucosa and barrier function.[38,39,40] Through these effect, we demonstrated that combination treatment has anti-inflammatory effect than single treatment by increasing specific bacteria inducing Treg.

In previous studies, altering gut microbiota leads to metabolite changes that impact IBD pathogenesis.[36] We found that combination treatment was associated with the metabolism such as purine and

glycine metabolism which play an important role in ribonucleic acids derivation and cellular metabolism (Figure 9A).[41] However, the specific functions of each microbial taxa in the gut microbiota need further evaluation. Furthermore, SCFAs such as acetate and butyrate play critical roles in the prevention of colitis.[42] Acetate have been reported to play a regulatory role in epithelial cell differentiation, stimulation of Treg as well as amelioration of mucosal inflammation in these intestinal conditions. Also, it is well known that butyrate exerts beneficial effect in colitis as it is the major energy source for the epithelium, induces the expansion of Tregs.[43] In this study, we found that combination treatment significantly increased acetate and butyrate level than single treatment (Figure 9B). Therefore, we suggest that combination treatment has effect of producing primary metabolic end products than single treatment, regulating immune responses and altering gut microbiota.

In conclusion, combination treatment has therapeutic effect of colitis than single treatment by regulating immune responses and altering gut microbiota, leading to ameliorate DSS-induced colitis. This finding suggests that patients suffering from IBD could potentially benefit from combination of IFX and *L. gasseri* KBL697 treatment.

VII. References

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Functional Changes in Gut Microbiota Leading to Improvement of Atopic Dermatitis. Front Mol Biosci. 2019; 6:1–12.

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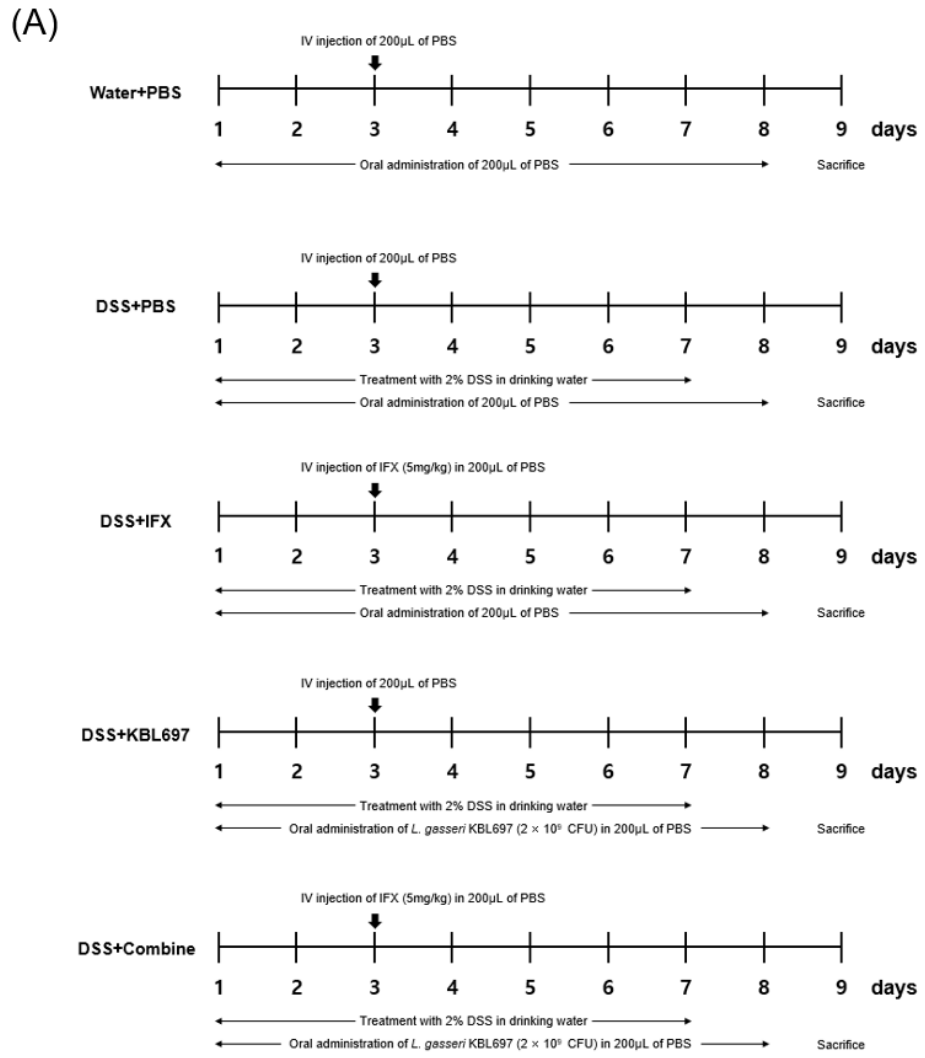

Supplementary figure 1. The overall experimental procedure of an *in vivo* dextran sodium sulfate (DSS)-induced colitis model

(A) Experimental design of *in vivo* DSS-induced colitis model.

국문초록

락토바실러스 가세리 균주와 인플릭시맵 병행처리의 염증성 장 질환 완화 효과

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락토바실러스 가세리 KBL697과 인플릭시맵은 BMDM, Caco-2 그리고 마우스 모델에서 대장염 증상 완화의 병행 효과가 있는 것을 발견했다. BMDM에 KBL697을 처리했을 때 염증성 사이토카인은 감소하고 염증 억제 사이토카인은 증가했다. 또한, Caco-2에 KBL697과 인플릭시맵을 병행처리 했을 때 밀착 연접이 단독처리 했을 때보다 강화되었다.

그 뿐만 아니라, DSS 대장염 마우스 모델에서 DAI, 장 길이, 조직학 검사를 통해 인플릭시맵과 KBL697의 병행처리가 단독처리보다 질병 완화에 좋은 효과를 있음을 증명했다. 병행처리는 단독처리보다 염증을 유도하는 miRNA를 감소시키고 선천 면역 반응을 조절하며 장벽 손상을 막았다. 게다가 장 조직에서 사이토카인을 분석한 결과 병행처리가 단독처리보다 Th1-, Th17- 타입의 사이토카인을 감소시키고 염증 억제 사이토카인 IL-10은 증가시켰으며, 장간막 림프샘에서 조절 T 세포의 비율을 증가시켰다.

병행처리는 면역반응 뿐만 아니라 장내 균 총에도 변화를 일으켰다. 병행처리는 단독처리한 그룹보다 장내 균의 다양

성이 증가하였고 장내 균의 군집 구조가 정상화된다는 것이 증명되었다. 그리고 대사체 분석 결과 병행처리는 단독처리보다 아세트산, 뷰티르산과 같은 짧은 사슬 지방산의 생성을 유도한다는 것을 밝혔다.

결론적으로 본 연구를 통해 락토바실러스 가세리 KBL697과 인플릭시맵의 병행처리가 단독처리보다 면역반응을 억제하고 장내 균총을 정상화함으로써 대장염을 완화하는 것을 발견했다. 이러한 발견은 락토바실러스 가세리 KBL697과 인플릭시맵의 병행처리가 대장염에 대한 새로운 치료방법이 될 수 있음을 시사한다.

주요 단어: 락토바실러스 가세리, 인플릭시맵, 대장염, 사이토카인, 대사체, 마이크로비옴

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