Oral Administration of *Lactobacillus plantarum* HY7714 Protects Hairless Mouse Against Ultraviolet B-Induced Photoaging

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Introduction

Various environmental stressors, including smoking and ultraviolet (UV) radiation, induce skin damage, resulting in skin aging [31, 36]. Out of the stressors that induce skin aging, direct exposure to UV radiation, especially its UVB component, is tightly related to a particular type of skin damage called photoaging, which is characterized by coarse and fine wrinkles, dryness, laxity, pigmentation, and increased skin thickness [11, 20, 24].

Ultraviolet (UV) irradiation alters multiple molecular pathways in the skin, thereby inducing skin damage, including photoaging. In recent years, probiotics have gained interest due to their beneficial effects on skin health, such as inhibiting atopic dermatitis and improving skin immunity or inflammation. However, little is known about the effects of probiotics on UVB-induced photoaging. In this study, we evaluated the effect of *Lactobacillus plantarum* HY7714 against UVB-induced photoaging in human dermal fibroblasts and hairless mice. The results showed that *L. plantarum* HY7714 treatment effectively rescued UVB-reduced procollagen expression through the inhibition of UVB-induced matrix metalloproteinase (MMP)-1 expression in human dermal fibroblasts. Data from a western blot showed that *L. plantarum* HY7714 inhibited the phosphorylation of Jun N-terminal kinase, thereby suppressing the UVB-induced phosphorylation and expression of c-Jun. Oral administration of *L. plantarum* HY7714 clearly inhibited the number, depth, and area of wrinkles in hairless mouse skin. Histological data showed that *L. plantarum* HY7714 significantly inhibited UVB-induced epidermal thickness in mice. Western blot and zymography data also revealed that *L. plantarum* HY7714 effectively inhibited MMP-13 expression as well as MMP-2 and -9 activities in dermal tissue. Collectively, these results provide further insight regarding the skin biological actions of *L. plantarum* HY7714, a potential skin anti-photoaging agent.

**Keywords:** Anti-wrinkle, *Lactobacillus plantarum*, photoaging, probiotic, ultraviolet B (UVB)
the collagenase matrix metalloproteinase (MMP)-1 by UVB exposure [25, 30]. UVB triggers cellular signaling pathways through cell surface receptors or secondary messengers, such as reactive oxygen species, to phosphorylate kinases, including Jun N-terminal kinase (JNK), p38, and extracellular regulated kinases (ERKs) [8, 15]. These kinases eventually stimulate transcriptional activities, thereby contributing to the regulation of target genes involved in skin photoaging, such as MMPs. Activator protein (AP)-1 is the major MMP transcriptional factor in UVB-induced skin. AP-1 is a homo- or heterodimer of Jun or Fos in mammalian cells, and the activity or expression of each subunit is stimulated by an upstream kinase such as MAPKs [2, 27]. MMPs are a family of zinc-dependent endopeptidases expressed in several cell types that degrade macromolecules from the extracellular matrix (ECM) [28]. Among these enzymes, MMP-1 (interstitial collagenase) plays an important role in the degradation of native types I and III collagen in human skin [25]. Once cleaved by MMP-1, collagen can be further broken down into small peptides by MMP-9, resulting in ECM degradation. Reduction in collagen fibers is an important mechanism involved in the development of skin aging [16].

The role of probiotics in regulating intestinal health has been widely studied for over a century [9]. After ingestion, probiotics exert their biological effects through adhesion to intestinal epithelial cells, and contribute to a decrease in harmful bacteria and intestinal inflammation. Modulation of the intestinal microbiota is one of the important functions of probiotics, which is deeply associated with the modulation of the intestinal immune system, improving bowel movement and decreasing allergy [13, 21]. However, in recent years, several lines of evidence suggest that some bacterial probiotics can modulate the skin immune system [3, 12, 31]. In human clinical trials, probiotic supplementation showed potential in the relief of atopic dermatitis and dry skin [7, 10, 14, 26]. Dietary supplements containing a specific probiotic with several components from natural plants protected against the early damage induced by UV exposure, through regulating immune cells and inflammatory cytokines in humans [3]. In addition to protecting against cutaneous photo damage, it has been suggested that a specific strain of lactic acid bacteria showed anti-aging effects such as reduction in wrinkle formation and improved elasticity in hairless mice [31]. However, only a few studies were designed to determine the anti-photoaging effects and mechanisms of probiotics on UVB-irradiated skin. Thus, the present study was designed to investigate the anti-photoaging activity and molecular mechanisms of Lactobacillus plantarum HY7714 on UVB-irradiated skin, both in vitro and in vivo.

Materials and Methods

Preparation of Bacteria for In Vitro and In Vivo Experiments

The four lactic acid bacterial strains used in the present study were isolated from healthy infant feces or breast milk. For the in vitro assay, these strains were inoculated in Man Rogosa Sharpe (MRS; BD; Sparks, MD, USA) broth, cultured at 37°C for 24 h, harvested using centrifugation (1,500 × g, 10 min), washed twice with sterile phosphate-buffered saline (PBS), and resuspended to a final concentration of 10⁹ CFU/ml. The bacteria were then heat-treated (100°C, 15 min) and stored at −20°C until use. For the in vivo assay, L. plantarum HY7714 was harvested as described above and resuspended at a final concentration of 1 × 10⁹ CFU/ml in sterile PBS.

Reagents

Antibodies against MMP-1, MMP-13, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against phosphorylated extracellular regulated kinases (ERKs), total ERKs, phosphorylated p38, total p38, phosphorylated JNKs, total JNKs, phosphorylated c-Jun, and c-Jun were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

Hs68 human dermal fibroblasts were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in monolayers at 37°C in a 5% CO₂ incubator in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

UVB-Exposure of Hs68 Cells

Hs68 cells were seeded on a six-well plate at a density of 2 × 10⁴ cells per well and grown in a culture medium for 24 h to reach 80% confluence. The cells were starved in serum-free medium for another 24 h and exposed to UVB at 50 mJ/cm² dose. The cells were then immediately treated with the test materials. UVB treatment was performed using Ultraviolet Crosslinkers (UVP; Upland, CA, USA) with a peak emission at 302 nm, and the distance from the UVB source to the plates was about 12.7 cm. Following irradiation, the cells were incubated for 15 min or 48 h and harvested for in vitro studies.

Enzyme-Linked Immunosorbent Assay (ELISA)

Hs68 cells were cultured in a 24-well plate (5 × 10⁴ cells/well) for 24 h. They were then treated with several lactic acid bacteria at 5 × 10⁶ CFU/ml for 24 h. The cell culture medium was collected, and type I procollagen was quantified using a procollagen type I C-peptide enzyme immunoassay kit (Takara, Shiga, Japan).

Western Blot Analysis

For in vitro western blotting, cells were grown to confluence in 6-well plates or 100 mm plates, washed two times with PBS, and
lysed in radioimmunoprecipitation assay (RIPA) buffer. The protein concentration was determined using a DC assay kit (Bio-Rad Corp., Hercules, CA, USA). Lysate protein (10 µg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to a polyvinylidene difluoride membrane. After blotting, the membrane was incubated with the specific primary antibody at 4°C overnight. Protein bands were visualized using a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody. For the in vitro test, to isolate proteins from the mouse skin, the dorsal skin of each mouse was excised and blended in RIPA buffer using a homogenizer. Proteins were analyzed as described above for the in vitro western blot assay. The relative amounts of proteins associated with specific antibodies were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Animals and Experimental Design
Five-week-old female hairless mice were purchased from Central Lab Animal Inc. (Seoul, Korea). The mice were maintained in climate-controlled quarters (at 24°C, 50% humidity) with a 12 h light/12 h dark cycle. The animal protocol used in this study was reviewed and approved based on ethical procedures and scientific care by the Ethics Committee at the R&BD Center of the Korea Yakult Company Limited (KYIAUC-11-04-0231-Y). The mice were divided into control group (n = 8), a UVB-only treatment group (n = 8), and a UVB plus L. plantarum HY7714 treatment group (n = 8). The mice in the control and UVB-only treatment groups were orally administered 100 µl of PBS. The mice in the UVB plus L. plantarum HY7714 treatment group were orally administrated 100 µl of PBS containing 1 × 10⁹ CFU of L. plantarum HY7714/mouse daily, 1 h prior to UVB irradiation.

Mouse UVB Irradiation
The distance from the UV source to the skin was about 12.7 cm, irradiation and performed using Ultraviolet Crosslinkers (UVP: Upland, CA, USA) with a peak emission at 302 nm. UVB radiation was applied to the back of the mice three times per week for 12 weeks. The starting dose of UVB radiation was 25 mJ/cm² (1 minimal erythematous dose [MED]) during the first week, and the dose was increased weekly by 1 MED (25 mJ/cm²) until reaching 4 MED (100 mJ/cm²), which was maintained until 12 weeks. Body weights were recorded weekly. Replica preparation was performed at the 12th week of radiation exposure.

Production of Replicas and Image Analysis
Replicas of mouse dorsal skin were constructed using SILFLO Corp., Hercules, CA, USA). Lysate protein (10 µg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to a polyvinylidene difluoride membrane. After blotting, the membrane was incubated with the specific primary antibody at 4°C overnight. Protein bands were visualized using a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody. For the in vitro test, to isolate proteins from the mouse skin, the dorsal skin of each mouse was excised and blended in RIPA buffer using a homogenizer. Proteins were analyzed as described above for the in vitro western blot assay. The relative amounts of proteins associated with specific antibodies were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Results

Lactic Acid Bacteria Increase Procollagen Production in Hs68 Cells
Decrease in type I collagen, the primary component of the ECM, which supports the skin dermis, is a main cause for photoaging [36]. Thus, we first examined the activity of various probiotics isolated from healthy infant feces or breast milk on procollagen production in Hs68 cells to select the probiotic that presented the strongest anti-photoaging activity. Among the probiotics tested, L. plantarum HY7714 potently increased procollagen secretion by 32% in the cells, whereas other probiotics had no significant effect (Fig. 1). All of the probiotics tested showed no cytotoxic activities with 10-fold more concentration in the Hs68 cell line (data not shown).

L. plantarum HY7714 Rescues UVB-Reduced Procollagen Expression and Suppresses UVB-Induced MMP-1 Expression in Hs68 Cells
UV radiation degrades collagen fibers and causes the collapse of the dermal structure, followed by an acceleration of the aging process. Because the induction of interstitial collagenase (MMP-1) by UVB exposure is a prominent factor in the loss of collagen fibers, resulting in coarse wrinkles, MMP-1 has been considered as the primary...
marker for skin photoaging [5]. Thus, to evaluate the effects of *L. plantarum* HY7714 on procollagen or MMP-1 expression in UVB-irradiated dermal fibroblasts, we performed western blot analysis. The result showed that UVB irradiation clearly inhibited procollagen expression in Hs68 cells, and that *L. plantarum* HY7714 effectively rescued procollagen expression in a dose-dependent manner (Fig. 2A).

UVB exposure also induced a 2-fold increase in MMP-1 expression compared with that of normal cells. In contrast, *L. plantarum* HY7714 notably suppressed UVB-induced MMP-1 expression in a dose-dependent manner (Fig. 2B).

**L. plantarum** HY7714 Suppresses UVB-Induced Phosphorylation of JNK/c-Jun, but Not ERK and p38, in Hs68 Cells

Numerous reports suggested that UVB irradiation activates MAPKs, including ERK, JNK, or p38, resulting in the induction of MMP-1 expression in dermal cells. Therefore, to elucidate the mechanisms involved in the effects of *L. plantarum* HY7714 on UVB-induced MMP-1 expression, we examined whether *L. plantarum* HY7714 blocked the activation of the ERK, JNK, or p38 pathways stimulated by UVB in Hs68 cells. Western blotting data showed that *L. plantarum* HY7714 considerably inhibited UVB-induced phosphorylation of JNK at doses of 5 × 10⁷ CFU/ml and 5 × 10⁸ CFU/ml. On the other hand, *L. plantarum* HY7714 had no effect on the phosphorylation of p38 expression, and the phosphorylation of ERKs was rather gradually increased dose dependently (Fig. 3A).

These results indicate that the inhibition of UVB-induced MMP-1 expression by *L. plantarum* HY7714 was mainly caused by the inhibition of JNK phosphorylation. MMP-1 expression is strongly regulated by the transcription factor AP-1, which is rapidly induced and activated by UV exposure in human skin in vivo. To examine this mechanism, we evaluated the expression and the phosphorylation of AP-1 subunits. The results showed that c-Jun phosphorylation and

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**Fig. 1.** Effects of several probiotics on procollagen expression in Hs68 cells.

The cells were treated with several probiotics at 1 × 10⁹ CFU/ml for 24 h. The culture supernatants were harvested, and type I procollagen protein levels in the supernatants were determined by ELISA. Data are representative of three independent experiments. LP 7714, *L. plantarum* HY7714; LR 24, *L. rhamnosus* 24; LR 25, *L. rhamnosus* 25; LP 26, *L. plantarum* 26. Asterisks (*) indicate significant differences (*p* < 0.05) between the control group and the probiotics-treated groups.

**Fig. 2.** Effect of *L. plantarum* HY7714 on UVB-reduced procollagen expression or UVB-induced MMP-1 expression in Hs68 cells.

The cells were treated with *L. plantarum* HY7714 at 5 × 10⁷ CFU/ml or 1 × 10⁹ CFU/ml, and simultaneously exposed to UVB (50 mJ/cm²) for 48 h. The level of procollagen or MMP-1 was determined by western blot analysis. Data are representative of three independent experiments. The protein levels were quantified using an image analysis program to evaluate the density of each band on the immunoblot, and the fold-change value was calculated.
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expression were significantly diminished after treatment with L. plantarum HY7714 in Hs68 cells. However, L. plantarum HY7714 did not inhibit c-Fos expression (data not shown).

**L. plantarum HY7714 Attenuates UVB-Induced Skin Wrinkle Formation in Hairless Mouse Skin**

In order to confirm the anti-photoaging activity of L. plantarum HY7714 in vivo, we orally administered L. plantarum HY7714 at a dose of 1 × 10⁹ CFU in 100 µl of sterile saline, or vehicle alone, to hairless mice for 12 weeks. In the UVB-only group, diamond-shaped thick and deep wrinkles were observed on the dorsal skin of the mice. In contrast, oral administration of L. plantarum HY7714 strongly reduced the wrinkle formation. To quantitatively analyze the number, area, and depth of wrinkles, we obtained the replicas from mouse dorsal skin at 12 weeks after UVB irradiation. The skin replicas were photographed and the three parameters were estimated using the skin VisioLine 650 system. These photograph data showed that wrinkle formation was alleviated by oral administration of L. plantarum HY7714 strongly prior to UVB irradiation (Fig. 4A). The number of wrinkles in mice from the UVB-only-treated group was increased by 2.5-fold compared with that of mice from the control group. However, L. plantarum HY7714 administration inhibited wrinkle numbers by 56% (Fig. 4B). Moreover, the total area and depth of wrinkles was suppressed by 48% and 83%, respectively, compared with that of the UVB-only-treated group (Figs. 4C and 4D).

**L. plantarum HY7714 Inhibits UVB-Induced Epidermal Hyperplasia in Hairless Mice Skin**

Since UVB irradiation induces the epidermis to become significantly thicker, we further evaluated the effect of L. plantarum HY7714 on UVB-induced epidermal thickening. To measure the thickness of the epidermis, biopsied skin specimens were stained with hematoxylin and eosin. The results showed that UVB-induced epidermal thickness of the dorsal skin was dramatically increased by 2.3-fold, due to chronic UVB exposure. On the other hand, oral administration of L. plantarum HY7714 strongly inhibited UVB-induced epidermal thickness by 65% (p < 0.01 vs. UVB-only treated group) (Fig. 5).

**L. plantarum HY7714 Inhibits UVB-Induced Activity or Expression of MMPs in Hairless Mouse Skin**

MMP-13, but not MMP-1, functions as a collagenase in mice, and is primarily induced in the epidermis before diffusing into the dermis and degrading collagen. To understand how L. plantarum HY7714 suppressed UVB-induced wrinkle formation, MMP expression and activities were examined using western blotting and zymography. In this study, repetitive UVB irradiation on mouse dorsal skin induced MMP-13 expression, and L. plantarum HY7714 administration significantly attenuated it (Fig. 6A). Because gelatinase, MMP-2, and MMP-9 degrade the interstitial collagen and basement, which results in skin wrinkling [18], we next evaluated whether L. plantarum HY7714 could...
affect UVB-induced MMP-2 and MMP-9 activities. Zymography results showed that oral administration of L. plantarum HY7714 effectively inhibited MMP-2 and MMP-9 activities in hairless mouse skin (Fig. 6B). Therefore, these results indicated that L. plantarum HY7714 could improve skin wrinkles by reducing collagen-degrading MMP expression or activities.

Discussion

There is growing interest in the dermal benefits of probiotics, such as for inhibiting atopic dermatitis [14, 29] and atopic eczema [6, 19] and improving skin immunity [3, 12]. In addition, recent studies revealed that oral administration of some probiotics can suppress skin damage through inhibiting skin inflammation and improving wrinkle formation or skin elasticity in mice [31]. These activities are diverse and depend on different strains. Although identification of the bioactive components of probiotics are very important to better understand their physiological functions on human health, this area of research remains poorly understood and needs to be investigated further. There is increasing evidence that cell wall components of lactic acid bacteria can inhibit MMP-1 expression in fibroblasts [37] or skin carcinogenesis in
hairless mice [35]. The metabolites and heat-treated bacteria from probiotics can also cause certain immune responses in the skin and improve atopic dermatitis in the NC/Nga mouse model [29, 32, 34]. In this study, we used live bacteria in the hairless mouse model. Thus, further studies are necessary to identify the probiotic components such as lipoteichoic acid or other metabolites responsible for the biological functions observed.

Numerous studies have shown that the MAPK signal transduction pathways are responsible for regulating a variety of cellular functions, such as cell inflammation, MMP expression, and type I collagen synthesis [38]. Among these, JNKs and p38 are generally considered as stress-activated MAPKs [5]. Several MMPs, especially MMP-1, MMP-2, and MMP-9, are known to be activated by AP-1. Upon UV irradiation of human skin, c-Jun protein is activated and combines with c-Fos to form an active form of the transcription factor AP-1 [17]. This UV-induced AP-1 initiates the transcription of MMP genes, encoding collagenase and gelatinase in both keratinocytes and fibroblasts [23, 33]. In particular, the protein c-Jun is a well-known nuclear substrate of JNK1, and c-Jun is an important component of various AP-1 subunits that bind to AP-1/ 12-O-tetradecanoyl-13-phorbol-acetate-responsive element enhancer elements in gene regulatory sites [22]. In this study, the JNK/c-Jun pathway was markedly increased by UVB, and L. plantarum HY7714 treatment subsequently prevented this JNK/AP-1 activation.

Collagen is the major structural component of the dermal tissue, and its degradation and degenerative changes cause wrinkle formation and aging appearance [4]. UV irradiation increased MMP expression in human skin, including MMP-1, MMP-2, and MMP-9 [16]. MMP-1 stimulates the cleavage of fibrillar collagen, and MMP-9 further degrades the cleaved collagen [15]. The gelatinases MMP-2 and MMP-9 can specifically degrade components of the epidermal basement membrane such as types IV and VII collagen [16]. It was reported that instant UV stimulation of the dermal tissue only increased MMP-9 levels, whereas constant UV exposure caused increases of both MMP-2 and MMP-9 levels in hairless mice [16]. Thus, MMP-2 as well as MMP-9 may play an important role in the photoaging process. Collagen-degrading MMP-2, -9, and -13 are primarily induced in the epidermis by UV irradiation, as shown by zymography (Fig. 6). Specifically, MMP-2 is highly expressed in the hairless mouse epidermis. These results show that MMP-2 might be one of the important markers of chronically induced photoaging.

In the present study, we found that L. plantarum HY7714 had a strong inhibitory effect on UVB-induced MMP-1 expression by inhibiting the JNK/c-Jun pathway in Hs68 cells. Oral administration of L. plantarum HY7714 also suppressed UVB-induced MMP-13 expression and MMP-2 and MMP-9 activities, thereby inhibiting wrinkle formation and epidermal thickening in the dorsal skin of hairless mice. These results demonstrate that L. plantarum HY7714
might be a good candidate as a skin anti-photoaging agent. However, the detailed molecular mechanisms and bioactive compounds responsible for the increased anti-photoaging effects of *L. plantarum* HY7714 remain to be elucidated.

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References


