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ARTICLE

The Synergic Anti-inflammatory Impact of *Gleditsia sinensis* Lam. and *Lactobacillus brevis* KY21 on Intestinal Epithelial Cells in a DSS-induced Colitis Model

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Abstract

We investigated the synergic anti-inflammatory activity of *Gleditsia sinensis* Lam. (GS) extract and *Lactobacillus brevis* KY21 both *in vitro* and *in vivo*. Western blot analysis and immunostaining showed that AKT phosphorylation that increased by the exposure of LPS were significantly decreased by the presence of either GS extract or *L. brevis* KY21. In addition, p65 intracellular transport was critically inhibited by GS extract and *L. brevis* KY21. We further studied these effects using an *in vivo* dextran sulfate sodium (DSS)-induced mouse model. Body weight, food intake, and clinical scores were dramatically decreased after treatment with DSS, whereas these effects were palliated by the addition of GS extract and *L. brevis* KY21. Importantly, transcription of genes encoding pro-inflammatory cytokines including IL-1β, TNF-α, and IFN-γ in mesenteric lymph nodes (MLN) and the spleen were increased by DSS treatment, whereas they were inhibited by the presence of GS extract and *L. brevis* KY21.

Keywords: anti-inflammatory activity, herbal extract, probiotic bacteria, DSS

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Introduction

Inflammatory bowel disease (IBD) which includes Crohn's disease and ulcerative colitis (UC), are a group of chronic diseases that result from a confluence of genetic and environmental factors (Podolsky, 2005). Although current reviews have summarized some of the evidence regarding the mechanisms behind IBD, these diseases are not yet fully understood.

The WHO defines probiotic bacteria as live organisms which provide a benefit to the host when provided in adequate quantities (FAO/WHO, 2002). The human gastrointestinal immune system could potentially be regulated by intestinal microbiota, and probiotics are currently being evaluated as an alternative means of manipulating this microbiota in patients with chronic inflammation (Madsen *et al.*, 2001). Probiotics have demonstrated their efficacy for a number of inflammatory conditions including arthritis (Bedaiwi and Inman, 2014). Importantly, probiotics have also been associated with the treatment of ulcerative colitis (UC) and Crohn's disease (CD) (Fujimori *et al.*, 2007).

In addition, it was well-established that several herbal compounds have been used to prevent and treat immune-associated disease in Asian countries. Among them, Astragali Radix (AR; Astragalus) appears to exert immunomodulatory effects by regulating the expression of cytokines such as IL-1α IL-1β, IL-6, inducible nitric oxide synthase (iNOS), and the production of nitric oxide (NO) (Lee *et al.*, 2005). Also, *Gleditsia sinensis* Lam. has been used in traditional medicine for treatment of swelling, carbuncles, and other skin problems (Chow *et al.*, 2003). The biological activity of these chemical compounds has been shown to be anti-mutagenic, anti-HIV, and anti-tumor (Li *et al.*, 2007; Yi *et al.*, 2015). The mechanism behind the anti-inflammatory effects of *G. sinensis*, however, remains unclear.

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Inflammation is a protective response triggered by infection or tissue injury that is controlled by pro-inflammatory mediators (Bamias *et al.*, 2012). Intestinal epithelial cells (IECs) in particular secrete mediators that are involved in immune responses to potentially pathogenic organisms, including antibacterial peptides such as defensins (Takahashi *et al.*, 2001). Although a number of signaling molecules including NF-κB (commonly consists of the p50 and p65 subunits) and AKT are involved in inflammation, the specific mode of action is currently still unclear. Equally importantly, several *in vivo* animal models of IBD have been designed, and amongst them the dextran sulfate sodium (DSS)-induced colitis model appears to represent the most accurate model of IBD since it mimics human IBD-like symptoms (Okayasu *et al.*, 1990).

The aim of this study was to evaluate the anti-inflammatory effects of probiotic strains and herbal extracts by measuring NF-κB translocation and AKT phosphorylation levels in LPS stimulated human intestinal epithelial HT-29 cells. In addition, we evaluated the anti-inflammatory effects of probiotics and natural compounds on DSS-induced chronic colitis in mice.

Materials and Methods

Gleditsia sinensis *Lam*

Fresh *Gleditsia sinensis* Lam. (GS) was purchased from a local herb mart, the Kyung-Dong market (Korea). The methanol GS extracts were kindly provided by the Natural Product and Metabolomics Laboratory at Korea University. These extracts were dissolved in dimethyl sulfoxide (DMSO) for cell treatment.

Bacteria and cell culture

Lactobacillus brevis KY21 (isolated from Korean infant feces), *L. salivarius* E4191 (isolated from Egyptian infant feces), and *Leuconostoc mesenteroides subsp. mesenterides* KDK411 (isolated from Kimchi) used in this study were generated in the Sae Hun Kim group (Food Microbiology Laboratory at Korea University, Korea). The strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth (Difco, USA) at 37ºC for 18 h. The stock cultures were maintained at -80ºC, using 50% glycerol as a cryoprotectant. The strains were sub-cultured three times prior to use.

The human epithelial cell line, HT-29, was obtained from the Korea Cell Line Bank (KCLB, Korea). The HT-29 cells were maintained at 37ºC with 5% CO_2 in RPMI 1640 medium (HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin (P-S; HyClone). The HT-29 cells were seeded on a six-well plate (Palcon, USA) at a density of 1×10^6 cells/well for western blot and confocal analyses. The HT-29 cells were pre-treated with 100 μg/mL of GS extract or 10^9 CFU/mL probiotic bacteria for 2 h and cultured with 1 μg/mL of LPS (*Escherichia coli* strain, 0111:B4, Sigma, USA).

Western blot analysis

The HT-29 cells were harvested, washed with PBS, and treated with cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100) added to 200 μg phenylmethylsulfonyl fluoride/mL, phosphatase inhibitor cocktail (Sigma), and protease inhibitor cocktail (EMD Chemicals. Inc., USA) at 4ºC for 1 h. Lysates were loaded on 10% polyacrylamide and transferred to an immobilon P membrane (Millipore Corporation, USA) and blocked with TBST including 5% skimmed milk (for total AKT) or TBST alone (for pAKT) at room temperature for 1 h. Afterwards, the membranes were incubated with purified total AKT antibody (Cell signaling, USA), pAKT antibody (#9611, Cell Signaling, USA), and then HRP-conjugated anti-mouse or anti-rabbit IgG (Amershamm, USA). Proteins were detected by the super signal Western blotting system and immunoreactive bands were visualized with an ECL system. The β-actin were used as internal control. The gel images were quantified using NIH Image J program (NIH http://rsb.info.nih.gov/ij/) and pAKT activity was determined by the ratio of pAKT/total AKT.

Confocal microscopy

HT-29 cells were seeded onto round coverslips and pretreated with GS extract (100 mg/mL) or probiotic bacteria (10^9 CFU/mL) for 2 h followed by a PBS wash to remove the unbound bacteria. Cultured HT-29 cells were stimulated by 1 μg/mL of LPS. After 24 h incubation, HT-29 cells were fixed in 4% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 30 min. The cell were permeabilized with 0.2% (w/v) Triton X-100 in PBS for 20 min. And then cells were washed three times with PBS and shaken while incubated with blocking solution (3% bovine serum albumin in PBS) for 1 h at room temperature. The cells were next incubated with primary antibodies to NF-κB p65 (Santa Cruz Biotechnology, 1:200) for 24 h, followed by three PBS washes to remove unbound primary antibody. This was followed by incubation with Alexa fluor 592 conjugated anti-rabbit IgG for 1 h at room temperature, and subsequent staining with 100 ng/mL

DAPI in PBS for 30 min. Finally, confocal images were obtained using a LSM5 EXCITER (carl-Zeiss, Germany).

Assessment of inflammation in DSS-induced colitis

Eight-week-old female BALB/c mice were purchased from SamTaKo (Daejeon, Korea). They were acclimatized for one week before the start of the experiment, and were housed individually in a room maintained at 22°C under a 12 h day/night cycle throughout the study. The mice were divided into six groups as follows and administered using oral gavage: (i) mice fed 4% DSS (molecular weight 5000; Wako Pure Chemical Industries, Ltd, Japan) mixed with water and DSS water *ad libitum*; (ii) mice fed 4% DSS plus 1 g GS extract/kg; (iii) mice fed DSS plus 10^9 CFU of *L. brevis*/kg in PBS; (iv) mice fed 50 mg sulfasalazine/kg (Sigma; positive control); (v) mice fed 4% DSS plus a combination of GS extract and *L. brevis* KY21; and (vi) mice fed PBS only (negative control).

A daily clinical assessment of DSS-induced colitis was performed, including measurement of food intake, body weight, rectal bleeding, an evaluation of stool condition, and the presence of blood in the stools. The mice were sacrificed on day 14 following the initiation of DSS treatment, and the length and weight of the colon were measured.

Histological evaluation

A histological examination was performed on samples of the distal colon from each animal. The samples were fixed in 10% buffered formalin, dehydrated in ethanol, and then embedded in paraffin. Four micron-thick sections were then prepared and stained with hematoxylin and eosin (H/E). All histological evaluations were performed in a blinded fashion using a validated scoring system (Dieleman *et al.*, 1998). In briefly, the sections were graded with a range from 0 to 3 for total inflammation and 0 to 4 for extent of crypt damage.

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from the mesenteric lymph nodes (MLN) and spleens of the mice using Trizole^{TM} reagent (Invitrogen, USA) according to the manufacturer's instructions, and was further purified using acidic phenol/chloroform extraction. cDNA was then generated using SuperScript III reverse transcriptase (Invitrogen). After cDNA synthesis, PCR was performed as follows: denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 35 cycles. The primer sequences used for RT-PCR are listed in Table 1. RT-PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide (EtBr) staining. All results were finalized after correction with GAPDH expression.

Statistical analysis

Differences between groups in each experiment were determined using Student's *t*-tests or ANOVA, followed by Duncan's test in SAS software package (ver. 9.1; SAS Inc., USA). Each result is representative of at least three independent biological replicates. A *p*-value of <0.05 in all replicate experiments was considered to be statistically significant.

Results and Discussion

Anti-inflammatory effects of GS extract and *L. brevis* KY21 on IECs

Inhibition of AKT phosphorylation in HT-29 cells

It has been well-established that pro-inflammatory cytokines including IL-8, a member of the CXC family of chemokines, are potent chemo-attractant that activates neutrophils (Baggiolini *et al.*, 1989). Especially, IL-8 is produced in response to stimulation by TNF-α (Abreu-Martin *et al.*, 1995). Inhibition of these two pathways resulted in a blockage of TNF-α-inducible IL-8 produc-

Table 1. Oligonucleotides used in this study. f indicates forward primers and r indicates reverse primers

Gene name	GenBank Accession No.	Sequence	Size (bp)
IL-1β	NM_008361.3	f: 5'-CCAGGATGAGGACATGAGCACC-3' r: 5'-ATCCACACTCTCCAGCTGCAGG-3'	358
TNF-α	NM_013693.2	f: 5'-GACGTGGAACTGGCAGAAGAGG-3' r: 5'-TGACGGCAGAGAGGAGGTTGAC-3'	502
IFN-γ	NM_008337.2	f: 5'-CTGAGACAATGAACGCTACACACTGC-3' r: 5'-AACAGCTGGTGGACCACTCGGAT-3'	433
GAPDH	NM_0020463	f: 5'-ATGACCACAGTCCATGCCATC-3' r: 5'-CCTGCTTCACCACCTTCTTG-3'	271

tion and also induced apoptosis (Osawa *et al.*, 2002). Importantly, TNF-α-induced IL-8 production was critically correlated with activation of the PI3K/AKT pathways that leading to cell survivals and immune response. Thus, we determined on the regulation of total AKT activity in the presence of GS extract or probiotic strains GS, KY21, KDK414, and E4191 using western blotting. The β-actin was employed as control. Unexpectedly, Western blot results showed that there was no significant difference on the total AKT activity among LPS treatment or GS extract/probiotic strains (ranged from 0.9 to 1.1 with the ration of pAKT/total AKT in all treatment; Fig. 1A).

Next, to determine the phosphorylation of AKT (pAKT), the cells were immunoblotted with phosphorylated AKT antibodies. As reported in Fig. 1A, the phosphorylation level of AKT was decreased by 4-folds when they exposed to 100 mg/mL of GS extract. However, the probiotic strains KDK414 and E4191 did not have any effect on AKT phosphorylation with the exception of *L. brevis* KY 21 (3-folds). These results show that GS and *L. brevis* KY 21 directly affect the regulation of pAKT, and these features can influence on the cascade response in inflammatory associated cytokine networks.

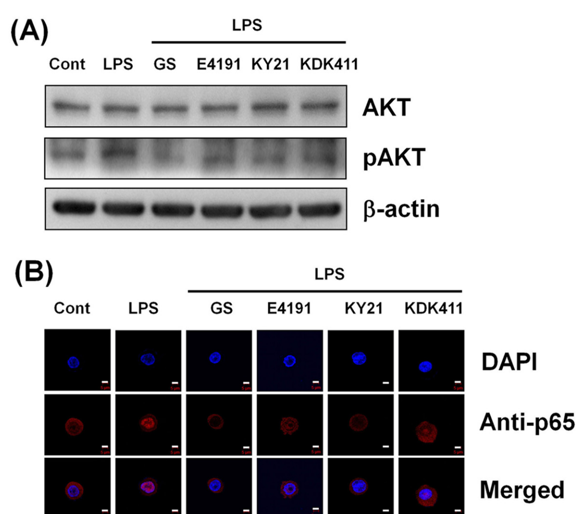

Fig. 1. Effects of GS extract and LAB strains on LPS-induced phosphorylation of AKT (A) and translocation of nuclear factor (NF)-κB p65 to the nucleus (B). HT-29 cells were pre-treated with either GS extract (100 μg/mL) or probiotic strains E4191, KY21, or KDK411 (10^9 CFU/mL) for 2 h, and then stimulated with LPS (1 μg/mL, 24 h). The level of AKT phosphorylation (pAKT) was decreased by GS extract in HT-29 cells assayed by Western blot. For p65 assay, HT-29 cells were pre-treated with either GS extract (100 μg/mL) or probiotic strains E4191, KY21, or KDK411 (10^9 CFU/mL) for 2 h, and then stimulated with 1 μg/mL LPS for 24 h. Immunofluorescence 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Bar scale is 5 mm. The result is representative of at least three independent biological replicates.

Inhibition of NF-κB translocation in HT-29 cells

In investigating the effects of anti-inflammatory agents on HT-29 cells stimulated by LPS, it is important to note that NF-κB exists in the cytoplasm, primarily as the p50 and p65 subunits and the IκBα inhibitory protein

Anti-inflammatory effects of GS extract and *L. brevis* KY21 on a DSS-induced colitis model

Body weight and food intake

As shown in Fig 2A, after the initiation of DSS-induced colitis, body weight was significantly lower in the DSS group compared with the DSS plus either 1 g GS extract/kg or 10^9 CFU *L. brevis* KY21/kg or combination of GS extract and *L. brevis* KY21 (GS+ KY21), and the 50 mg/kg sulfasalazine (Sigma)-treated mice. In addition food intake was significantly lower in DSS-treated mice than in DSS plus GS extract, 10^9 CFU of *L. brevis* KY21/kg, and 50 mg sulfasalazine/kg (positive control)-treated mice on day 5 after the initiation of DSS treatment (Fig. 2B). Importantly, the combination of GS extract and *L. brevis* KY21 was most effective in alleviating weight loss and decreased food intake. This suggests that GS and *L. brevis* KY21 administration may help to prevent severe weight loss in cases of DSS-induced colitis.

Observation of intestinal injury

Consistent with previous reports, oral administration of 4% DSS in drinking water to WT BALB/c mice induced moderate colitis, which was first manifest by diarrhea followed by bloody stools. A common feature of the DSS-induced colitis model is a significant decrease in colon length (Rumi *et al.*, 2004). The length of the colon in DSS with GS, *L. brevis* KY21, GS+KY21, and sulfasalazine-treated mice was compared to that of DSS plus PBS (Fig. 2C and 2D). The percentage of mice with increased colon length in the DSS with GS group (24%), KY21 group (18%), GS+KY21 group (27%), and sulfasalazine group (34%) were significantly increased compared with DSS alone group. The weight of the colon was evaluated in proportion to colon length. The colonic weight/length ratio was higher in the DSS-treated mice compared with those treated with GS extract, KY21, GS+KY21, and sulfasalazine (data not shown). This ratio is a possible marker of tissue edema (Mizoguchi and Mizoguchi, 2008). In addition, mice supplemented with either GS extract or *L.*

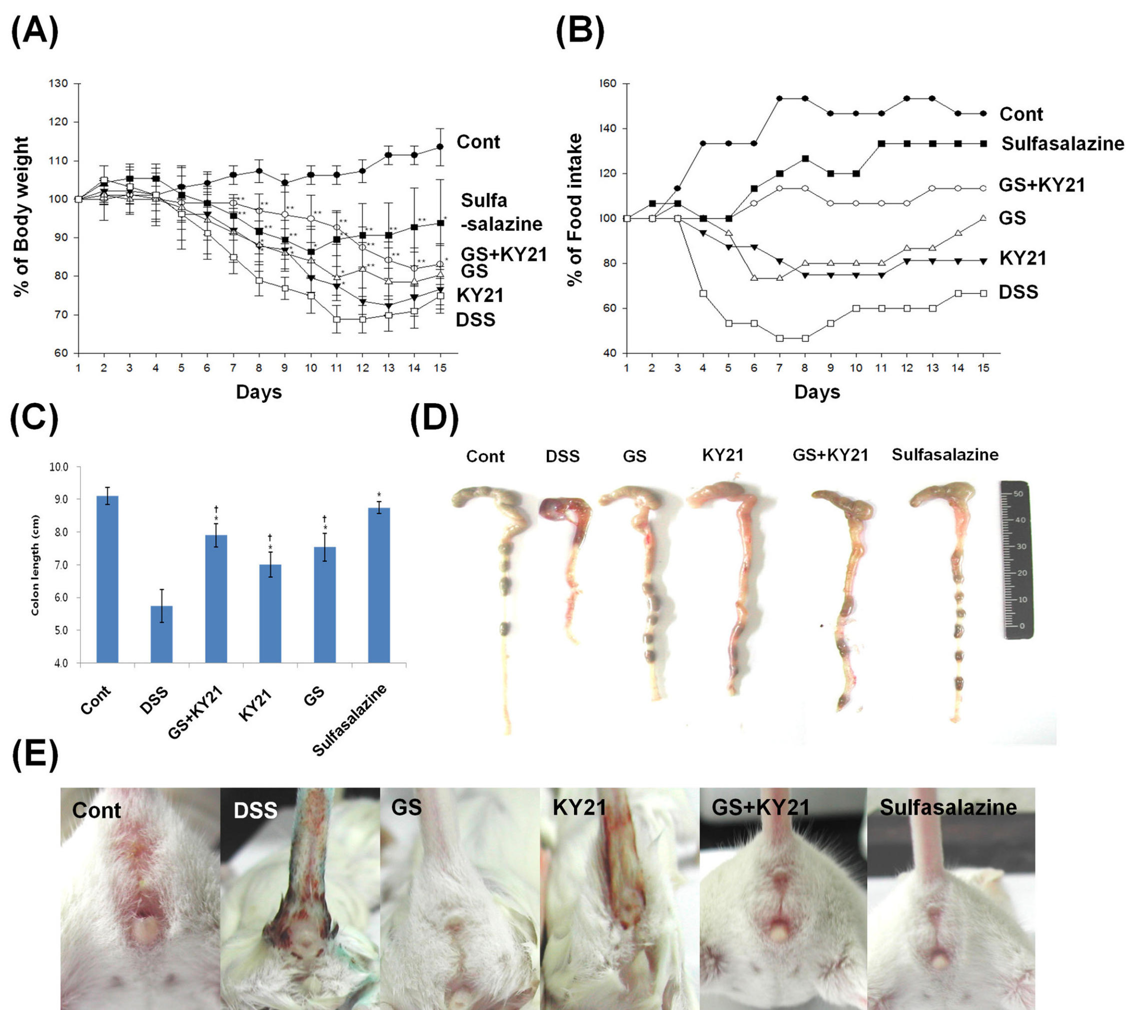

Fig. 2. Effects of GS extract and *L. brevis* KY21 on body weight (A), food intake (B), colon length (C and D) and rectal bleeding (E) in DSS-induced mice. The mice were fed 4% DSS (mixed with water; *ad libitum*), 4% DSS plus GS extract (1 g/kg), 4% DSS plus 10^9 CFU/kg *L. brevis* KY21, 4% DSS plus combination of GS extract and *L. brevis* KY21, or 4% DSS plus 50 mg kg^{-1} sulfasalazine (SS; Sigma, USA) during day 14. The weight of each individual mouse was then followed daily. In addition, colon length was determined on day 14 after the start of DSS treatment. The data represent means±SEM. *$p<0.05$ versus the DSS group, **$p<0.01$ versus the DSS group, and $^{\dagger}p<0.05$ versus the untreated group (n=5 mice/group).

brevis KY21 showed significantly lower susceptibility to DSS-induced colitis than control mice, which was manifest by severe intestinal bleeding (Fig. 2E). Consistent with body weight and food intake, a synergistic impact of GS extract and *L. brevis* KY21 was observed with respect to protection from intestinal injury. Our findings demonstrate that treatment with GS extract and *L. brevis* KY21 resulted in significant protection against intestinal injury in cases of DSS-induced colitis.

Histological analysis and scores for intestinal mucosal wounds

A 10 mm colonic tissue sample was used for the histological assessment. Inflammation and crypt damage on H&E-stained sections were assessed using the standard method previously described (Dieleman *et al.*, 1998). Damage to the colonic mucosa is shown by increased histological severity scores and decreased crypt area (Howarth *et al.*, 1998). In the healthy control group, the crypt structure in the mucosal layer was regular, and the thickness of the submucosal and muscular layers was normal. In the UC control group, the crypt structure was irregular because of cryptitis and crypt disappearance, complete crypt loss, destruction of epithelial cells, and severe inflammatory cell infiltration, though the GS extract and GS+KY 21-treated groups did not exhibit these features (Fig. 3A). This result suggests that combined supplementation with GS extract and *L. brevis* KY21 notably prevented epithe-

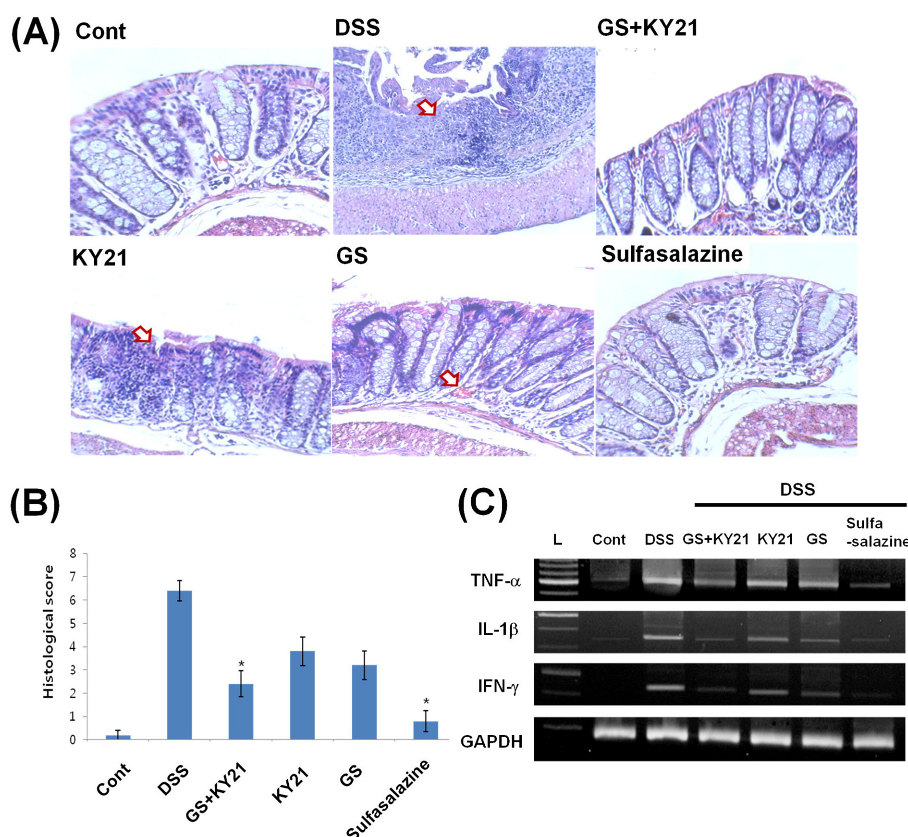

Fig. 3. Histological staining (A), scores (B), and mRNA expression of cytokine-encoded genes in mesenteric lymph nodes and the spleens using RT-PCR (C) in cases of DSS-induced colitis supplemented with GS extract, *L. brevis* KY21, and sulfasalazine. The mice were fed 4% DSS (mixed with water; *ad libitum*), 4% DSS plus GS extract (1 g/kg), 4% DSS plus 10^9 CFU/kg *L. brevis* KY21, 4% DSS plus combination of GS extract and *L. brevis* KY21, or 4% DSS plus 50 mg kg^{-1} sulfasalazine (SS; Sigma, USA) during day 14. The colons were excised on day 14 after DSS treatment was initiated and stained with hematoxylin and eosin (H/E). Magnification × 200. The total histological scores were derived from the severity of total inflammation and extent of crypt damage. The data represent means±SEM. *p<0.05 versus the DSS group. L: DNA ladder marker (Invitrogen).

lial damage by the exposure of DSS.

mRNA expression of cytokine encoded genes in mesenteric lymph nodes and the spleen

The mesenteric lymph nodes and spleen were used to evaluate the presence of TNF-α, IFN-γ, and IL-1β. These pro-inflammatory cytokines play an important role in the killing of tumor cells (Mantovani *et al.*, 1992), and were increased by treatment with DSS (Fig. 3C). Cytokines induced by LAB are shown to play key roles in immune-regulation. Several studies have reported that some specific strains of lactobacilli can induce pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-12, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) as well as anti-inflammatory cytokines such as IL-10 and transforming growth factor β (Christensen *et al.*, 2002). Sulfasalazine blocks the phosphorylation of the inhibitor kB (IkB) (Weber *et al.*, 2000). Similarly, sulfasalazine inhibited all of these cytokines, whereas GS and *L. brevis* KY21 significantly inhibited the expression of TNF-α, IFN-γ, and IL-1β. Interestingly, the combination of GS extract and *L. brevis* KY21 was most effective in inhibiting the transcription of pro-inflammatory cytokines genes. This result demonstrated that ingestion of GS extract and *L. brevis* KY21 were able to inhibit the transcriptions of pro-inflammatory cytokine genes observed in DSS-induced colitis specifically, and prevent the initiation of the inflammatory response.

In conclusion, we investigated the synergic anti-inflammatory effects of GS extract and *L. brevis* KY21, both *in vitro* and *in vivo*. Our results showed that the combination of GS extract and *L. brevis* KY21 improve the recovery from intestinal damage and prevent reactivation of experimental colitis, supporting the potential use of this new protective or preventive strategy for IBD in the food industry.

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