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Weissella koreensis KJ, Which Increases Gut Tight Junction Protein Expression, Alleviates TNBS-Induced Colitis by Suppressing Inflammatory Cytokines

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Abstract: Inflammatory bowel disease (IBD), a chronic inflammatory disease, results from dysregulation of the immune responses. The IBD prevalence rate was 321.2 per 100,000 people in 2021 and, compared with that in 2006 (200 per 100,000 people), had increased at a rate of +46%. Therefore, the development of a safe and new treatment for IBD is urgently needed. *Weissella koreensis*, a strain of lactic acid bacteria (LABs), was isolated from kimchi and shown to inhibit a pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α). Its anti-inflammatory effect was further assessed using a mouse model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). The administration of TNBS significantly increased myeloperoxidase (MPO) expression, macroscopic score, and colonic shortening. Oral administration of *W. koreensis* KJ suppressed the TNBS-induced response and significantly inhibited the expression of the pro-inflammatory cytokines TNF-α, interleukin (IL)-1β, and IL-6 in the intestinal tissues. In particular, *W. koreensis* KJ reversed the TNBS-induced decrease in the expression of these tight junction proteins. Therefore, since *W. koreensis* KJ isolated from kimchi, which increases gut tight junction proteins, attenuating colitis by suppressing inflammatory cytokines, it can be used as a therapeutic candidate for treating colitis such as IBD.

Keywords: *Weissella koreensis*; colitis; 2,4,6-trinitrobenzene sulfonic acid (TNBS); gut permeability; tight junction protein

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease caused by an immune imbalance and includes two main forms: Crohn's disease (CD) and ulcerative colitis (UC) [1]. IBD is recognized as a significant health problem worldwide due to its increasing prevalence, substantial impact on quality of life, and high healthcare costs. In particular, the prevalence of IBD was 321.2 per 100,000 people in 2021, a 46% increase compared to that in 2006 [2].

IBD involves a complex interplay of genetic, environmental, and immune factors, such as genetic susceptibility, immune response, microbial factors, environmental triggers, and intestinal barrier dysfunction [3]. Therefore, establishing an appropriate treatment strategy is very important.

A leaky gut increases gut permeability [4]. Weakened tight junctions can induce an increase in gut permeability, allowing various macromolecules to pass through the intercellular gap [4]. Increased gut permeability is observed with conditions such as aging, asthma, food allergy, rheumatoid arthritis, Crohn's disease, and ulcerative colitis [5–7]. A leaky gut is also associated with the gut–brain–liver axis and has been described in a variety of liver and brain diseases, including metabolic-dysfunction-associated steatohepatitis (MASH) and dementia [8]. Therefore, the regulation of gut permeability is identified as a therapeutic target for various immune diseases.

Gut permeability is regulated by the expression of tight junction proteins, such as zonula occludens (ZO), occludin, and claudins [9]. When the expression of tight junction proteins is decreased in the gut, gut permeability increases, thereby increasing blood absorption of toxins by gut bacteria such as lipopolysaccharide (LPS), resulting in systemic inflammation [9]. LPS induces the NF-κB pathway, which activates the biosynthesis of a wide range of inflammatory cytokines in macrophages [10]. Among inflammatory cytokines, TNF-α and IL-1β are important mediators of inflammation [11]. These cytokines play an important role in promoting the inflammatory response [12,13]. Thus, regulation of gut permeability is required for suppression of inflammation.

Intrarectal injection of 2,4,6-trinitrobenzenesulfonic acid (TNBS) induces colitis similar to IBD [14]. TNBS-induced colitis in mice has been widely used as an experimental model for studying the mechanism of IBD and evaluating drug candidates for its treatment [15]. TNBS-induced colitis has been a very useful model for testing many current therapies for IBD, including sulfasalazine and anti-TNF-alpha antibodies [16,17]. In addition, a leaky gut was confirmed in an animal model of colitis induced by TNBS to suppress the expression of tight junction proteins [18]. Therefore, a TNBS-induced colitis mouse model is suitable as an animal model for studying intestinal permeability.

The health benefits of kimchi, a traditional Korean food manufactured by fermenting vegetables with lactic acid bacteria (LABs), including Lactobacilli, have been demonstrated in various studies [19–21]. These bacteria alleviate dysbiosis of the gut microbiome [22], immune imbalances [22], and intestinal [14,19] and systemic inflammation [23]. Among the LABs isolated from kimchi, Lactobacilli have been used in many health foods due to their health-promoting effects, which have been proven in several studies [24]. However, there are various LABs in addition to *Lactobacillus* in kimchi, and further research on them is required.

Since there is a close relationship between a leaky gut and inflammation [25], we hypothesized that an LAB that suppresses inflammatory cytokines increases the expression of leaky-gut-related tight junction proteins. Consequently, LABs that inhibit TNF-α, a representative inflammatory cytokine, were isolated from kimchi, and their gut tight junction protein-modulating effects were confirmed in a TNBS-induced colitis mouse model.

2. Materials and Methods

2.1. Materials

TNBS, sodium thioglycolate, Roswell Park Memorial Institute medium, and LPS from *Escherichia coli* O111:B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer used as a lysis buffer for mouse colon tissue and myeloperoxidase (MPO) activity assay kits were procured from Abcam (Cambridge, UK). Enzyme-linked immunosorbent assay (ELISA) kits for detecting TNF-α (cat. no. DY410), IL-6 (cat. no. DY406), and IL-1β (cat. no. DY401) were sourced from R&D Systems (Minneapolis, MN, USA). Antibodies against-ZO-1 (cat. no. ab96587), occluding (cat. no. ab222691), and β-actin (cat. no. #3700) were provided by Abcam (Cambridge, UK) and Cell Signaling Technology (Beverly, MA, USA). A Pierce enhanced chemiluminescence (ECL) Western blotting substrate and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Welgene (Daegu, Republic of Korea). The Gram staining kit for bacterial staining was supplied by BioMerieux (Grenoble, France), and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation and Preparation of LABs from Kimchi

Twenty LAB strains were isolated from kimchi, a representative traditional fermented food in Korea. The sample was serially diluted with De Man–Rogosa–Sharpe (MRS) broth (BD, Radnor, PA, USA) diluent and then plated on MRS agar, followed by incubation at 37 °C for 48 h under anaerobic conditions. Finally, a single colony was streaked on MRS agar. The isolated LAB strains were confirmed by using 16S ribosomal DNA sequencing

and Gram staining. For the in vitro assay, pelleted bacteria suspended in 1 mL of saline were stored at 72 °C for 30 min to become inactivated. Heat inactivation was performed to evaluate the anti-inflammatory effects of various LAB strains under the same conditions in vitro. Because live LABs have different conditions such as growth rate for each strain, heat inactivation is an inevitable choice. For the in vivo assay, LABs cultured in an MRS liquid medium (1 to 2 OD at 600 nm) were centrifuged (5000× *g* for 20 min) and the pellet was washed twice with saline. The finally obtained bacterial cells (1×10^8 CFU and 1×10^9 CFU) were suspended in 1% glucose and administered to mice via the oral route.

2.3. Viability in Gastric and Intestinal Juice

To measure viability in artificial gastric juice and intestinal juice, *W. koreensis* KJ was first inoculated in artificial gastric juice, incubated at 37 °C for 3 h, and then centrifuged to obtain bacterial cells. Then, the obtained cells were added to artificial intestinal fluid and incubated again at 37 °C for 3 h. The number of live *W. koreensis* KJ was determined by plating on MRS agar.

Artificial gastric juice was prepared by adding pepsin (1 mg/mL) to MRS broth adjusted to a pH of 2.5 with 1 N HCl. The artificial intestinal fluid was adjusted to a pH of 6.8 with Gram broth containing mucin (0.1%), pancreatin (0.04%), bile salt (0.2%), trypsin (0.04%), and NaCl (0.85%).

2.4. Ability to Attach to Intestinal Epithelial Cells

To measure the adhesion ability of Caco-2, a human intestinal cell line, Caco-2 cells from the Korean Cell Line Bank (KCLB) were cultured at 37 °C with 5% CO_2 using DMEM containing 10% (*v*/*v*) FBS, 1 mM sodium pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The number of Caco-2 cells was adjusted to 1.0×10^5 cells/mL, and the cells were seeded to form a monolayer in 6-well culture plates supplemented with antibiotic-free DMEM containing 10% FBS. After inoculation, the cells were pre-incubated for 2 h at 37 °C and 5% CO_2.

W. koreensis KJ cultured in the MRS liquid medium at 37 °C for 24 h was centrifuged to collect only the cells, which were then washed with phosphate-buffered saline (pH 7.0); the number of *W. koreensis* KJ in the DMEM was adjusted to 1.0×10^8 CFU/mL. Caco-2 cells were pre-incubated with a *W. koreensis* KJ suspension and cultured at 37 °C for 2 h. Then, the unattached cells were removed, and the attached cells were detached with trypsin-EDTA solution, washed with phosphate-buffered saline (pH 7.0), and cultured on MRS agar plates to determine the number of *W. koreensis* KJ.

The cells were washed twice with phosphate-buffered saline (PBS) (pH 7.0) and centrifuged (7000× *g*, 10 min) at 37 °C for 24 h in MRS broth to measure auto-aggregation. The cell suspension (2 mL) was vortexed vigorously for approximately 10 s and incubated at 37 °C for 2 h. The supernatant (1 mL) was collected, and the absorbance at 600 nm was measured. The self-cohesive force (%) was determined by using the equation [(1 − absorbance after incubation for 2 h/absorbance before incubation) × 100] [26].

2.5. Preparation of Macrophages

Mouse peritoneal macrophages were obtained following a previously described method by injecting male Institute of Cancer Research (ICR) mice intraperitoneally with thioglycolate [14]. After washing the peritoneal cavity with RPMI 1640 (5 mL) to obtain peritoneal fluid, it was centrifuged at 300× *g* for 5 min. Culture plates were seeded with approximately 1×10^6 cells, which were incubated in RPMI 1640 containing 10% FBS and 1% antibiotic-antimycotic at 37 °C and washed twice. Macrophages were used as plate-attached cells. To select LABs that inhibit inflammatory cytokines, mouse peritoneal macrophages were treated with LPS (100 ng/mL) and/or heat-inactivated LABs for 24 h in accordance with a previously described method [14]. The level of inflammatory cytokines in the cell culture supernatant was confirmed using ELISA.

2.6. Animals

The induction of colitis using TNBS was primarily performed in five-week-old male ICR mice [27,28]. Therefore, five-week-old male ICR mice (weighing 26–28 g) in a specific-pathogen-free state were purchased from the Orient Lab, Animal Inc. (Seoul, Republic of Korea). The mice were bred in controlled environmental conditions of 50 ± 10% humidity under 20–22 °C temperature, and they were acclimatized to a 12-h light/dark cycle in an animal breeding room prior to the experiments. During the experiments, lighting conditions were maintained consistent with the acclimation environment. They were given ad libitum access to standard laboratory feed (Samyang, Republic of Korea) and drinking water. Each group in all the experiments consisted of 6 mice (6 mice per cage). The experiments were conducted in accordance with Eulji University Animal Ethics Regulations (IACUC) (approval no. EUIACUC 22-12).

2.7. Induction and Evaluation of Colitis in Mice

The mice were divided into 5 experimental groups for in vivo experiments: (1) a normal control group treated with vehicle only; (2) a control group administered only TNBS; (3, 4) two separate groups of mice treated with *W. koreensis* KJ at 1×10^8 or 1×10^9 CFU/mouse after TNBS treatment; and (5) a positive control group of mice administered TNBS and sulfasalazine (50 mg/kg).

Intrarectal administration of TNBS for colitis induction followed previously described methods [14]. Induction of colitis was performed via intrarectal injection of a 2.5% (*w*/*v*) TNBS solution (100 µL) in 50% (*v*/*v*) ethanol into the colon of the mice. For intrarectal injection of the TNBS solution, the needle was inserted approximately 3.5–4 cm proximal to the anus area. To achieve an even distribution of the TNBS solution throughout the colon, the mice were held vertically for 30 sec following the injection. After treatment with TNBS, *W. koreensis* KJ (1×10^8 CFU/mouse or 1×10^9 CFU/mouse) or sulfasalazine (50 mg/kg) was administered orally once a day for 3 days. The mice were sacrificed 18 h after the final dose. After the intestines were removed, they were immediately cut vertically and gently flushed with saline. The degree of colitis was measured according to a previously presented macroscopic scoring guide [14]: 0, free of ulcer or inflammation; 1, free of ulceration or hyperemia observed in localized areas; 2, ulceration without hyperemia; 3, inflammation and ulceration in one area; 4, inflammation and ulceration were found in ≥2 sites; 5, ulceration spreading over >2 cm. For tissue staining, some of the colon tissue was stored in 4% paraformaldehyde (PFA), and the rest was stored at −80 °C until the end of the experiments.

2.8. MPO Activity Assay

Measurement of MPO activity in the homogenized mouse colon tissue was performed according to the kit's instructions. One unit of MPO activity is expressed as 1 unit/mg protein, defined as the amount of enzyme required to break down 1 µmol peroxide/mL.

2.9. Immunoblot Analysis and ELISA

After dissolving the colon tissue with RIPA lysis buffer, it was centrifuged for 20 min at 15,000× *g* and 4 °C to perform immunoblot analysis and cytokine analysis. The protein concentration of the supernatant collected after centrifugation of the protein lysate was determined using a protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA). For 1 h and 30 min, 50 µg of the supernatant was electrophoresed using a 10% (*w*/*v*) polyacrylamide gel. Then, proteins were transferred to a nitrocellulose membrane. Following blocking with 1% BSA for 30 min, the membrane was washed with PBS-Tween 20 for 5 min three times each and cultured overnight with the primary antibodies (ZO-1, occludin, and claudin-1) at a ratio of 1:1000. The next day, after washing the membrane three times for 10 min each, it was incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at a ratio of 1:2500. An enhanced chemiluminescence (ECL) reagent was used to visualize the protein bands. The quantity of luminescence was

determined using the Quantity 1 version 4.6.7 software (Bio-Rad Laboratories, Hercules, CA, USA).

For cytokine analysis, the supernatants from peritoneal macrophages and colon homogenates were added to 96-well ELISA plates. According to each recommended protocol, the levels of expression of TNF-α, IL-1β, and IL-6 were measured using ELISA kits [14]. The assay was performed following the manufacturer's instructions. The standards and samples were added to the wells and incubated for 2 h at room temperature. The wells were washed with the washing buffer provided, and the standards and samples were incubated with biotin conjugate for 2 h. After washing the plates, the standards and samples were incubated with streptavidin–HRP for 40 min. Then, after washing, the standards and samples were incubated with tetramethyl-benzidine (TMB) substrate solution in the dark at room temperature for approximately 20 min. The stop solution was added, and absorbance at 450 nm was read immediately using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.10. H&E Staining of Colon Tissue

To examine mucosal defects and inflammatory lesions, the separated colons were fixed in 10% buffered formalin and then made into paraffin blocks. These paraffin blocks were cut to a thickness of 5 mm, stained with hematoxylin and eosin, and observed with an optical microscope (BX60, Olympus Co., Tokyo, Japan).

2.11. Immunohistochemistry (IHC) Staining

To proceed with IHC staining, the colon tissue was immersed in 4% paraformaldehyde at 4 °C for 2–4 h and post-fixation was performed. The tissue was stored in 30% sucrose, which completely penetrated the tissue, and then cut with a cryostat (Leica, Nussloch, Germany) to a thickness of 10 μm. IHC was subsequently performed. To assess the level of tight junction protein expression, rabbit polyclonal anti-ZO-1 IgG, rabbit polyclonal anti-occludin IgG, and rabbit polyclonal anti-claudin IgG were used as the primary antibodies. The antibodies were diluted 1:100. Subsequently, the tissue sections were incubated with the secondary antibody and avidin and biotinylated peroxidase complex. The color development of the antibody was induced with 3,3′-diaminobenzidine (DAB, Merck, Germany) using an ABC kit (Vector Laboratories, CA, USA). After drying, the samples were sealed in Permount (Merck, Germany) using ethanol and xylene, and observed using an optical microscope (BX60, Olympus Co., Tokyo, Japan).

2.12. Statistical Analysis

All experimental data are presented as the mean ± standard deviation. One-way ANOVA was performed, followed by post hoc analysis using Dunnett's comparison tests, using SPSS 20.0 software (IBM SPSS version 20. 0. 0 for Windows, IBM Co., Armonk, NY, USA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. W. koreensis KJ Suppresses the Expression of Pro-Inflammatory Cytokines in LPS-Stimulated Peritoneal Macrophages

Among the 20 LAB strains (heat-treated) isolated from kimchi, KJ significantly inhibited the level of TNF-α in LPS-stimulated peritoneal macrophages, as demonstrated by the results of the TNF-α inhibitory assay (Supplementary Figure S1). Further confirmation showed that KJ inhibited TNF-α in a concentration-dependent manner (Figure 1). KJ decreased the level of TNF-α in the supernatant at a concentration of 1×10^5 CFU/well by 65.7% when compared to the LPS-treated group. Based on the results of Gram staining and 16S ribosomal DNA sequencing, KJ was identified as *W. koreensis*.

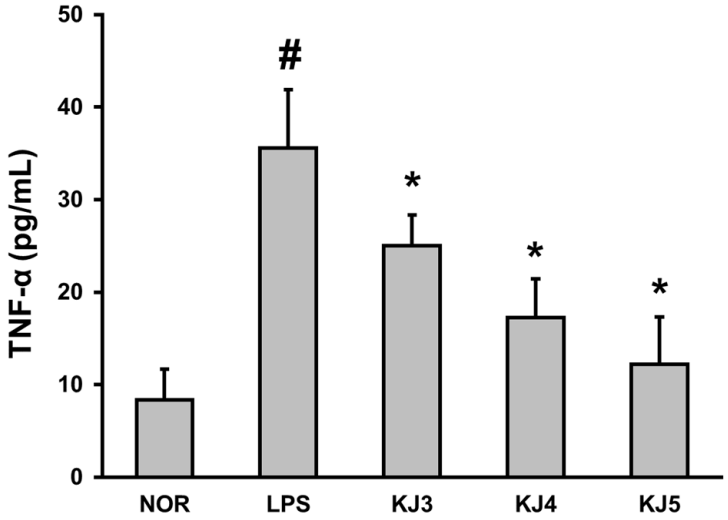

Figure 1. TNF-α inhibitory effect of *W. koreensis* KJ in mouse peritoneal macrophage cells treated with LPS. Mouse peritoneal macrophage cells (1×10^6 cells/well) were treated with 100 ng/mL LPS in the presence or absence of heat-inactivated LABs (KJ3, 1×10^3; KJ4, 1×10^4; and KJ5, 1×10^5 CFU/well) for 20 h. To determine the level of TNF-α, the culture supernatants were used for ELISA. The mean ± SD ($n = 3$) shows the enzyme activity values. # Significant difference compared to the normal control (NOR) group ($p < 0.05$). * Significant difference compared to the LPS-treated group ($p < 0.05$).

3.2. Characterization of W. koreensis KJ for Application in Humans

To evaluate the potential application of *W. koreensis* KJ in humans, its resistance to artificial digestive juices and adhesion to Caco-2 cells were confirmed. Table 1 shows the live cell counts of *W. koreensis* KJ after culturing in artificial digestion solutions. *W. koreensis* KJ exhibited a resistance of approximately $2.5 \pm 1.3 \times 10^7$ CFU/mL to artificial gastric fluid at a pH of 2.5. After culturing *W. koreensis* KJ in artificial intestinal fluid, $1.5 \pm 0.4 \times 10^8$ CFU/mL of viable cells were detected; this result means that there is resistance to the artificial intestinal fluid.

Table 1. Resistance of *W. koreensis* KJ to artificial digestive juices and adhesion to intestinal epithelial Caco-2 cells.

	Viable Cell Counts (CFU/mL)			Auto-Aggregation (%)
	Gastric Juice	Intestinal Juice	Adhesion	
Weissella koreensis KJ	$2.5 \pm 1.3 \times 10^7$	$1.5 \pm 0.4 \times 10^8$	$3.2 \pm 0.9 \times 10^5$	40.3 ± 4.5

Numbers of *W. koreensis* KJ were counted by plating serial dilutions (in diluted anaerobic broth, pH 7.2) on MRS and BL agars, followed by anaerobic incubation at 37 °C for 48 h. All values are expressed as the mean ± SD ($n = 6$).

The possibility of intestinal adhesion was confirmed by measuring the adhesion ability of *W. koreensis* KJ to Caco-2, a human intestinal epithelial cell line. *W. koreensis* KJ attached to Caco-2 cells at a level of $3.2 \pm 0.9 \times 10^5$ CFU/mL (Table 1). This result was similar to the previously reported *Lactobacillus sakei* levels [14].

In addition, the self-cohesive force, which indicates the cluster ability among bacterial cells of the same kind, correlates with the ability of a strain to adhere to intestinal epithelial cells [29]. The self-cohesive force of *W. koreensis* KJ was measured to be 40.3 ± 4.5% (Table 1). This result was also similar to the reported *Lactobacillus sakei* levels [14].

3.3. W. koreensis KJ Ameliorates TNBS-Induced Colitis in Mice

The anti-colitis effects of *W. koreensis* KJ were studied using a TNBS-induced colitis mouse model. The mice administered TNBS exhibited weight loss, severe colon inflammation, and colon shortening (Figure 2B,C). Histological evaluation of the colons of the TNBS-treated mice revealed a high degree of intestinal edema associated with the destruction of colonic epithelial cells and robust infiltration of the mucosal superficial layer (Figure 2D). Oral administration of *W. koreensis* KJ alleviated weight loss, colon shortening, inflammation, and colon thickening (Figure 2). In addition, in the histological examination, it was observed that *W. koreensis* KJ reduced the level of intestinal edema and inhibited immune cell infiltration and epithelial cell destruction (Figure 2D).

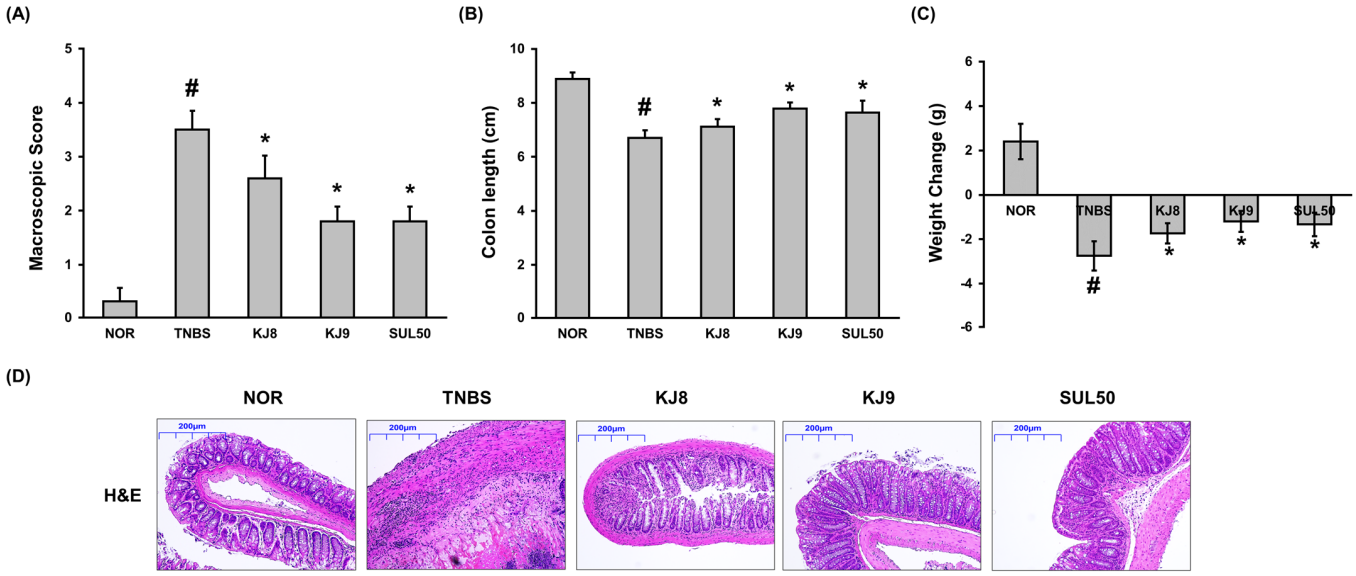

Figure 2. Effects of *W. koreensis* KJ in TNBS-induced colitis mice: (**A**) macroscopic score; (**B**) effect on colon length; (**C**) effect on weight change; and (**D**) histological examination of colon tissues, stained with hematoxylin and eosin (H&E). TNBS was administered intrarectally to each of the TNBS-treated, *W. koreensis* KJ-treated, and SUL50-treated groups. After TNBS injection, *W. koreensis* KJ (TNBS, saline only; KJ8, 1×10^8 CFU/mouse of *W. koreensis* KJ; KJ9, 1×10^9 CFU/mouse of *W. koreensis* KJ; SUL50, 50 mg/kg sulfasalazine) was administered orally for 3 days. The NOR group was treated with vehicle instead of *W. koreensis* KJ. All experimental values are expressed as the mean ± SD ($n = 6$). # Significant difference compared to the normal control group ($p < 0.05$). * Significant difference compared to the TNBS-treated group ($p < 0.05$).

Furthermore, *W. koreensis* KJ inhibited the activity of MPO, a biomarker of TNBS-induced oxidative damage (Figure 3A). Treatment with *W. koreensis* KJ at 1×10^9 CFU/mouse inhibited MPO to a level similar to that of sulfasalazine. Additionally, *W. koreensis* KJ significantly lowered the expression of TNF-α, IL-1β, and IL-6, which are inflammatory cytokines induced by TNBS (Figure 3B–D) *W. koreensis* KJ significantly inhibited each inflammatory cytokine in a concentration-dependent manner, and the effects were equal to or higher than that of sulfasalazine. These results demonstrate that *W. koreensis* can attenuate TNBS-induced colitis.

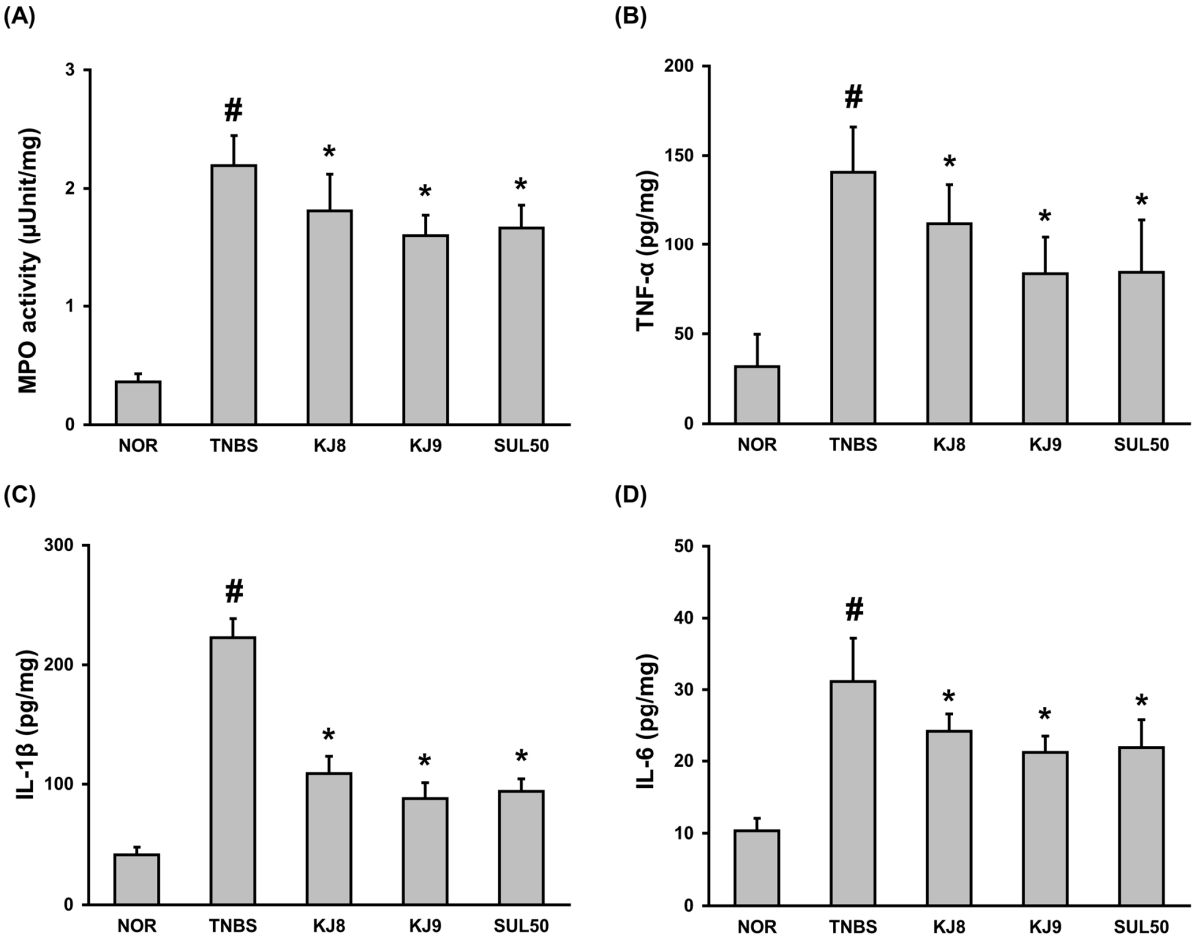

Figure 3. Effects of *W. koreensis* KJ on MPO activity (**A**) and pro-inflammatory cytokines TNF-α (**B**), IL-1β (**C**), and IL-6 (**D**) in TNBS-induced colitis mice. TNBS was administered intrarectally to each of the TNBS-treated, *W. koreensis* KJ-treated, and SUL50-treated groups. After TNBS injection, *W. koreensis* KJ (TNBS, saline only; KJ8, 1×10^8 CFU/mouse of *W. koreensis* KJ; KJ9, 1×10^9 CFU/mouse of *W. koreensis* KJ; SUL50, 50 mg/kg sulfasalazine) was administered orally for 3 days. The normal control (NOR) group was treated with vehicle instead of *W. koreensis* KJ. All experimental values are expressed as the mean ± SD ($n = 6$). # Significant difference compared to the normal control group ($p < 0.05$). * Significant difference compared to the TNBS-treated group ($p < 0.05$).

3.4. W. koreensis KJ Increases the Expression of Intestinal Tight Junction Proteins

Administration of TNBS destroyed the epithelial tissue of the colon, leading to a state in which a leaky gut was possible (Figure 2D). Since a leaky gut is associated with tight junction proteins, the expression level of intestinal tight junction proteins was confirmed via IHC (Figure 4A). The measured tight junction proteins were determined as ZO-1, occludin, and claudin-1 by referring to a previous study by Lim and Kim [30].

At 20× magnification, the expression of ZO-1, occludin, and claudin-1 in the colon tissue of the TNBS-treated mice was significantly suppressed compared with that in the normal colon tissue (Figure 4A). Oral administration of *W. koreensis* KJ successfully reversed the TNBS-induced decrease in expression of ZO-1, occludin, and claudin-1 (Figure 4A). Moreover, it effectively inhibited TNBS-induced destruction of the intestinal epithelial tissue (Figure 2D). The immunoblot results for tight junction proteins also demonstrate the effect of *W. koreensis* KJ. The levels of tight junction proteins, including ZO-1, occludin, and claudin-1, in the intestinal homogenate decreased in the *W. koreensis* KJ-administered group compared to the TNBS-treated group (Figure 4B). The immunoblot of each protein, including beta-actin, was performed separately using the same lysate, and relative comparison of expression was conducted by examining the consistency of the β-actin band. In addition,

the trend could be reconfirmed through band quantification. These results indicate that *W. koreensis* KJ can directly regulate tight junction proteins.

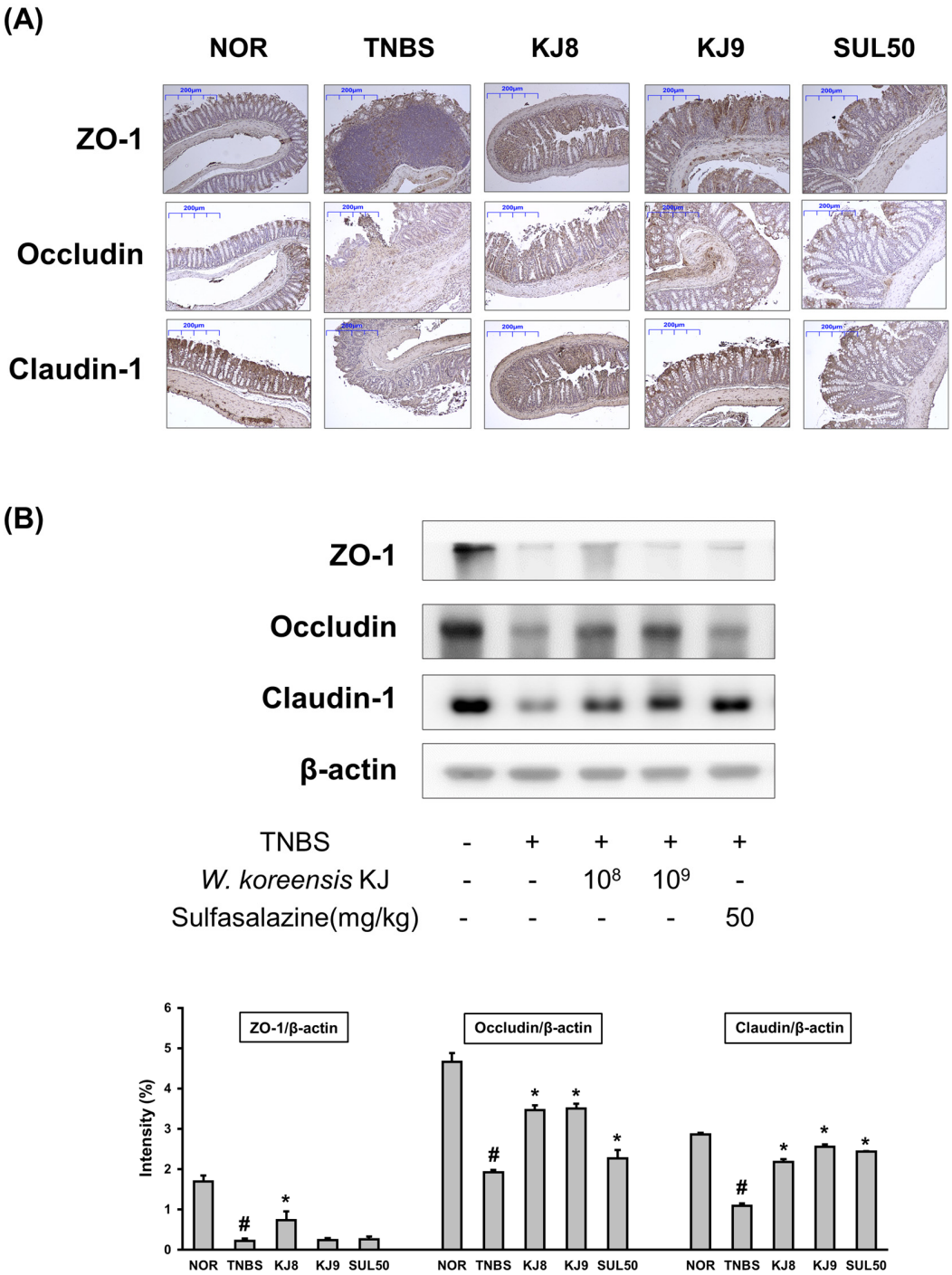

Figure 4. Effects of *W. koreensis* KJ on the expression of gut tight junction proteins in TNBS-induced colitis mice. (**A**) Histological examination of colon tissues immunostained with anti-ZO-1, anti-occludin, and anti-claudin antibodies. (**B**) Detection of ZO-1, occludin, and claudin-1 using Western blot. TNBS was administered intrarectally to each of the TNBS-treated, *W. koreensis* KJ-treated, and SUL50-treated groups. After TNBS injection, *W. koreensis* KJ (TNBS, saline only; KJ8, 1×10^8 CFU/mouse of *W. koreensis* KJ; KJ9, 1×10^9 CFU/mouse of *W. koreensis* KJ; SUL50, 50 mg/kg sulfasalazine) was administered orally for 3 days. The normal control (NOR) group was treated with vehicle instead of *W. koreensis* KJ. # Significant difference compared to the normal control group ($p < 0.05$). * Significant difference compared to the TNBS-treated group ($p < 0.05$).

4. Discussion

IBD, including Crohn's disease and ulcerative colitis, is a representative inflammatory disease occurring in the intestinal tract that is caused by immune and gut microbiome imbalances [31,32]. IBD is difficult to treat because of the complex etiology between genetic, environmental, or microbial factors and the immune response [33]. IBD is characterized by chronic relapsing intestinal inflammation. Although 5-aminosalicylic acid is used as a treatment regimen, there is an unmet medical need because IBD relapse occurs frequently [34]. Therefore, new treatments for IBD are urgently needed. Currently, IBD treatment strategies focus on inflammation control; however, they are associated with several limitations. Inhibition of the inflammatory response can relieve emergency symptoms, but its effect on the control of the immune response via the interaction between the host and intestinal microbes, which is considered to be the main cause of IBD, is restricted [35]. Therefore, we focused on leaky gut as a new treatment target.

A leaky gut is observed in patients with IBD and is mainly caused by decreased expression of tight junction proteins, including ZO-1, occludin, and claudin-1 [36,37]. A leaky gut has considerable influence on the aggravation of IBD, but there is still debate as to whether it is a cause or a result of IBD [5,38]. Nevertheless, there are studies showing that pro-inflammatory cytokines caused by factors such as stress increase intestinal permeability and induce changes in the composition of intestinal microorganisms, leading to the development of IBD. There are also studies showing that increased intestinal permeability due to IBD is a cause of depression. These are the bidirectional effects of the gut–brain axis and demonstrate that intestinal permeability is important in the etiology and complications of IBD [39,40]. Additionally, there is a study showing that a leaky gut caused by inhibition of tight junction proteins may induce colitis and liver damage. This shows that the gut–liver axis may be involved in causing complications of colitis [41].

Therefore, it is important to develop a treatment that can suppress leaky gut in IBD while controlling the immune response. LABs can suppress the population of harmful bacteria through the regulation of the intestinal microbiome and inhibit the inflammatory response caused by LPS released from Gram-negative bacteria [40]. Thus, LABs are considered to be a material capable of controlling various etiologies of IBD and inhibiting the associated leaky gut, offering a potential new treatment target for IBD.

Of course, there may be objections to applying LABs to the treatment of IBD because there are results showing that LABs induce the production of inflammatory cytokines. *Lactobacillus plantarum* HY7712 (HY7712) isolated from kimchi is a representative example. HY7712 induces the production of TNF-α in mouse peritoneal macrophages. However, it inhibits NF-κB in mouse peritoneal macrophages of LPS [20]. HY7712 increases the immunity of mice with cyclophosphamide-induced immunosuppression [20] while suppressing the immunity of mice with autoimmune diseases [42]. This demonstrates the role of LABs in regulating immunity in certain situations, which may be a result of the normalization of an altered intestinal microflora. LABs with immunomodulatory effects have the potential to alleviate immune diseases, including IBD.

This study aimed to obtain an LAB that can be used for novel treatment strategies. As a result, we isolated *W. koreensis* KJ from kimchi, which is capable of controlling tight junction protein expression while exerting anti-inflammatory effects.

The administration of TNBS induces colitis in mice. Weight loss is predominantly induced, and colon shortening is an anatomical feature seen in TNBS-induced mice. These symptoms are very similar to symptoms seen in human colitis, and the TNBS-induced colitis model is widely used for drug evaluation [15]. In our study, *W. koreensis* KJ reversed TNBS-induced weight loss and colon shortening (Figure 2B,C). These results show the effect of *W. koreensis* KJ in alleviating colitis symptoms.

W. koreensis KJ, which inhibits TNF-α expression in peritoneal macrophages, induced inflammatory response by LPS (Figure 1) and suppressed inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in TNBS-induced colitis mice (Figure 3B–D), as well as suppressed MPO activity (Figure 3A). In particular, recent studies have shown that IL-6 is

a very important cytokine in regulating the balance between IL-17-producing Th17 cells and regulatory T cells (Treg). Th17 cells play an important role in the pathogenesis of immune diseases, while Tregs function to suppress excessive effector T-cell responses. IL-6 induces the development of Th17 cells from naïve T cells. In contrast, IL-6 inhibits Treg differentiation. Dysregulation or overproduction of IL-6 is a major cause of immune diseases, including IBD. Given the important role of IL-6 in altering the balance between Tregs and Th17 cells, controlling IL-6 activity is a potentially effective approach for the treatment of IBD [43]. The IL-6 inhibitory ability of *W. koreensis* KJ shows its potential in alleviating IBD through the regulation of Th17/Treg. Additionally, MPO is an enzyme that is highly expressed in granules as a biomarker of TNBS-induced oxidative damage and catalyzes the formation of highly reactive oxygen species (ROS) [14]. The MPO inhibitory ability of *W. koreensis* KJ suggests the possibility of inhibiting oxidative damage caused by inflammation.

The TNBS-treated mice showed a high degree of intestinal edema associated with destruction of colonic epithelial cells, and strong infiltration of the mucosal superficial layer. *W. koreensis* KJ inhibited the destruction of intestinal epithelial tissue in mice with TNBS-induced colitis (Figure 2D). Furthermore, TNBS reduced the expression of ZO-1, occludin, and claudin-1, which are representative tight junction proteins (Figure 4A,B). These results show the effect of TNBS in inducing a leaky gut. *W. koreensis* KJ increased the expression of intestinal tight junction proteins, which was reduced by TNBS (Figure 4A,B). Although the IHC and immunoblot results are not completely consistent, it is evident that *W. koreensis* KJ regulates gut tight junction proteins. The difference in results may be due to the difference between the IHC-analyzed site and the immunoblot-analyzed site. These results demonstrate the regulatory effect of *W. koreensis* KJ on tight junction proteins, suggesting that *W. koreensis* KJ can reverse a leaky gut.

In this study, *W. koreensis* KJ was identified as a suitable LAB for the development of a new IBD treatment strategy through simultaneous suppression of the inflammatory response and leaky gut. Based on these effects, the potential application of *W. koreensis* KJ in the human body was evaluated, and it showed suitable characteristics in the results. Because *W. koreensis* KJ is stable in artificial gastric and intestinal juices (Table 1), when it is orally administered to humans, it is expected to be stable in the stomach and the gut. In addition, *W. koreensis* KJ adhered well to Caco-2, a human intestinal epithelial cell line, and its self-cohesive force was well confirmed (Table 1). The level of adhesion was similar to that of *Lactobacillus sakei* [14]. Therefore, based on the results of evaluating the potential of *W. koreensis* KJ as a functional food, *W. koreensis KJ* is expected to survive, colonize, and proliferate well in the human intestine.

Among the LABs that have been identified, *Lactobacillus* and *Bifidobacterium* have been mainly studied and developed as products. This study, which studied the efficacy of *W. koreensis* isolated from kimchi, a traditional Korean fermented food, will serve as a basis for research on other LABs in addition to *Lactobacillus* and *Bifidobacterium*.

In future research, changes in the gut microbiota population caused by *W. koreensis* KJ will be investigated. These studies will serve as references for the effects of this LAB, including its anti-inflammatory effects by causing changes in the gut microbiota population [1].

In conclusion, *W. koreensis* KJ alleviates colitis by simultaneously inhibiting the inflammatory response and leaky gut. The inhibitory effect of *W. koreensis* KJ on inflammatory cytokines demonstrates its suppression of inflammatory responses, and its effect on increasing the expression of tight junction proteins demonstrates the inhibition of leaky gut. In addition, the stability of *W. koreensis* KJ in artificial digestive fluids and its intestinal epithelial cell adhesion prove its applicability to the human body. Therefore, *W. koreensis* KJ can be developed as a functional food material for the treatment of IBD. Additionally, since *W. koreensis* KJ can be isolated from kimchi, a traditional Korean fermented food, kimchi can be used as a source for this functional LAB.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres15020047/s1, Figure S1: TNF-α inhibitory effect of the 20 LAB strains (heat-treated) isolated from kimchi.

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