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Lactobacillus plantarum HY7714 Restores TNF-α Induced Defects on Tight Junctions.

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ABSTRACT: In addition to intestinal balance, probiotics are known to have beneficial effects on skin inflammation, metabolic diseases, and emotions. Previously, we have reported the skin anti-aging effects of *Lactobacillus plantarum* HY7714 (HY7714) in a clinical trial. To prove the protective skin effects of HY7714 through the intestinal tight junction (TJ), we investigated the effects of HY7714 on the intestines through tumor necrosis factor (TNF)-α induced TJ defects in Caco-2 cells. Specifically, 24 h treatment with HY7714 restored the decreased expression of zonula occludens-1, occludin, and claudin-1 compared to the TNF-α-treated groups ($P<0.05$). It also attenuated the level of pro-inflammatory cytokines interleukin-6, 8, and 1β. Further, increases in the mRNA levels of Elk-1, nuclear factor-κB, and myosin light chain kinase expression induced by TNF-α were recovered by HY7714. These findings imply that HY7714 improves intestinal barrier integrity and is a potential therapeutic agent for dysfunctions derived from TJ defects.

Keywords: lactic acid bacteria, Caco-2 cells, tight junctions, anti-inflammation

INTRODUCTION

Many studies have investigated additional beneficial effects of probiotics, other than gut health, including the immune system (1), metabolic diseases (2), and brain function (3). Probiotics also appear to improve skin health (4). In orally fed mice, probiotics suppressed ultraviolet (UV)-induced wrinkle formation and skin elasticity by regulating elastase activity and interleukin (IL)-1β levels (5,6). In human clinical trials, probiotic supplementation contributed to alleviation of atopic dermatitis (7-10). We previously reported that HY7714 was efficacious in skin protection from wrinkle formation and transepidermal water loss. In hairless mice, UVB-induced wrinkle-related genes matrix metalloproteinase (MMP)-13, MMP-2, and MMP-9 were suppressed, and wrinkle formation was inhibited by oral administration of HY7714 (11). UV irradiation-induced decreases in skin hydration and ceramide level were restored by HY7714 feeding (12). It has been confirmed that oral consumption of HY7714 increases skin hydration and alleviates facial wrinkling in clinical trials (13).

Intercellular junctional complexes maintain the intestinal epithelium integrity, and this barrier maintains intestinal homeostasis and provides protection from the external environment (14,15). Among junctional complexes, tight junctions (TJ) play an essential role to strengthen this system and regulate intestinal permeability. Their maintenance is controlled by various stimuli, such as cytokines, pathogens, and their toxins (16). Tumor necrosis factor (TNF)-α enhances intestinal permeability and disrupts TJ integrity via a myosin light chain kinase (MLCK)-dependent process and/or regulation of tight junction protein expressions, such as zonula occludens (ZO)-1, occludin, and claudins, through nuclear factor (NF)-κB and extracellular regulated protein kinase (ERK) 1/2 signaling pathways (17-23). Therefore, suppression of TNF-α induced reaction could stabilize TJ integrity and intestinal permeability and its relative dysfunctions (24).

A pro-inflammatory cytokine is a signaling molecule secreted from immune cells and promotes inflammation (25). It has been found to regulate tight junction permeability in the intestinal epithelium. IL-6 induces claudin-2 expression and decreases ZO-1 stability (26,27). IL-1β suppresses the level of occludin (28), and IL-8 per-

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turbs TJ function (29).

We investigated the mechanism of the Lactobacillus for supporting the skin protection effects of HY7714 through the intestines by regulating TJ permeability and integrity, using Caco-2 human intestinal monolayer cells.

MATERIALS AND METHODS

Isolation and preparation of lactic acid bacteria (LAB)

Lactobacillus plantarum HY7714 was isolated from the breast milk of healthy women. For the *in vitro* assay, it was inoculated in de Man-Rogosa-Sharpe broth (BD Biosciences, Franklin Lakes, NJ, USA), cultured at 37°C for 20 h, harvested by centrifugation (1,500 *g*, 10 min), washed twice with sterile phosphate-buffered saline, and resuspended to a final concentration of 1×10^{10} colony-forming unit (CFU)/mL. Subsequently, the bacteria were heat-treated (100°C, 15 min) and stored at −20°C until further use.

Cell culture

Caco-2 human intestinal adenocarcinoma monolayer cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and were cultured in Eagle's minimum essential medium supplemented with 20% fetal calf serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in a humidified atmosphere of 5% CO_2 : 95% air at 37°C. Cells were harvested with trypsin/ethylenediaminetetraacetic acid, transferred to a 6-well plate (1×10^5 cells/well), and grown for 21 days to reach differentiation. Growth medium was refreshed every 2~3 days.

HY7714 effects on TJ and inflammatory cytokines

Fully differentiated Caco-2 cells were serum-deprived overnight and treated for 24 h with 1×10^8 CFU/mL of heat-treated *L. plantarum* HY7714 and 100 ng/mL of recombinant human TNF-α (Sigma-Aldrich Co., St. Louis, MO, USA). Cells without HY7714 and TNF-α treatments were designated as the normal group (NG), and those treated with TNF-α only as the negative group (Neg). All tests were performed with nine repeats.

Enzyme-linked immunosorbent assay (ELISA)

After a 24 h treatment, the amount of TJ proteins [ZO-1, occludin, and claudin-1 (CUSABIO, Houston, TX, USA)] and inflammatory cytokines [IL-6, IL-8, and IL-1β (BD Biosciences)] in the culture medium of Caco-2 cells was measured by ELISA kit according to the manufacturer's instructions.

RNA extraction

RNA was extracted from the adherent component of Caco-2 cells after 24 h treatment and collected using an Easy-spin Total RNA Extraction kit (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions.

Reverse transcription and quantitation of gene expression using real-time polymerase chain reaction (PCR)

Total RNA (2 μg) was reverse transcribed to cDNA using the Omniscript RT kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cDNA served as a template for PCR amplification using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and specific TaqMan primers (Applied Biosystems; Table 1) under the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. For assessment of relative quantities, the gene amounts were first normalized to a housekeeping gene, glyceraldehyde 3 phosphate dehydrogenase, followed by normalization to NG. Data are representative of three independent experiments.

Statistical analysis

Experiments were performed at least three times, and data are presented as the mean±standard deviation (SD) of triplicate preparations. Differences between groups were evaluated with the unpaired Student's *t*-test, and they were deemed statistically significant at $P<0.05$.

RESULTS

L. plantarum HY7714 rescues TNF-α induced reduction of TJ proteins in Caco-2 cells.

ZO-1, occludin, and claudin-1 are essential structural proteins of TJ and regulate its permeable barrier. The expression of ZO-1 decreased after TNF-α treatment by 23.8% relative to NG ($P<0.01$) and was restored with

Table 1. Primers used for reverse transcription PCR

Gene	Catalog number	Reference	Exon boundary	Assay location
Elk-1	Hs00901847_m1	NM_001114123.2	3~4	535
		NM_001257168.1	3~4	535
		NM_005229.4	2~3	429
NF-κB	Hs00230071_m1	NM_001005474.2	6~7	1,322
		NM_031419.3	5~6	1,455
ZO-1	Hs01551861_m1	NM_001301025.1	13~14	1,762
		NM_001301026.1	13~14	1,777
		NM_003257.4	12~13	1,998
		NM_175610.3	12~13	1,998
GAPDH	Hs02758991_g1	NM_001256799.2	6~7	752
		NM_001289745.1	7~8	810
		NM_001289746.1	6~7	704
		NM_002046.5	7~8	718

NF-κB, nuclear factor-κB; ZO-1, zonula occludens-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

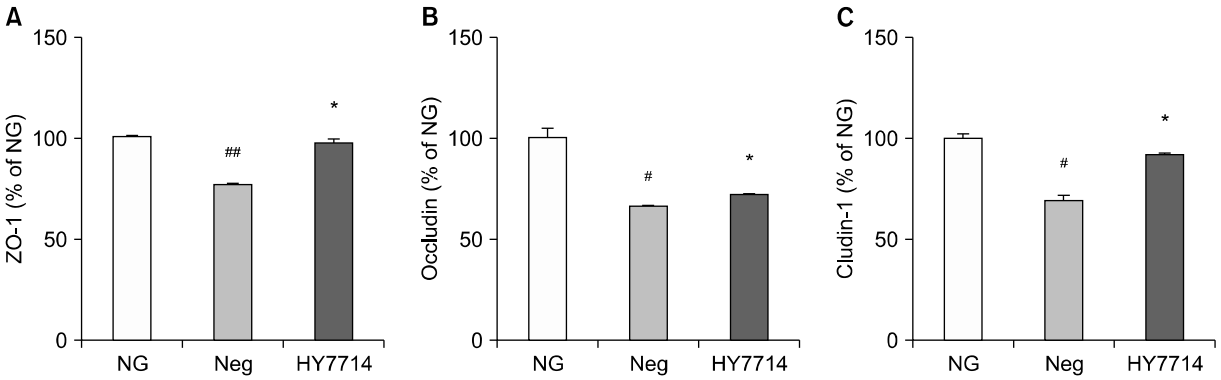

Fig. 1. The effect of HY7714 on (A) zonula occludens (ZO)-1, (B) occludin, and (C) claudin-1 expression in tumor necrosis factor (TNF)-α-induced Caco-2 cells. Cells without TNF-α and *Lactobacillus plantarum* HY7714 treatments are designated as the normal group (NG), TNF-α only treatment as the negative group (Neg), and the TNF-α and *L. plantarum* HY7714 treatment (HY7714) group. Values are expressed as % of NG and mean±SD (n=9). Hashtags indicate a significant difference between NG and Neg at $^{\#}P<0.05$ and $^{\#\#}P<0.01$. Asterisks indicate a significant difference between Neg and HY7714 at $^{*}P<0.05$.

HY7714 treatment ($P<0.05$ vs. Neg) (Fig. 1A). Occludin and claudin-1 expressions also decreased after TNF-α treatment by 34% and 31.3%, respectively, relative to NG ($P<0.05$) and were rescued by HY7714 treatment ($P<0.05$ vs. Neg) (Fig. 1B and 1C).

L. plantarum HY7714 decreases TNF-α induction of pro-inflammatory cytokines.

Pro-inflammatory cytokines promote intestinal inflammation and induce TJ permeability (25). TNF-α treatment increased IL-6 expression by 2.9-fold compared to NG ($P<0.01$), which was decreased by treatment with HY7714 ($P<0.01$) (Fig. 2A). Other cytokines IL-8 and IL-1β were also induced 4.8- and 9.6-fold by TNF-α treatment, respectively, compared to NG ($P<0.01$) and reduced with HY7714 treatment ($P<0.05$ and $P<0.01$, respectively) (Fig. 2B and 2C).

Effects of *L. plantarum* HY7714 on TNF-α-induced activation of Elk-1 and NF-kB pathways

Elk-1 and NF-κB pathways promote TJ permeability in intestinal epithelial cells (23). TNF-α induced a 20% and 22% increase in Elk-1 and NF-κB expression, respectively, in Caco-2 cells compared with NG ($P<0.05$ and $P<0.01$). Further, these were attenuated by HY7714 treatment ($P<0.05$ and $P<0.01$ vs. Neg) (Fig. 3A and 3B).

L. plantarum HY7714 attenuates TNF-α-induced MLCK expression.

MLCK expression (induced by Elk-1 and NF-κB) facilitates MLC phosphorylation, which stimulates disruption of actin-myosin structures (30). In addition, MLCK drives paracellular permeability in the intestines (31,32). TNF-α induced a 16% increase in MLCK expression in Caco-2 cells compared with NG ($P<0.05$) (Fig. 3C), which was attenuated by HY7714 treatment ($P<0.05$ vs. Neg).

DISCUSSION

Intestines have a defensive barrier from the external environment, and this permeable barrier is sealed by TJ

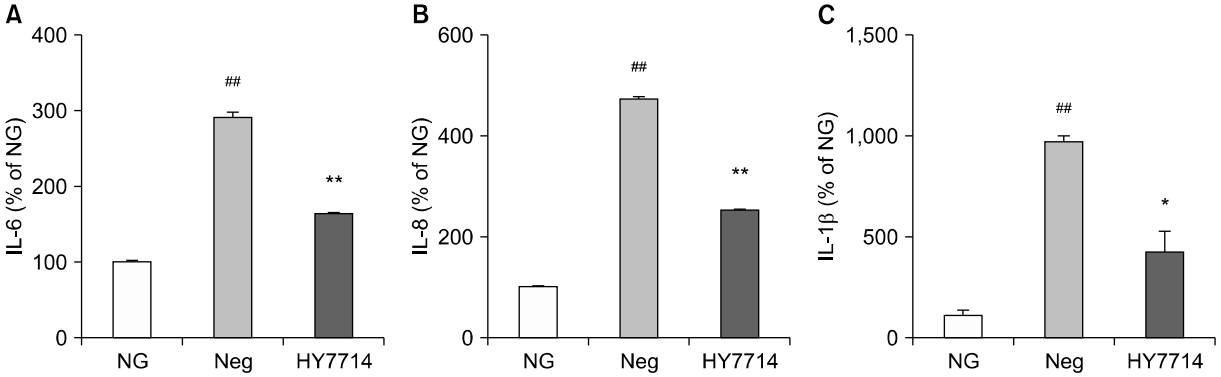

Fig. 2. The effect of HY7714 on (A) interleukin (IL)-6, (B) IL-8, and (C) IL-1β expression in tumor necrosis factor (TNF)-α-induced Caco-2 cells. Cells without TNF-α and *Lactobacillus plantarum* HY7714 treatments are designated as the normal group (NG), TNF-α only treatment as the negative group (Neg), and the TNF-α and *L. plantarum* HY7714 treatment (HY7714) group. Values are expressed as % of NG and mean±SD (n=9). Hashtags indicate a significant difference between NG and Neg at $^{\#\#}P<0.01$. Asterisks indicate a significant difference between Neg and HY7714 at $^{*}P<0.05$ and $^{**}P<0.01$.

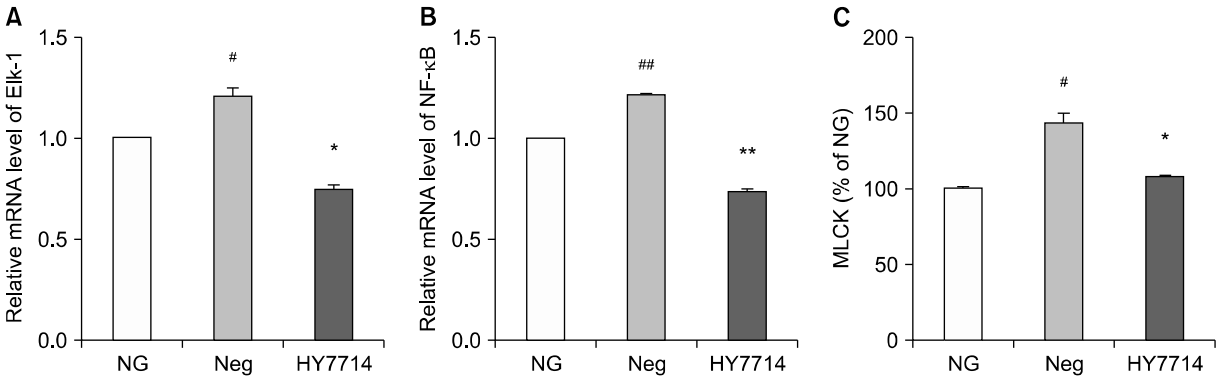

Fig. 3. mRNA expression of (A) Elk-1, and (B) nuclear factor (NF)-κB genes in Caco-2 cells treated with HY7714. Values normalized to glyceraldehyde-3-phosphate dehydrogenase are expressed as fold of NG and mean±SD (n=9). (C) The effect of HY7714 on myosin light chain kinase (MLCK) expression in TNF-α-induced Caco-2 cells is expressed as % of NG and mean±SD (n=9). Cells without TNF-α and *Lactobacillus plantarum* HY7714 treatments are designated as the normal group (NG), TNF-α only treatment as the negative group (Neg), and the TNF-α and *L. plantarum* HY7714 treatment (HY7714) group. Hashtags indicate a significant difference between NG and Neg at $^{\#}P<0.05$ and $^{\#\#}P<0.01$. Asterisks indicate a significant difference between Neg and HY7714 at $^{*}P<0.05$ and $^{**}P<0.01$.

structures, which consist of TJ proteins (e.g., ZO-1, occludins, claudins, and junctional adhesion molecules) and the actin cytoskeleton (1,2,33). When its integrity is disrupted, perturbations may occur in the inflammatory and immune system, which is related with diseases such as diarrhea, edema, and cancer (34,35). The ERK1/2 and NF-κB pathways are led by activated Elk-1 and NF-κB p65, which bind to the MLCK promoter region to activate MLCK gene transcription (23,36). These pathways increase the TJ permeability by up-regulating MLC phosphorylation, which promotes disassembly of actin-myosin complexes (37). The ERK1/2 and NF-κB pathways also suppress expression of tight junction proteins (23) and decrease the integrity of TJ, thus increasing permeability. In this study, TNF-α increased the levels of Elk-1 and NF-κB and MLCK expression in Caco2-cells. When treated with HY7714, this induction was suppressed around the NG level.

Cytokines are mostly produced from immune cells when activated by antigens or oxidative stress. They trigger the secretion of other cytokines (38-40) and oxidative stresses, which stimulate an immunoresponse (41,42). They also negatively impact TJ integrity (25). IL-6 acts as a modulator of gut barrier functions and increases TJ permeability. Mice injected with IL-6 showed increases in claudin-2 expression, which reduced TJ integrity through the MEK/ERK and PI3K pathways in intestinal epithelial cells (26). IL-8 and IL-1β are also responsible for TJ permeability by causing intestinal barrier dysfunctions (28,29). HY7714 showed potential protective activity for TJ integrity as its treatment recovered IL-6, IL-8, and IL-1β overexpression induced by TNF-α.

In recent years, several beneficial effects of probiotics other than gut health have been reported. Probiotics have been shown to suppress *Helicobacter pylori* (43) and regulate immune systems (1), metabolic diseases (2), and emotions (3). Previously, we reported skin anti-photoaging efficacy of HY7714 ingestion in an *in vivo* assay. HY7714 has been shown to control gene expression related to skin hydration and wrinkles (11,12). Further, it has demonstrated efficacy in human clinical trials. Participants treated with 1×10^{10} CFU/d of HY7714 for 12 weeks showed improvements in skin hydration, facial wrinkling, elasticity, and skin gloss versus before supplementation (13). Further studies are needed to reveal the effects of HY7714 in the intestines through changes in the microbiota and pro-inflammatory cytokines after HY7714 ingestion.

HY7714 protects against TNF-α-induced defects on TJ by regulating MLCK pathway-associated Elk-1 and NF-κB activation and restores ZO-1, occludin, and claudin-1 protein expression. It also repressed IL-6, IL-8, and IL-1β, which impair the tight junction barrier. These results implicate HY7714 as a candidate therapeutic agent for dysfunctions related to TJ damage.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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