RESEARCH ARTICLE

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Mitigating Effects of Tenebrio *molitor* Larvae Powder Administration in Mice with Dextran Sodium Sulfate (DSS)-Induced Colitis

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Abstract

Background: Ulcerative colitis (UC) is an inflammatory bowel disease that affects people worldwide. The causes of UC are diverse, and symptoms include diarrhea, weight loss, anemia, rectal bleeding, and bloody stools. Tenebrio *molitor* larvae have recently gained attention as edible insects with various physiological and medical effects. Research on the anti-inflammatory effects of ingesting Tenebrio *molitor* larvae powder (TMLP) is being actively conducted. In this study, TMLP was administered to mice with dextran sodium sulfate (DSS)-induced colitis to investigate its effects in reducing colitis symptoms. **Methods:** Mice were initially given 3% DSS in water to induce colitis and then feed containing 0%, 2%, or 4% TMLP. Pathologic changes in colon tissues were assessed by histology, and neutrophil levels were measured by myeloperoxidase (MPO) assay. Levels of IL-1β, IL-6, and TNF-α were measured using real-time PCR and ELISA assays, and IκB and NF-kB protein levels were measured by western blotting. **Result:** Disease Activity Index (DAI) scores and MPO activity were reduced in TMLP-treated mice, and colon length increased as much as normal mice. Pathologic changes in the colon tissues of DSS-induced mice were attenuated, and the expression of inflammatory cytokine genes IL-1β, IL-6, and TNF-α decreased. Concomitant decreases in the protein expression of IL-1β and IL-6 were confirmed using ELISA. Western blotting revealed that levels of phosphorylated forms of IκB and NF-κB also decreased. **Conclusion:** These results show that feeding TMLP to DSS-induced mice inhibited the typical inflammatory pathway of colitis. Therefore, TMLP shows potential as a food additive that can help treat colitis.

Keywords: Tenebrio- colitis- inflammation

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Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that affects the large intestine. UC presents with characteristic symptoms, including diarrhea, weight loss, anemia, rectal bleeding, abdominal pain, and bloody stool, and affects millions of people around the world (Nunes et al., 2019; Wang et al., 2018). The incidence of IBD has been increasing rapidly in industrial countries for several decades. Europe and North America have the highest reported prevalences (Nunes et al., 2019). The exact cause of IBD is unknown; however, various factors, including environmental, genetic, immune system, and intestinal microbiome abnormalities, have been suggested. Mesenteric immune system dysfunction is thought to play a direct role in UC development (Yan et al., 2018). Murine models of UC are essential for identifying the underlying mechanisms of IBD incidence and evaluating potential treatments (Chassaing et al., 2014).

Dextran sodium sulfate (DSS)-induced colitis models are often used in IBD research because colitis manifests quickly, the chemical is easy to handle, and disease induction is reproducible (Chassaing et al., 2014). DSS is a water-soluble, negatively charged sulfated polysaccharide with a molecular weight of 5-1,400 kDa. Animals given DSS lose weight and develop soft stools or diarrhea. Colitis with rectal bleeding and the resulting inflammatory response typically appear 5-7 days after administration of 3%-5% DSS in mice. The characteristics of colitis in DSS-treated mice show pathophysiologic characteristics similar to human colitis and are often used as an experimental animal model to conduct preclinical research on UC (Kim et al., 2012; Hu et al., 2020).

Edible insects are an environmentally sustainable alternative to livestock because they are rich in protein and nutritional value. Insects have been used as food resources or tools to facilitate plant reproduction. However, insects have the potential to be applied in various fields. Insect

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extracts are used worldwide in civilian medicine to fight diseases, and now, specific substances from insects can be isolated and biologically manipulated to treat diseases (van Huis, 2013; Stull, 2018; Ratcliffe et al., 2013). For example, the arthropods *Scolopendra* spp. and *Beauveria bassiana* are used to treat arthritis and stroke, respectively (Pemberton, 1999). Administration of maggot-derived protein to DSS-induced rats decreased the disease activity index (DAI) and significantly attenuated the severity of UC. Maggot-derived proteins have also shown potential for improving other UC symptoms, such as weight loss, colon shortening, colon histology, and inflammatory signaling pathway activation (Wang et al., 2018). These results suggest that insects are a new source of novel medicines.

Tenebrio molitor larvae (TML), also known as mealworms or yellow mealworms, are edible insects and good sources of protein with nutritional value, digestibility, flavor, and functionality. TML is a sustainable source of protein, a replacement for bean powder or fish powder, and is sometimes used as feed for pets and zoo animals (Hon et al., 2020). TML plays important roles in preventing skeletal muscle atrophy by stimulating myoprotein synthesis; inhibiting myoprotein degradation (Lee et al., 2021); affecting antioxidant, anticoagulant, and anti-inflammatory activities; and promoting skin wound recovery by increasing collagen synthesis, re-epithelialization, and angiogenesis in mice (Kim et al., 2021).

TML is thought to have infinite potential as a raw material for medicines; however, few studies have evaluated its use in reducing inflammation caused by colitis in animal models. Therefore, this study assessed whether *Tenebrio molitor* larvae powder (TMLP) mitigated colitis symptoms in DSS-induced mice.

Materials and Methods

Experimental animals and DSS-induced colitis

Nine-week-old male ICR mice were obtained from Damul Science (Daejeon, Korea), and TMLP was provided by the Korea Institute of Utility Insects (Goksong, Korea). Mice were randomly divided into four groups (n = 5): Control (standard water and feed), DSS (3% DSS in water + standard feed), DSS-TMLP 2% (3% DSS in water + 2% TMLP in standard feed) and DSS-TMLP 4% group (3% DSS in water + 4% TMLP in standard feed). Standard feed, experimental feed, and water were supplied for the initial 7 days of the experiment, and then 3% DSS in water, standard feed, and experimental feed were given to the appropriate groups for 7 subsequent days (Figure 1). Feed and water were freely accessible. Weights were measured on the day 3% DSS was initiated and at the end of the experiment. Environmental conditions were maintained at 22 ± 1°C, relative humidity of 50 ± 5%, and a 12-hour light/dark cycle. Animal testing was approved by the Chonnam University Animal Experiment Ethics Committee (CNU IACUC-YB-2022-03).

DAI scoring

DAI scoring was conducted as previously published (Park et al., 2022).

DAI scores range from 0 to 4 with the following definitions:

0: normal stool, no bloody stool, no anal hemorrhage, and a weight loss rate of <1%/week

1: slightly watery stool, red stool, no anal hemorrhage, and a weight loss rate of ≥1%/week and <3%/week

2: watery stools, bloody stools, anal hemorrhage, and a weight loss rate of ≥3%/week and <5%/week

3: diarrhea, clear red bloody stool, anal hemorrhage, and a weight loss rate of ≥5%/week and <7%/week

4: diarrhea, clear red bloody stool, anal hemorrhage, and a weight loss rate of ≥7%/week.

Three observers calculated the average DAI score of each group.

Tissue sample collection

Mice were anesthetized by injecting 2% 2,2,2-tribromoethanol (Sigma-Aldrich, St. Louis, MO, USA) into the abdominal cavity. The colon was excised from the appendix to the rectum to measure its length. Colon tissue samples were collected at approximately 1 cm from the rectum and fixed with 10% neutral formalin (Sigma-Aldrich, St. Louis, USA). The remaining tissue was stored at −80°C until analysis. The spleen was extracted and weighed.

Histology

Histological assays were performed as previously published with slight modifications (Park et al., 2022). Formalin-fixed colon tissues were cut to a thickness of 4 mm and stained with hematoxylin and eosin. The degree of colon mucosal proliferation and the histology of the crypts were evaluated using an optical microscope at 100X magnification. The degree of tissue damage was assessed as previously published15 using the sum of the following scoring criteria: Crypt histology (normal: 0; severe damage: 3), tissue inflammation (normal: 0; severe infiltration: 3), goblet cell prevalence (normal: 0; loss: 1).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of leukocyte infiltration (Faith et al., 2008), was assayed using an MPO colorimetric activity assay kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. Colon tissue samples were thoroughly washed with saline, homogenized using Precellys 24 homogenizer (MP Biogenizer, OH, USA), and centrifuged (12,000 × g, 10 minutes, 4°C) to collect the supernatant. MPO activity was obtained using a standard curve and measured at 540 nm using a Multiskan EX microplate reader (Thermo Fisher Scientific, MA, USA).

Real-time PCR of inflammatory cytokines

RNA was extracted from homogenized colon tissue samples using a Nucleospin RNA Plus kit (Machere-Nagel, Duren, and Germany). cDNA was synthesized from RNA using cDNA Synthesis Master Mix (LeGene Biosciences, CA, USA), and real-time PCR was performed with TOPreal™ qPCR 2X PreMIX (SYBR Green, UDG 175 plus with low ROX) (100 ng/ul). The sequence of

primers used was as follows.

IL-1β F: CAACCAACAAGTGATATTCTCCATG
IL-1β R: GATCCACACTCTCCAGCTGCA
IL-6 F: CAGAGATACAAAGAAARGATGGATGCT
IL-6 R: CAGAAGACCAGAGGAAATTTTCAATA
TNF-α F: CCACGCTCTTCTGTCTACTGAACTT
TNF-α R: TGAGAGGGAGGCCATTTGG
GAPDH F: CTGGAGAAACCTGCCAAGTA
GAPDH R: AGTGGGAGTTGCTGTTGAAG

Enzyme-linked immunoassays (ELISA)

Colon tissue samples were homogenized with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, USA) to extract proteins. Each ELISA was performed with 60 μg of extracted protein using an IL-1 beta or IL-6 Mouse ELISA kit (Sigma-Aldrich, USA) according to the manufacturer's instructions.

Western blot analyses

Western blotting was performed as previously published with slight modifications (Park et al., 2022). Colon tissues were homogenized with RIPA buffer. Proteins were then mixed with Novex™Tris-Glycine SDS sample buffer (2×) (Thermo Fisher Scientific, MA, USA), incubated at 95°C for 5 minutes, and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride transfer membrane (Thermo Fisher Scientific, MA, USA) and incubated in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skim milk. Primary antibodies (1:1,000 dilution) were applied to the membrane and incubated at 4°C for 12 hours. The primary antibodies used in this study were anti-β-actin (Thermo Fisher Scientific, USA), anti-NF-κB p65 (Thermo Fisher Scientific, USA), anti-phospho-NF-κB p65 (Thermo Fisher Scientific, USA), anti-IκB (Thermo Fisher Scientific, USA), and anti-phospho-IκB (Thermo Fisher Scientific, USA). Membranes were then washed three times in TBST for 15 minutes and incubated with the appropriate HRP-conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG at 1:5,000 dilutions) in TBST for 2 hours. Reactive proteins were visualized under UV using the Pierce™ ECL western blotting substrate (Thermo Fisher Scientific, USA). The intensities of protein bands were quantified using ImageJ software (NIH Image, MD, USA). β-actin was used as the loading control.

Statistical analysis

The results obtained from each experiment were analyzed by Duncan's multiple range tests, and significance was defined as a p value <0.05, using the analysis of variance performed in GraphPad Prism software version 5.0. Results are reported as means ± standard deviations (SD).

Results

Evaluation of DAI score, colon length, spleen weight, and body weight

To investigate the effects of TMLP on DSS-induced colitis, mice were fed 3% DSS in water with feed as described above. Mice started to have loose stools approximately 3 days after ingesting DSS. Watery diarrhea with bloody stools were observed in all groups except for the control group at 7 days. DAI scores at the end of the experiment were significantly higher relative to the control group. In the DSS-TMLP 2% and DSS-TMLP 4% groups, this increase was attenuated (Figure 2A). Colon lengths of the DSS group were significantly shorter than the control group. However, colon lengths in the DSS-TMLP 2% and DSS-TMLP 4% groups were significantly higher than those in the DSS group and similar to those of the control group (Figure 2B and 2E). Spleen weights were significantly higher in the DSS group relative to the control group. Although there were no significant differences in spleen weights between the DSS-TMLP groups and the DSS group, the values were lower in the DSS-TMLP groups (Figure 2C). Body weights showed no significant differences between any pair of groups but were trending higher in control, DSS-TMLP 2%, and DSS-TMLP 4% groups at the end of the experiment (Figure 2D).

Histology analyses

Tissues from the DSS group had severe colonic ulcers and tissue damage with inflammatory cell infiltration, connective tissue ruptures, goblet cell loss, and severe crypt structure changes. Relative to the DSS group, the DSS-TMLP 2% and DSS-TMLP 4% groups had less intestinal epithelial destruction, inflammatory cell infiltration, and goblet cell loss (Figure 3A). When histology scores were evaluated, those of the DSS group were significantly higher than those of the control group; however, relative to the DSS group, those of the DSS-TMLP 2% and DSS-TMLP 4% groups were significantly lower and showed a dose-dependent response (Figure 3B).

Myeloperoxidase activity and inflammatory cytokine gene expression

MPO activity was significantly higher in the DSS group relative to the control group, indicating that DSS induced severe inflammation in mouse colons. Administration of TMLP reduced the intensity of DSS-induced inflammation.

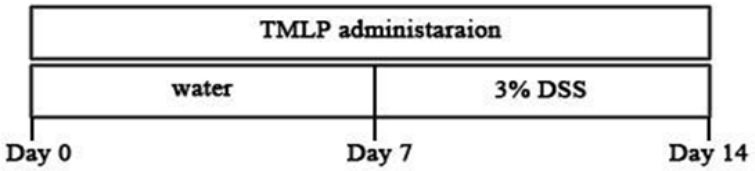

Figure 1. Experimental Diet Overview. Mice were given 3% DSS in water for 7 days to induce colitis. For the subsequent 7 days of the 14-day experiment, two groups of mice were given feed containing 2% or 4% TMLP.

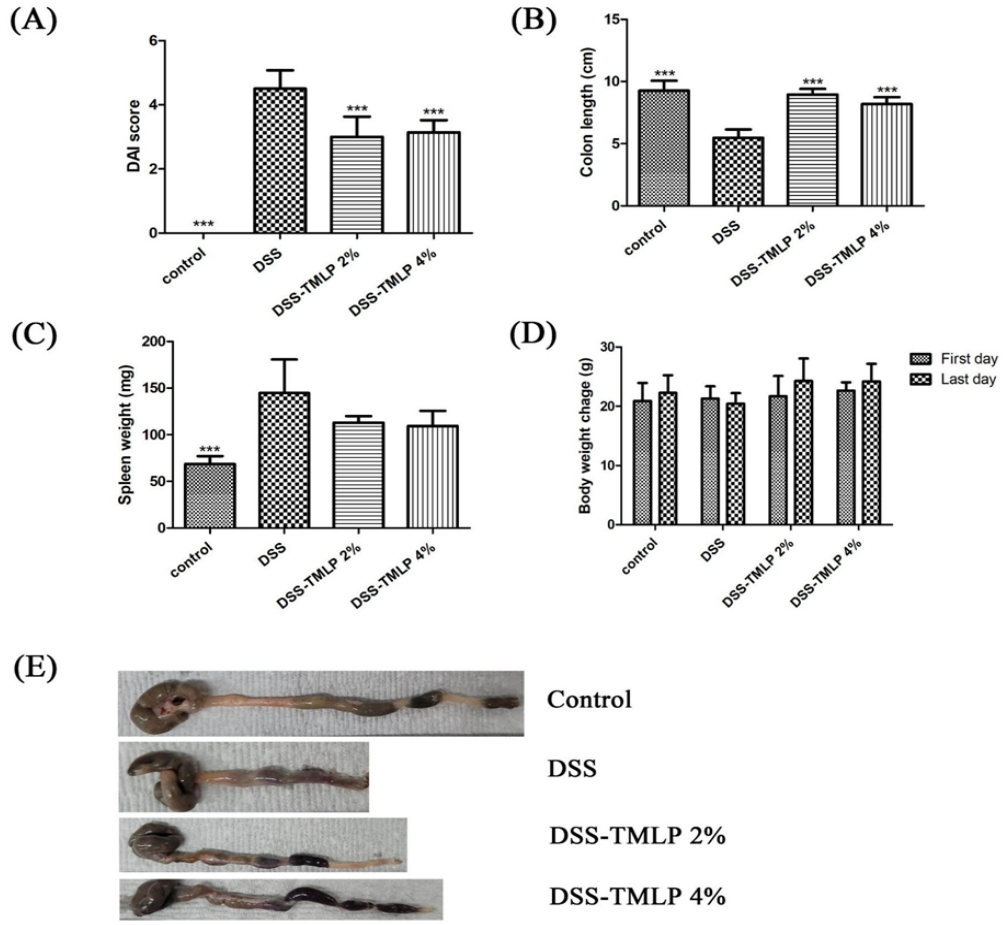

Figure 2. DAI Score, Colon Length, Spleen Weight, and Body Weight in DSS-induced Mice Treated with TMLP. (A) DAI scores, (B) colon length, (C) spleen weight, (D) body weight, and (E) typical colon length for each group. The data are presented as mean ± SD. TMLP group data are compared with those of the DSS group using Dunnett's multiple comparison test. ***$p < 0.01$

MPO activity was significantly lower in the DSS-TMLP 2% and DSS-TMLP 4% groups compared to the DSS group and was similar to that of the control group (Figure 4A). Expression levels of IL-1β, IL-6, and TNF-α were significantly higher in the DSS group compared to the control group and significantly lower in the DSS-TMLP 2% and DSS-TMLP 4% groups relative to the DSS group (Figsure 4B-D). In particular, in the DSS-TMLP 2% and DSS-TMLP 4% groups, IL-1β expression levels were similar to or slightly less than that of the control group

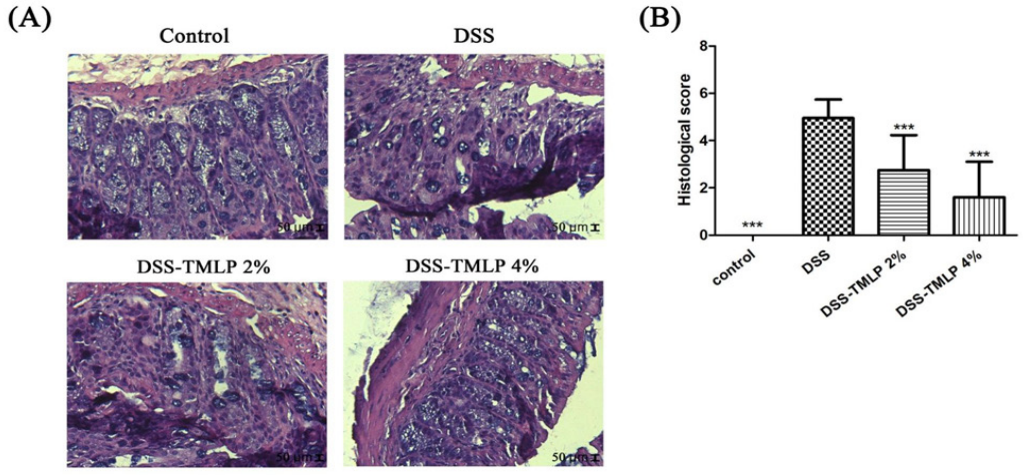

Figure 3. TMLP Alleviates Tissue Damage from DSS-Induced Colitis. Tissues were observed under optical microscopy after staining with hematoxylin and eosin (H&E). (A) Representative optical microscope images of colon tissue samples and (B) histological scores. The data are presented as mean ± SD. TMLP group data are compared with those of the DSS group using Dunnett's multiple comparison test. ***$p < 0.01$

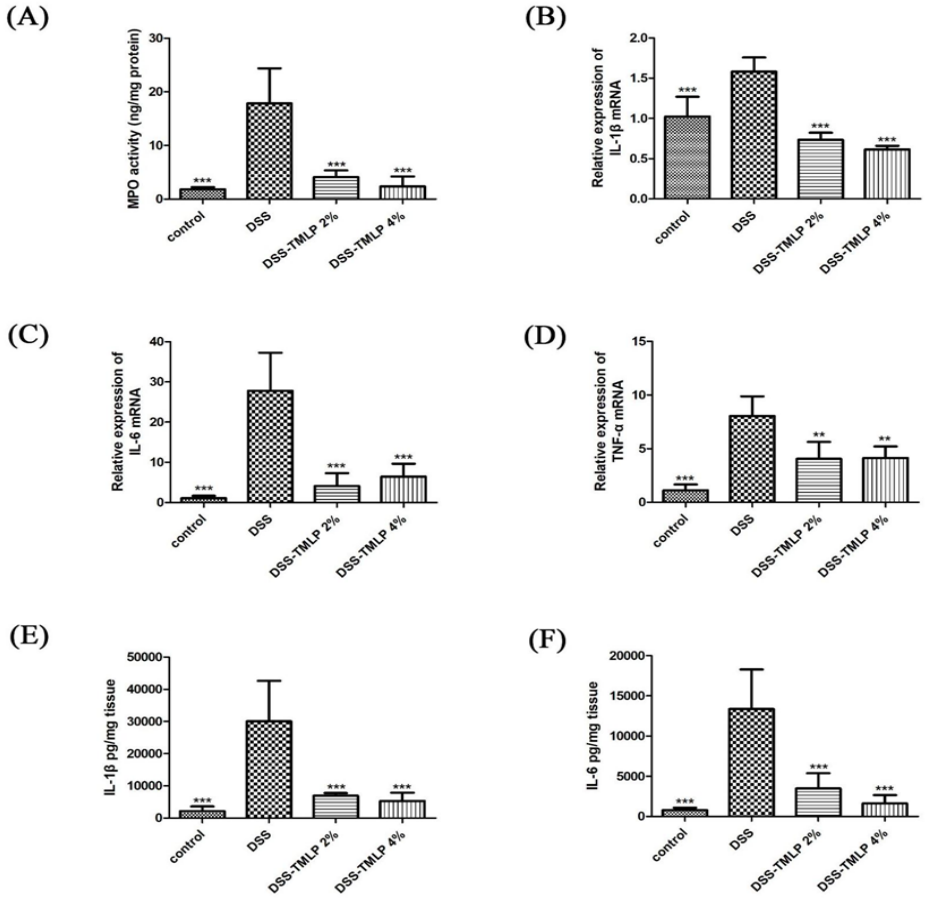

Figure 4. MPO Activity and Pro-Inflammatory Cytokine Gene and Protein Expression Levels in Colon Tissues of Mice with DSS-Induced Colitis. (A) MPO activity, (B) Relative IL-1β gene expression, (C) Relative IL-6 gene expression, (D) Relative TNF-α gene expression, (E) IL-1β protein expression, and (F) IL-6 protein expression. The data are presented as mean ± SD. TMLP group data are compared with those of the DSS group using Dunnett's multiple comparison test. **p < 0.05, and ***p < 0.01

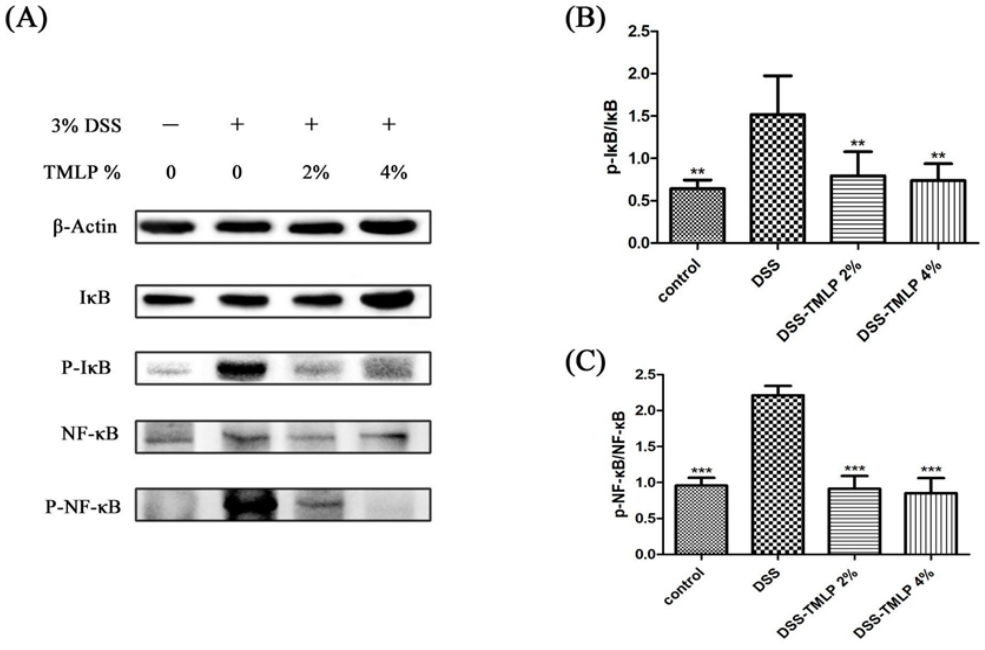

Figure 5. Western Blot Analysis of p-IκB/IκB and p-NF- κB/NF- κB. (A) Western blot bands of β-actin, IκB, p-IκB, N-κB and p-NF-κB, (B) p-IκB/IκB ratios, and (C) p-NF-κB/NF-κB ratios. The relative density of each signaling band was calculated. β-actin was used as loading control. The data are presented as mean ± SD TMLP group data are compared with those of the DSS group using Dunnett's multiple comparison test. **p < 0.05, and ***p < 0.01.

(Figure 4B).

Inflammatory cytokine analysis by ELISA

Protein levels of IL-1β and IL-6 in colon tissues were significantly higher in the DSS group compared to the control group, indicating severe inflammation. Relative to the DSS group, levels of IL-1β and IL-6 proteins in the DSS-TMLP 2% and DSS-TMLP 4% groups were significantly lower, indicating that the magnitude of DSS-induced colitis was attenuated to levels similar to that of the control group (Figures 4E and 4F). These results suggest that TMLP treatment reduced the severity of DSS-induced colitis.

Western blot analysis

Inflammatory pathway activity is expressed by changes in the ratios of the phosphorylated to unphosphorylated forms of IκB (p-IκB/IκB) and NF-κB (p-NF-κB/NF-κB) proteins. DSS activates the NF-κB pathway by increasing p-IκB/IκB and p-NF-κB/NF-κB in colonic tissues. However, administration of TMLP significantly decreased p-IκB/IκB and p-NF-κB/NF-κB ratios in the DSS-TMLP 2% and DSS-TMLP 4% groups (Figures 5A-C).

Discussion

Although the exact cause of IBD has not been identified, genetic predisposition, epithelial barrier defects, immune response abnormalities, environmental changes, and intestinal microbiome abnormalities have been proposed. IBD and UC occur in the inner mucosal layer of the colon and rectum, and long-term disease increases the risk of developing osteoporosis and colorectal cancer (Yang et al., 2019; Seyedian et al., 2019). Drugs such as 5-aminosalicylic acid, steroids, and immunosuppressants are typically used to treat UC, and surgical measures such as colectomy are performed for some patients. Estimates of the annual direct and indirect costs associated with UC are EUR 12.5-29.1 billion in Europe and USD 8.1-14.9 billion in the United States. The research directions for UC include the development of new drugs and treatments (Ungaro et al., 2017).

The administration of 3% DSS to mice in drinking water induces acute and chronic colitis while ensuring survival. In this study, the effects of administering TMLP to mice with DSS-induced colitis were studied. Mice with DSS-induced colitis have significantly higher DAI scores, shorter colons, and more substantially damaged intestinal mucosa than controls (Xu et al., 2021). In this study, DAI scores were lower in the DSS-TMLP 2% and DSS-TMLP 4% groups relative to the DSS group. Colon length shortening was less substantial in the DSS-TMLP 2% and DSS-TMLP 4% groups compared to the DSS group. Microscopically, the DAI scores corresponded to inflammation, and the inflammation observed in the DSS-TMLP 2% and DSS-TMLP 4% group tissues was less than that observed in the DSS group. These results suggest that the administration of TMLP helps prevent acute damage to intestinal mucosa caused by DSS.

MPO activity indicates the degree of neutrophil infiltration within the intestinal tissue, and is highest in inflamed tissue (Faith et al., 2008). In the DSS group, MPO activity was significantly higher than in the control group, indicating an increase in neutrophilic inflammation. However, relative to the DSS group, MPO activity levels in the DSS-TMLP 2% and DSS-TMLP 4% groups were significantly lower, with levels similar to those of the control group. These results suggest that TMLP attenuates DSS-induced inflammation by inhibiting neutrophil infiltration.

IL-1β, IL-6, and TNF-α genes are activated in the early stage of inflammation5. These inflammatory cytokines cause immune dysfunction and local inflammation, damage the colon mucosa, and intensify the clinical symptoms of UC (Wang et al., 2021). In this study, IL-1β, IL-6, TNF-α expression levels were higher in the DSS group relative to the control group; however, relative to the DSS group, IL-1β, IL-6, TNF-α expression levels were lower in the DSS-TMLP 2% and DSS-TMLP 4% groups. IL-1β and IL-6 protein levels followed the same pattern. These results suggest that the administration of TMLP helps to decrease intestinal inflammation.

In UC patients, inflammation of the colonic mucosa is caused by the overactivation of effector immune cells that produce high levels of inflammatory cytokines, resulting in damage to colon tissue. The classical inflammatory pathway involves the phosphorylation of IκB and NF-κB. Phosphorylation is a crucial regulator of the immune system, and higher p-IκB/IκB or p-NF-κB/NF-κB ratios are associated with significant UC and promote the expression of various inflammatory genes (Atreya et al., 2008). In this study, p-IκB/IκB and p-NF-κB/NF-κB ratios were significantly higher in the DSS group compared with the control group, but in the DSS-TMLP 2% and DSS-TMLP 4% groups, these ratios were significantly lower relative to the DSS group. In summary, these results suggest that dietary administration of TMLP inhibits the progression and exacerbation of intestinal inflammation by inhibiting the NF-κB signaling pathway.

TMLP contains minerals and vitamins such as fatty acids, calcium, zinc, iron, magnesium, riboflavin, pantothenic acid, biotin, chitin, and chitosan (Choi et al., 2020; Son et al., 2021). TMLP has recently shown potential as a medicinal insect, mediating anti-inflammatory (Zielińska et al., 2017), wound healing (Zielińska et al., 2021), and malignant tumor suppression26 effects. Biotin deficiencies induce colitis by activating the NF-κB inflammatory pathway, and biotin supplements are reported to alleviate exacerbated colitis (Jayawardena and, Dudeja, 2020; Skupsky et al., 2020). Riboflavin prevents inflammation of intestinal mucosa in mice with colitis (Levit et al., 2018). It has been proposed that biotin and riboflavin, both components of TMLP, are involved in controlling colitis. Chitosan has a wide range of bioactivities, including anti-cancer, anti-inflammatory, anti-obesity, antioxidant, and nerve protection effects, and is attracting considerable attention in pharmacological and medical applications (Anil, 2022). Chitin extracts alleviate intestinal tissue damage in mice with DSS-induced colitis. Chitin exerts an anti-inflammatory effect by regulating the composition and cytokines in the colonic environment, affecting resident bacterial species (Nagatani et al., 2012).

Because TMLP contains high levels of chitin and chitosan, these components may have exerted anti-inflammatory effects in mice fed TMLP.

In this study, DSS-induced mice were fed TMLP to relieve colitis through anti-inflammatory effects. The various pharmacological effects of TMLP suppressed colitis symptoms in this animal model. However, this study could not show which specific components in TMLP showed anti-colitis effects. Therefore, further in vivo studies of TMLP in food supplements and clinical studies and anti-colitis studies of specific constituents are needed.

Author Contribution Statement

Bo Mi Park: Data collection & Draft thesis; Bock Gie Jung: Data analysis; Jin-A Lee: Revision of the paper; Bong Joo Lee: Supervision.

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Experimental Ethics

Animal testing in this study was approved by the Chonnam National University Animal Experiment Ethics Committee (CNU IACUC-YB-2022-03).

Conflict of interest

The authors declare no conflict of interest.

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