A Mixed Formulation of Lactic Acid Bacteria Inhibits Trinitrobenzene-Sulfonic-Acid-Induced Inflammatory Changes of the Colon Tissue in Mice

Yeon Suk Cha¹, Jae-Gu Seo², Myung-Jun Chung², Chung Won Cho¹, and Hyun Joo Youn¹*

¹School of Biological Sciences, College of Biomedical Sciences and Engineering, Inje University, Gimhae 621-749, Republic of Korea
²Research and Development Center, Cell Biotech Co., Ltd., Gimpo 415-871, Republic of Korea

Introduction

Lactic acid bacteria (LAB) are probiotics that provide numerous beneficial effects on the host body, especially on the intestine. Combining several strains of LAB, we prepared a formulation containing four different LAB and studied its anti-inflammatory activity both in vitro and in vivo. The formulation significantly reduced NO production from RAW 264.7 cells treated with bacterial lipopolysaccharide, indicating that the formulation might include anti-inflammatory activity. The formulation also suppressed inflammatory change induced by trinitrobenzene sulfonic acid (TNBS) in mice, where oral or rectal administration of the formulation protected the colon tissue from the damage by TNBS. Expressions of the IL-6 and FasL genes appeared to be down-regulated by the formulation in TNBS-treated colon tissues, suggesting that the suppression of those genes may be involved in the anti-inflammatory activity of the formulation.

Keywords: Anti-inflammation, probiotics, nitric oxide, IBD, IL-6, FasL

Received: March 31, 2014
Revised: June 1, 2014
Accepted: June 2, 2014
First published online June 9, 2014
*Corresponding author
Phone: +82-55-3203265;
Fax: +82-55-336-7706;
E-mail: mbhjyoun@inje.ac.kr
pISSN 1017-7825, eISSN 1738-8872
Copyright © 2014 by The Korean Society for Microbiology and Biotechnology
process, such as regulatory T cells (Treg) [13]. By increasing IL-10 levels, some LAB were successfully used for IBD patients as an adjunct therapy [12]. In our previous study, a mixed LAB formulation composed of four different LAB was shown to inhibit the development of atopic dermatitis in mice [24], which prompted us to examine the effectiveness of the mixed LAB formulation on other autoimmune-type inflammatory diseases such as IBD. Here, we have studied the anti-inflammatory activity of the mixed LAB formulation in vitro with the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells, and also in vivo using the trinitrobenzene-sulfonic-acid (TNBS)-induced mouse IBD model.

Materials and Methods

Mice and Chemicals

6-week-old female ICR mice were purchased from Hyochang Science (Daegu, Korea) and handled in accordance with the protocols approved by the Inje University Animal Resource Center. Mice were housed in polyethylene cages containing clean wood shavings and given rodent chow and tap water ad libitum. Murine macrophage RAW 264.7 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). Most of the cell culture reagents and chemicals, including TNBS, dexamethasone (Dex), lipopolysaccharide (LPS), Griess reagent, and Dulbecco’s modified Eagle’s medium (DMEM), were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY, USA) and the TACS MTT Assay kit was from R&D Systems (Minneapolis, MN, USA). SV Total RNA Isolation System and SYBR Green master mix were obtained from Promega (Madison, WI, USA). Omniscript Reverse Transcriptase for cDNA synthesis was purchased from Qiagen (Hilden, Germany). The primers for polymerase chain reaction (PCR) were synthesized by Bionics Co. (Seoul, Korea). Optimal cutting temperature (OCT) compound for tissue section preparation was purchased from Sakura (Tokyo, Japan) and Harris hematoxylin and eosin for tissue staining were from Youngdong Pharmaceutical (Yongin, Korea) and Sigma Chemical Co., respectively. The four strains of LAB were obtained from Cell Biotech Co., Ltd. (Gimpó, Korea).

Preparation of the Mixed LAB Formulation

The LAB formulation used in this study, named ATP formulation, was prepared as described previously in the study of the effects of the formulation on an atopic dermatitis model [24]. To make 1 g of ATP formulation, a total of 5 × 10^7 viable cells composed of each 1.25 × 10^7 viable cells of Bifidobacterium lactis (KCTC 11904BP), Lactobacillus casei (KCTC 12398BP), Lactobacillus plantarum (KCTC 10752BP), and Lactobacillus rhamnosus (KCTC 12202BP) were mixed with the filler material containing polydextrose, fructooligosaccharide, glucooligosaccharide, and magnesium stearate, which constituted 85% (w/w) of the formulation.

In Vitro Anti-Inflammatory Activity of ATP Formulation

RAW 264.7 macrophage cells were plated with DMEM/10% FBS and allowed to adhere to the well. The cells were then treated with ATP formulation at the concentrations of 0.02 (1 × 10^3 colony forming units (CFUs)), 0.1 (5 × 10^4 CFUs), and 0.5 mg/ml (2.5 × 10^5 CFUs), and those treated with 0.01 µM dexamethasone were used as an anti-inflammatory control. After an hour of sample treatment, the cells were then exposed to LPS (0.1 µg/ml) for an additional 15 h. The amounts of NO produced were calculated from the nitrite accumulations in the wells using the Griess reaction [8]. Briefly, 100 µl of each culture supernatant was mixed with an equal volume of Griess reagent at room temperature for 10 min, and the nitrite concentration was calculated using the sodium nitrate standard curve. The amount of live cells in each culture was also determined using the TACS MTT Assay (R&D Systems) as described by the manufacturer. Briefly, culture supernatant of each well was replaced by fresh DMEM and followed by the treatment of the MTT reagent until purple precipitates became evident. Following resolution of the precipitates with the detergent solution, absorbance at 595 nm was measured.

In Vivo Anti-Inflammatory Activity of ATP Formulation

The anti-inflammatory activity of the ATP formulation was studied in vivo using TNBS-induced IBD model mice [9].

IBD was induced by the rectal injection of 130 µl of 2% TNBS (dissolved in 50% ethanol) into the descending colon through insertion of a rubber cannula via the anus while the mouse was under anesthesia. The mice were kept in a vertical position for 30 sec and then returned to their cages. Two days after TNBS injection, the mice were sacrificed for the examination of inflammatory changes in the colon tissues. The suppressive effects of the ATP formulation on the inflammatory changes by TNBS have been studied with two independent protocols, using the rectal or the oral administration of the fraction. For the study of the rectal administration of ATP formulation on TNBS-induced colitis, solutions containing the ATP formulation mixed in 2% TNBS were injected into the colon and the degrees of inflammatory changes of the treated tissues were examined. In this protocol, mice were divided into five groups: a control group injected with vehicle (filler) and 50% ethanol; a TNBS-treated control group injected with vehicle and 2% TNBS; and three ATP formulation-treated groups injected with 1 mg (5 × 10^6 CFUs), 2 mg (1 × 10^7 CFUs), or 4 mg (2 × 10^7 CFUs) of ATP formulation mixed in 2% TNBS, respectively. The effects of oral administration of the ATP formulation on the TNBS-induced bowel inflammation were examined using the mice pre-treated with the oral feeding of ATP formulation prior to the rectal injection of TNBS. Briefly, mice were fed with 4 mg (2 × 10^6 CFUs) of ATP formulation three times a day for two consecutive days, which were then followed by the rectal injections of 2% TNBS at 2 h after the final feeding of ATP formulation. In this experiment, mice were divided into three groups: a control group with oral administration of vehicle and rectal injection of 50% ethanol; a TNBS-treated control group with...
A Mixed LAB Formulation Inhibits TNBS-Induced Inflammation

October 2014

Vol. 24

No. 10

oral administration of vehicle and rectal injection of 2% TNBS; and a ATP formulation-treated group with oral administration of 4 mg of ATP formulation and rectal injection of 2% TNBS. All groups in both the rectal and the oral protocols included at least three mice.

Degrees of inflammatory changes of the colon tissues were examined by comparing the thickness of the colon wall, necrotic damage of the colon surface, and histologic analysis of the colon tissue sections. Colon segments from the region 4 cm proximal to the anus were excised and subjected to the measurement of colon wall thickness and the observation of hemorrhagic necrosis on the colon wall surface. The excised colon tissue segments were then preserved in 10% formaldehyde, and the fixed tissues were embedded in OCT compound prior to being sectioned with a cryostat. The 5 µm sections were then stained with hematoxylin and eosin (HE staining) and examined under a light microscope for any disruption of the epithelial mucosa structure and the infiltration of inflammatory cells into the colon tissues.

Effects of ATP Formulation on the Gene Expression in the Colon Tissue

Modulation of the gene expression in the colon tissues treated with TNBS by the ATP formulation was examined using the reverse-transcriptase polymerase chain reaction (RT-PCR). RNAs were prepared from the colon tissue sections of the mice fed with 4 mg of ATP formulation three times a day for two consecutive days and then treated with the rectal injections of 2% TNBS. The RNAs from the tissues of the mice fed with the filler material and treated with 2% TNBS were used as the vehicle-treated control. Total RNAs were prepared using the SV Total RNA Isolation System as described by the manufacturer and then subjected to complementary DNA (cDNA) synthesis with an Omniscript RT kit (Qiagen, Germany). The cDNAs were then amplified using a number of primer sets for the mouse genes known to be involved in the inflammatory process, including the IL-6, FasL, and Fas genes. The primer set TGCACTTGCAGAAAACAATC and TGG TCTTGTGCTTTAGCC was used for IL-6. The primer set CAACAC AAATCTATCTGTCCAGTAAGTGCA was synthesized for FasL, and the set AGGAATTCGTGTGAA CATGGAACCC and AGAAGCTTCACTCCACAGTCTTCGT was for Fas. The primer set GGCAACAGGTTGTGATGG and ACGGTGCGCTTGGGTTC was selected for β-actin. PCRs were carried out using the following conditions: one cycle of 94ºC for 5 min; 30 cycles of denaturation at 94ºC for 4 sec, annealing at 60ºC for 4 sec, and elongation at 72ºC for 45 sec; and an extension cycle at 72ºC for 10 min.

Statistical Analysis

Data were evaluated using Student’s t-test, and p < 0.05 was considered as statistically significant.

Results

Effect of ATP Formulation on NO Release from the LPS-Treated RAW 264.7 Cells

NO production from RAW 264.7 cells treated with LPS is a useful model system for the study of inflammation in vitro, where the ATP formulation significantly suppressed NO production in a dose-dependent manner (Fig. 1A). Compared with the NO amount from the cells treated with LPS alone, the cells treated with 2 or 4 mg/ml of ATP formulation showed an almost 2-fold decrease in NO production (Fig. 1A-e and 1A-f), which suggests that the formulation may include anti-inflammatory activity. In order to see if the ATP formulation could influence the amount of NO production in the cultures through the modulation of cell growth, the viabilities of the RAW 264.7 cells in the cultures were examined with the MTT reduction assay. As shown in Fig. 1B, there were no significant

![Fig. 1. NO production from RAW 264.7 cells treated with ATP formulation (A) and viabilities of the cells in the cultures (B).](image-url)
differences in the viable cell density between the culture conditions (Fig. 1B), indicating that the concentrations of ATP formulation used in the study just modulated the NO production process without affecting the number of cells in the cultures. This result shows that the ATP formulation exhibits anti-inflammatory activity in vitro, and suggests that the formulation may play a role in the development of inflammatory diseases in vivo.

**Suppression of the TNBS-Induced Bowel Inflammation by the Rectal Injection of ATP Formulation**

Whereas the mice treated with TNBS alone showed IBD-like changes such as inflammation and hemorrhage of the colon tissue around the TNBS-treated area, those receiving the rectal injection of the mixture of TNBS and ATP formulation did not show any of those inflammatory changes (Fig. 2). The colon walls from the mice treated with TNBS alone showed significant thickening (Fig. 2A-b), whereas those from the mice co-injected with TNBS and 2 or 4 mg ATP formulation did not present any significant colon wall thickening (Fig. 2A-d and 2A-e), indicating that the ATP formulation protected the colon tissue from the TNBS-induced edematous change. Examination of the colon wall surface revealed that the co-injection of TNBS and ATP formulation blocked necrotic damage induced by TNBS (Fig. 2B). Whereas the colon wall surfaces of the mice treated with TNBS alone appeared to be very dark, probably due to the hemorrhagic necrosis of the colon tissues (Fig. 2B-b), those of the mice co-injected with TNBS and 2 or 4 mg ATP formulation (Fig. 2B-d and 2B-e) turned out to be as clean as the walls of the TNBS-untreated control mice (Fig. 2B-a). All these observations indicate that the ATP formulation prevented the development of inflammatory changes induced by TNBS in a dose-dependent manner.

**Suppression of the TNBS-Induced Bowel Inflammation by the Oral Administration of ATP Formulation**

The effects of the oral feeding of ATP formulation on the TNBS-induced colitis were also examined in order to see whether ATP formulation modulated the bowel tissue of the mice to suppress the inflammatory changes by TNBS. In this protocol, mice were pre-treated with the oral feeding of 4 mg of ATP formulation three times a day for two consecutive days and then treated with rectal injection of TNBS. When examined for wall thickness, surface necrosis, epithelial mucosa integrity, and inflammatory cell infiltration, the mice pre-treated with the oral feedings of ATP formulation showed significant inhibition of the inflammatory changes induced by TNBS (Fig. 3). Compared with the TNBS-treated control mice (Fig. 3A-b), mice fed with the ATP formulation showed significant reduction in the colon wall thickness (Fig. 3A-c), almost to the level of the TNBS-untreated controls (Fig. 3A-a). Examination of the colon wall surfaces also did not show any significant necrotic damage by TNBS in the mice pre-treated with the formulation (Fig. 3B). Whereas the mice treated with rectal injection of TNBS alone developed strong dark areas on the wall surfaces due to the hemorrhagic necrosis of the colon tissues (Fig. 3B-b), those fed with the ATP formulation prior to TNBS treatment did not show any damage on the wall surfaces (Fig. 3B-c) as in those of the TNBS-untreated controls (Fig. 3B-a). The oral feeding of ATP formulation also suppressed both the ulcerative damage of the colon...
epithelial mucosa and the leukocyte infiltration into the colon tissue induced by the rectal injection of TNBS (Fig. 3C). The colon epithelial mucosa of the mice treated with TNBS presented complete destruction of epithelial structure (Fig. 3C-b), whereas those of mice fed with the ATP formulation did not show any damage by TNBS (Fig 3C-c). These findings indicate that the oral feeding of ATP formulation could modulate the colon tissue to block the TNBS-induced inflammatory development and tissue damage.

**ATP Formulation May Suppress the TNBS-Induced Bowel Inflammation Through the Down-Regulation of IL-6 and FasL Expression**

We have examined the effect of the ATP formulation on the transcription levels of pro-inflammatory or immunomodulatory genes in the colon tissue in order to have an idea on the anti-inflammatory activity of the formulation. After mice were treated with oral feeding of the ATP formulation or vehicle and following rectal injection of TNBS, RNAs were prepared from the colon tissue sections near the TNBS injection sites and gene expression changes were examined (Fig. 4). The reverse-transcriptase PCR study showed that the colon tissues from the mice treated with ATP formulation and TNBS showed strong down-regulation of IL-6 and FasL gene expression (Fig. 4A-b), compared with the expression levels of the genes in the mice treated with TNBS alone (Fig. 4A-a).
Discussion

NO produced by macrophages plays a role in the inflammatory response, and thus NO production from LPS-treated RAW 264.7 macrophage cells is a good model system for the study of inflammation in vitro [21]. Using this in vitro system, we were able to identify that the ATP formulation containing four probiotic LAB strains significantly suppressed NO production in a dose-dependent manner, indicating that the formulation presents anti-inflammatory activity. Considering that IBD includes complex autoimmune diseases involving increased luminal activities of nitric oxide and inflammatory processes [2], we decided to study if the formulation could block inflammatory development in mice treated with TNBS, a mouse model of a human IBD. In both studies, using the rectal injection and the oral feeding of ATP formulations, the formulation prevented the colon wall thickening as well as hemorrhagic necrosis induced by TNBS. Histologic studies of the colon tissues also showed that the formulation blocked ulcerative damage of the colon epithelial mucosa and leukocyte infiltration into the colon tissue. Since the known characteristics of IBD involve NO production, colon wall thickening, hemorrhagic necrosis, leucocytes infiltration, and epithelial mucosa disruption [2, 11], all these findings clearly indicate that the ATP formulation effectively inhibited the inflammatory process and damage induced by TNBS. The findings from the oral-feeding protocol, where the feeding of ATP formulation blocked the inflammatory changes by the rectal injection of TNBS, suggest that the feeding of ATP formulation provided enough probiotics into the colon tissue and modulated the micro-environment of the tissue to resist inflammatory changes induced by the rectal injection of TNBS.

Considering that cytokines and other immune-modulators are involved in the regulation of inflammatory response in IBD [28, 30], we have examined the genes whose expressions were modulated by the ATP formulation, in order to imagine how the formulation showed the anti-IBD activity. Among the genes up-regulated by TNBS, the expressions of IL-6 and FasL appeared to be suppressed by the feeding of ATP formulation, while the expression of Fas remained unchanged. Since the expression levels of IL-6 and FasL were increased in human IBD [1, 32], the down-regulations of those genes by the ATP formulation in TNBS-treated tissues could explain the protective anti-inflammatory activity of the formulation. It is also interesting that FasL is involved in the production of IL-6 [31]. IL-6 is a pro-inflammatory cytokine and plays an important role in the inflammatory development of IBD [17], which indicates that the ATP formulation blocks inflammatory changes by TNBS through modulation of the IL-6-induced inflammatory process. In addition to the pro-inflammatory activity of IL-6, IL-6 could also contribute to the survival of tissue-damaging effector T cells and the perpetuation of chronic intestinal inflammation [1], which also supports the protective effect of the down-regulation of IL-6 by the ATP formulation. In addition to the role of FasL on the IL-6 expression [31], FasL could be directly involved in the TNBS-induced tissue damage. Since FasL is a death receptor signaling effector-T-cell-mediated cell death [27, 29], the down-regulation of FasL by the ATP formulation could be responsible for the reduction of the interaction of FasL on the colon tissue cells with Fas on the infiltrating lymphocytes [15]. In addition to the direct role of FasL on cell death, the decreased FasL expression by the ATP formulation could not provide a co-stimulatory signal for the Fas-expressing effector T cell development and might not lead to effector T cell activation [14, 18, 25].

In this study, a formulation containing mixed LAB strains was found to have some protective effect on the development of inflammatory changes in an IBD model, which could be exploited to develop probiotic formulations for the treatment or prevention of IBD. Since the long-term consumption of IBD medications itself becomes a great threat to patients [26], protective formulations like the ATP formulation also can be used as an adjunct therapy for IBD patients to reduce the amount of the medications or potentiate the effect of the conventional treatments.

Acknowledgments

This work was supported by a 2011 Inje University research grant. The authors wish to express their thanks to Dr. Jung Suk Hwang of Changwon Samsung Hospital for her help in the histologic analysis of the mouse colon tissues.

References
