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Effect of oral administration of *Lactobacillus plantarum* HY7714 on epidermal hydration in ultraviolet B-irradiated hairless mice

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Running title: Probiotics and UVB-induced reduction in skin hydration

Abstract

In this study, we evaluated the effect of *Lactobacillus plantarum* HY7714 on skin hydration in human dermal fibroblasts and in hairless mice. In Hs68 cells, *L. plantarum* HY7714 not only increased serine palmitoyltransferase (SPT) mRNA level, but also decreased ceramidase mRNA level. In order to confirm the hydrating effects of *L. plantarum* HY7714 *in vivo*, we orally administered vehicle or *L. plantarum* HY7714 at a dose of 1×10^9 CFU/day to hairless mice for 8 weeks. In hairless mice, *L. plantarum* HY7714 decreased UVB-induced epidermal thickness. In addition, we found that *L. plantarum* HY7714 administration suppressed the increase in transepidermal water loss (TEWL) and decrease in skin hydration, which reflects barrier function fluctuations following UV irradiation. In particular, *L. plantarum* HY7714 administration increased the ceramide level compared to that in the UVB group. In the experiment on SPT and ceramidase mRNA expressions, *L. plantarum* HY7714 administration improved the reduction in SPT mRNA levels and suppressed the increase in ceramidase mRNA levels caused by UVB in the hairless mice skins. Collectively, these results provide further insight that *L. plantarum* HY7714 can be a potential candidate for preserving skin hydration levels against UV irradiation.

Key words: Skin hydration, *Lactobacillus plantarum*, photoaging, probiotic, ultraviolet B

Introduction

Exposure to ultraviolet radiation (UVR) cannot be avoided on earth. Prolonged exposure to UVR is dangerous for human health. Photodamage, caused by single or repeated exposure to UVR, is recognized as the initial step of photocarcinogenesis [3]. Skin damage by UV irradiation can be subdivided into acute and chronic photodamage: 1) Acute exposure is dangerous to the skin, causing DNA damage and connective tissue degradation and 2) accumulated damage by chronic exposure causes premature skin aging (photoaging) [9, 11]. UVR causes direct damage to cellular DNA, tissue inflammation, immune response suppression, and free radical formation with the consequent oxidation of proteins, lipids, and DNA [28, 32]. UV light is divided into UVA, UVB, and UVC depending on the wavelength range. UVC is absorbed by the ozone layer, whereas UVA and UVB pass through the ozone layer and can affect the skin [21, 30, 34]. UV exposure causes several skin diseases, including skin cancer and premature aging. UVA and UVB are believed to initiate these processes. In particular, UVB irradiation is closely related with photoaging, which is characterized by coarse and fine wrinkles, dryness, laxity, pigmentation, and increased skin thickness [29]. UVB is mostly absorbed by the cellular components in the epidermis, causing dermal remodeling mediators such as cytokines or bacterial components to diffuse from the epidermis to the dermis and stimulate the production of elastin and glycosaminoglycans [36].

Probiotics are defined as “living microorganisms that, when administered in adequate amounts, confer health benefits on the host” [8]. Most studies on probiotics focus on *Lactobacillus* spp. *Lactobacilli*, lactic acid bacteria associated with fermented foods, mainly contribute to raw food preservation via acidification, along with contributing to product characteristics such as flavor and texture [19]. The intake of probiotics gives us preventive-curative effects against diseases,

including intestinal dysfunctions, gastrointestinal infections, inflammatory bowel disease, and, possibly, colon cancer [24]. Recently, photoprotective effects by specific nutrients have been demonstrated to be successful in preventing certain damages caused by UVR [5, 26]. Specifically, probiotics consumption has been considered as a new strategy in systemic photoprotection [5]. Dietary supplements containing a specific probiotic with several natural plant components protected against the early damage induced by UV exposure by regulating immune cells and inflammatory cytokines in humans [6]. In addition, the positive effects of probiotic consumption on atopic eczema and the re-establishment of skin homeostasis after UV irradiation suggest a gut-skin axis that can be sensitively modulated by therapeutic means [1, 35].

A specific strain of lactic acid bacteria has been shown to have anti-aging effects on wrinkle formation and to improve skin elasticity in hairless mice [31]. However, only few studies have been designed to determine the effects and mechanisms of probiotics on epidermal hydration of the UVB-irradiated skin. Thus, the present study was designed to investigate the protective effects of lactic acid bacteria on epidermal hydration, both *in vitro* and *in vivo*.

Materials and Methods

Preparation of bacteria for *in vitro* and *in vivo* experiments

The four strains of lactic acid bacteria (HY7714, *L. plantarum* HY7714 (Stock No. HY7714); N27, *L. plantarum* 27 (Stock No. N27); L51, *L. gasseri* 51 (Stock No. L51); and L82, *L. gasseri* 82 (Stock No. L82)) used in the present study were isolated from the feces of healthy infants or from breast milk. For the *in vitro* assay, these strains were inoculated in de Man–Rogosa–Sharpe (MRS; BD; Sparks, MD, USA) broth, cultured at 37°C for 20 h, harvested using centrifugation

(1,500 g, 10 min), washed twice with sterile phosphate-buffered saline (PBS), and resuspended to a final concentration of 1×10^{10} CFU/mL. The bacteria were then heat-treated (100°C, 15 min) and stored at -20°C until further use. For the *in vivo* assay, *L. plantarum* HY7714 was harvested as described above and resuspended at a final concentration of 1×10^9 CFU/mL in sterile PBS.

Cell culture

Hs68 human dermal fibroblasts were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. Hs68 cells were cultured in a 24-well plate (5×10^4 cells/well) for 24 h. The cells were then treated with several lactic acid bacteria at a density of 5×10^7 CFU/mL for 24 h. The cell culture medium was collected, and hyaluronic acid was quantified using enzyme immunoassay kit. For mRNA assay, Hs68 cells were cultured in a 6-well plate (2×10^5 cells/well) for 24 h. The levels of mRNA were measured after the cells were treated with HY7714 at 2×10^9 CFU/well for 24 h.

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, 55% relative 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 Ltd (KYIACUC-2014-00024-Y). The mice were divided into control group (n = 8), Con; UVB-only treatment group (n = 8), UVB; and UVB plus *L. plantarum*

HY7714 treatment group (n = 8), HY7714. The mice in the control and UVB-only treatment groups were orally administered 100 μ L of PBS. The mice in the UVB plus *L. plantarum* HY7714 treatment group were orally administered 100 μ L of PBS containing 1×10^9 CFU of *L. plantarum* HY7714/mouse daily, 1 h prior to UVB irradiation.

Mouse UVB irradiation

The UVB radiation source emitted wavelengths with a peak emission at 302 nm using Ultraviolet Crosslinkers (Upland, CA, USA). The backs of the mice were exposed to UVB radiation three times per week for 8 weeks. The starting dose of UVB radiation was 25 mJ/cm² (1 minimal erythematous dose (MED)) and was increased weekly by 1 MED (25 mJ/cm²) until it reached 4 MED (100 mJ/cm²), which was maintained for 8 weeks. Body weights were recorded weekly. Replica preparation was performed on the 8th week of radiation exposure.

Histological examination

The dorsal skin samples (1×0.4 cm²) removed at week 10 were fixed in 10% buffered formalin for at least 24 h, progressively dehydrated in solutions containing an increasing percentage of ethanol (70%, 80%, 95%, and 100%, vol/vol), embedded in paraffin under vacuum, and sectioned at 4- μ m thickness. Hematoxylin and Eosin (H&E) staining was used for routine examination of the tissues and quantification of epidermal hyperplasia. The thickness of the epidermis was measured at three randomly selected locations per slide by using an optical microscope (Leica DMLB, Buffalo Grove, IL, USA) with a magnification of 200 $\times$.

Measurement of skin hydration and skin transepidermal water loss (TEWL)

Skin hydration and TEWL were measured after irradiation. Skin hydration was measured using a corneometer CM 820 (Courage & Khazaka Electronic GmbH, Cologne, Germany) and was automatically calculated and expressed in arbitrary units (A.U.), following the method described by Berardesca [17]. TEWL was measured quantitatively using a Tewameter[®] (TM300, Courage & Khazaka). TEWL was automatically calculated and expressed in g/h/m² [18]. Skin hydration and TEWL were measured on the back of the mice in a room with standardized temperature and humidity conditions (24°C and 55% relative humidity).

Preparation of epidermis samples

After 6 weeks, all mice were sacrificed by cervical dislocation. For separation of the epidermis and dermis, whole skin samples (1 × 2 cm²) were incubated at 4°C overnight in dispase II prepared in PBS. The epidermis sheet was then isolated by scraping with forceps.

Quantitative real-time polymerase chain reaction (PCR) analysis of serine palmitoyltransferase (SPT) and ceramidase mRNA expression

Total RNA was extracted from the dorsal skin tissues and isolated using the QIAGEN RNA Prep Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. From each sample, 2 µg of RNA was reverse-transcribed using MuLV reverse transcriptase, 1 mM dNTP, and 0.5 µg/µL oligo (dT12-18).

Real-time quantitative PCR was performed in 96-well plates by using the Applied Biosystems (Foster City, CA, USA) Prism 7500 Sequence Detection System; each 20-µL reaction mixture consisted of 10 µL of SYBR[®] Green Master Mix (Applied Biosystems) and 0.8 µL of 10 pmol/L forward and reverse primers specific for mouse SPT, ceramidase, and GAPDH. The primer

sequences were as follows: mouse SPT, 5'-TACGACAGCCTCTTGCTGGT-3' (forward), 5'-GGAGAATTGGCCTTTGGAAG-3' (reverse), gene accession number NM011479; mouse ceramidase, 5'-TCCGTGATGGCTAAGGACAC-3' (forward), 5'-ACAGAAGTCCCGGAGGA A-3' (reverse), gene accession number NM175731; and mouse glyceraldehyde 3'-phosphate dehydrogenase (GAPDH), 5'-TTGTCAAGCTCATTTCCTGGTATG-3' (forward), 5'-GCCAT GTAGGCCATGAGGTC-3' (reverse). Human PCR primers used in this study were purchased from the Applied Biosystems Corporation: SPT: Hs00370543_m1, ACER1 (alkaline ceramidase 1): Hs00370322_m1, and keratin 5: Hs00361185_m1. Thermal cycling was initiated by denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. For assessment of relative quantities, the gene amounts for SPT and ceramidase were normalized first to that of a housekeeping gene, GAPDH or keratin 5, and then to that in the normal control group (Con).

Tissue homogenates

Epidermis were rinsed in ice-cold PBS (0.02 mol/L, pH 7.0–7.2) to remove the excess dispase II thoroughly and homogenized on ice in 600 µL of PBS by using a bead homogenizer. The samples were then subjected to two freeze-thaw cycles for further degradation of cell membranes, and centrifuged for 5 min at 5,000 *g*. The supernatants were collected and assayed immediately, or stored at –20°C.

Determination of ceramide content

The concentrations of the homogenates were determined using a protein assay kit (Bio-Rad Corp.; Hercules, CA, USA). A 96-well plate was coated with 10 µg/well homogenates and

incubated overnight at 4°C. After washing, the wells were blocked with 200 µL/well assay diluent (BD Biosciences, Pharmingen, CA, USA) and incubated with monoclonal anti-ceramide antibody for 2 h at room temperature. Ceramide levels were visualized using 3,3',5,5'-Tetramethylbenzidine solution after hybridization with a horseradish peroxidase-conjugated secondary antibody.

Determination of hyaluronic acid and filaggrin content

The contents of hyaluronic acid and filaggrin in the skin samples were measured by hyaluronic acid ELISA kit (Echelon Bioscience Inc, Salt Lake City, UT, USA) and filaggrin ELISA kit (DLdevelop, Wuxi, China), according to the manufacturer's instructions by using epidermis homogenates.

Statistical analysis

Where appropriate, data are expressed as mean $\pm$ SEM, and the Student's *t*-test was used for multiple statistical comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

Results

Lactic acid bacteria increase SPT mRNA level and decrease ceramidase mRNA level in Hs68 cells

We first examined the effect of different lactic acid bacteria, isolated from feces of healthy infants or from breast milk, on hyaluronic acid production in Hs68 cells. In the hyaluronic acid production assay, none of the strains of lactic acid bacteria showed significant effect on

hyaluronic acid production in Hs68 cells when compared to that in the Con group (68.0 ± 2.0 ng/mL) (Fig. 1A). Next, we investigated the effects of the different lactic acid bacteria on SPT and ceramidase mRNA levels in Hs68 cells. *L. plantarum* HY7714 administration increased STP mRNA levels by 2.74 fold ($p < 0.05$, Fig. 1B) and decreased ceramidase mRNA levels by 0.47 fold ($p < 0.05$, Fig. 1C) compared to that in the Con group, while other lactic acid bacteria had no effects on both STP and ceramidase mRNA levels.

***L. plantarum* HY7714 reduces UVB-induced epidermal thickness**

In order to confirm the hydrating effects of *L. plantarum* HY7714 *in vivo*, we orally administered either *L. plantarum* HY7714 at a dose of 1×10^9 CFU/day or the vehicle alone to hairless mice for 8 weeks. The effects of oral *L. plantarum* HY7714 administration on histological alterations produced in the epidermis by UV exposure were studied in skin sections of hairless mice. An increase in epidermal thickness was observed in UVB group ($78.9 \pm 6.9 \mu\text{m}$) compared with that in the Con group ($29.3 \pm 1.3 \mu\text{m}$) ($p < 0.001$). However, the epidermal thickness induced by UVB was decreased ($50.3 \pm 4.5 \mu\text{m}$) in the *L. plantarum* HY7714 group ($p < 0.01$, Fig. 2).

***L. plantarum* HY7714 rescues UVB-induced reduced skin hydration and suppresses UVB-induced TEWL**

Since oral *L. plantarum* HY7714 administration reduced UVB-induced epidermal thickness, we further evaluated the effects of oral *L. plantarum* HY7714 administration on skin hydration and TEWL in hairless mice. UVB irradiation induced a decrease in skin hydration (19.5 ± 3.5 A.U.) and a s increase in TEWL (11.6 ± 1.0 g/h/m²), compared with that in the Con group (43.3 ± 9.2 A.U. and 4.04 ± 0.42 g/h/m², respectively). *L. plantarum* HY7714 group improved reduced skin

hydration by UVB (31.1 ± 1.3 A.U., $p < 0.001$, Fig. 3A) and suppressed UVB-induced TEWL (7.72 ± 0.40 g/h/m², $p < 0.01$, Fig. 3B).

***L. plantarum* HY7714 induced ceramide level in UVB-irradiated hairless mice skin**

The effects of UVB irradiation on ceramide, hyaluronic acid, and filaggrin levels were investigated in the back of hairless mice. UVB reduced the level of ceramide (0.84 ± 0.10 O.D) compared to that in the Con group (1.00 ± 0.02 O.D., Fig. 4A), but did not have any effect on the levels of hyaluronic acid and filaggrin. *L. plantarum* HY7714 group showed increased level of ceramide (1.15 ± 0.03 O.D.) compared with that in the UVB group, but showed no difference in other indices (Fig. 4B, 4C).

***L. plantarum* HY7714 improved UVB-induced reduction in SPT mRNA level and suppressed UVB-induced increase in ceramidase mRNA level in hairless mice**

We confirmed the effects of oral *L. plantarum* HY7714 administration on SPT and ceramidase mRNA levels in hairless mice. UVB irradiation decreased STP mRNA level by 0.23 ± 0.02 times and increased ceramidase mRNA level by 1.64 ± 0.19 times, compared to that in the Con group ($p < 0.05$ and $p < 0.05$, respectively). Interestingly, oral administration of *L. plantarum* HY7714 rescued STP mRNA level by 0.57 ± 0.10 times (Fig 5A) and suppressed ceramidase mRNA level by 0.99 ± 0.14 times, compared to that in the Con group ($p < 0.05$ and $p < 0.05$, respectively) (Fig. 5B).

Discussion

Probiotics can provide benefits to the human skin by modulating immune and inflammatory

responses as well as by having a positive influence on the human gut or dermal fibroblasts [12, 20, 37]. In animal studies, oral administration of probiotics can modulate inflammatory immune responses to alleviate allergic and inflammatory diseases [14]. A study on the gut–skin relationship reported that phenols produced by the gut bacteria accumulate in other organs, including the skin, in hairless mice and induce a skin disorder [16]. Thus, we hypothesized that oral administration of the strain HY7714 may provide benefits to skin by having a positive influence to the intestinal microflora.

Based on the preliminary screening experiments for determining the levels of hyaluronic acid, SPT mRNA, and ceramidase mRNA in Hs68 cells, as shown in Fig. 1, *L. plantarum* HY7714 was selected as a candidate for consecutive studies among the various probiotics isolated from the feces of healthy infants or from breast milk. In a sequential UVB-irradiated hairless mice study, we investigated the dietary effects of *L. plantarum* HY7714 on epidermal hydration in UVB-irradiated hairless mice. In hairless mice, oral intake of *L. helveticus*-fermented milk whey decreased TEWL and prevented the onset of so sodium dodecylsulfate-induced dermatitis [4]. It is also reported that oral intake of *L. rhamnosus* decreased TEWL and skin inflammation [17]. These findings were consistent with our results.

Next, we investigated possible mechanisms underlying the effects of *L. plantarum* HY7714 on ceramide, hyaluronic acid, and filaggrin expression—properties intimately related to skin hydration. *L. plantarum* HY7714 administration induced ceramide expression as compared to that in the UVB group. Ceramides play a key role in maintaining the structural integrity of the epidermal barrier and epidermal hydration [7, 13, 33]. Epidermal ceramides are essential not only for protection against desiccation, but also for protection against microbial infections [23]. Higher susceptibility to pathogenic infections and various skin disorders like atopic dermatitis or

harlequin ichthyosis can be explained by reduced ceramide levels [2, 38]. In chronic UV-irradiated skin of hairless rat, decreased ceramide levels were observed owing to abnormal sphingomyelinase [25]. It is also reported that high ceramidase expression causes a decrease in ceramide levels in the epidermis [15]. These findings were consistent with our results. Ceramide levels are modulated by a balance between the activity and expression of generating enzymes and degrading enzymes such as SPT in the *de novo* synthesis pathway and ceramidase, respectively [10, 22]. We investigated the effects of *L. plantarum* HY7714 on SPT and ceramidase mRNA expression in the epidermis. mRNA expression level does not represent production amount of specific proteins from mRNA. However, reported studies showed that mRNA expression level of SPT and ceramidase was consistently correlated with protein level of those [18, 27]. Thus, we investigated SPT and ceramidase mRNA expression in hairless mice. *L. plantarum* HY7714 administration suppressed the decrease in SPT mRNA expression and the increase in ceramidase mRNA expression, induced by UVB irradiation. Therefore, our results indicate that *L. plantarum* HY7714 can alleviate the damage of skin barrier function by regulating ceramide-metabolizing enzymes.

Collectively, we determined the UV-protective effects of *L. plantarum* HY7714, which decreases UVB-induced epidermal thickness, skin hydration loss, and TEWL, as observed in UVB-irradiated hairless mice model. This could be mediated by an increased *de novo* synthesis of ceramides due to higher SPT mRNA expression as well as a decreased degradation due to lower ceramidase mRNA expression. Further studies revealing the molecular mechanisms of HY7714 effects on skin protection are needed.

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Figure legends

Fig. 1. Effect of probiotics on hyaluronic acid, SPT, and ceramidase mRNA levels in Hs68 cells

The cells were treated with several probiotics for 24 h. (A) Culture supernatants were harvested, and the content of hyaluronic acid was determined by ELISA. (B) Human SPT and (C) ceramidase mRNA levels were determined by real-time PCR. Data are representative of two independent experiments. HY7714, *L. plantarum* HY7714; N27, *L. plantarum*; L51, *L. gasseri* 51; L82, *L. gasseri* 82. * indicates significant differences ($p < 0.05$) between the control group and the probiotic-treated group.

Fig. 2. Effect of oral administration of *L. plantarum* HY7714 on epidermal thickness in UVB-irradiated hairless mice skin

(A) Hematoxylin and eosin-stained skin tissue sections. Images are representative of results from eight tissue samples (Magnification, 200×). (B) Bars represent the mean of epidermal thickness (μm), as calculated from eight animals (three measurements/section). Results are presented as mean $\pm$ SEM ($n = 8$). Con, the control group; UVB, the group treated with UVB alone; HY7714, the group treated with UVB and *L. plantarum* HY7714. # indicates significant differences ($p < 0.001$) between the control group and the group treated with UVB alone. ** indicates significant differences ($p < 0.01$) between the group treated with UVB alone and the group treated with *L. plantarum* HY7714.

Fig. 3. Effect of oral administration of *L. plantarum* HY7714 on skin hydration and TEWL

in UVB-irradiated hairless mice skin

(A) Skin hydration and (B) TEWL. Data are presented as mean $\pm$ SEM (n = 8). Con, the control group; UVB, the group treated with UVB alone; HY7714, the group treated with UVB and *L. plantarum* HY7714. # indicates significant differences ($p < 0.001$) between the control group and the group treated with UVB alone. *** and ** indicate significant differences ($p < 0.001$ and $p < 0.01$, respectively) between the group treated with UVB alone and the group treated with *L. plantarum* HY7714.

Fig. 4. Effect of oral administration of *L. plantarum* HY7714 on ceramide, hyaluronic acid, and filaggrin expression in UVB-irradiated hairless mice skin

(A) Ceramide, (B) hyaluronic acid, and (C) filaggrin. Data are presented as mean $\pm$ SEM (n = 8). The epidermis was homogenized, and the expression of ceramide, hyaluronic acid, and filaggrin in the homogenates was determined by ELISA. Con, control group; UVB, group treated with UVB alone; HY7714, group treated with UVB and *L. plantarum* HY7714. # indicates significant differences ($p < 0.001$) between the control group and the group treated with UVB alone. *** and ** indicate significant differences ($p < 0.001$ and $p < 0.01$, respectively) between the group treated with UVB alone and the group treated with *L. plantarum* HY7714.

Fig. 5. Effect of oral administration of *L. plantarum* HY7714 on SPT and ceramidase mRNA levels in UVB-irradiated hairless mice skin

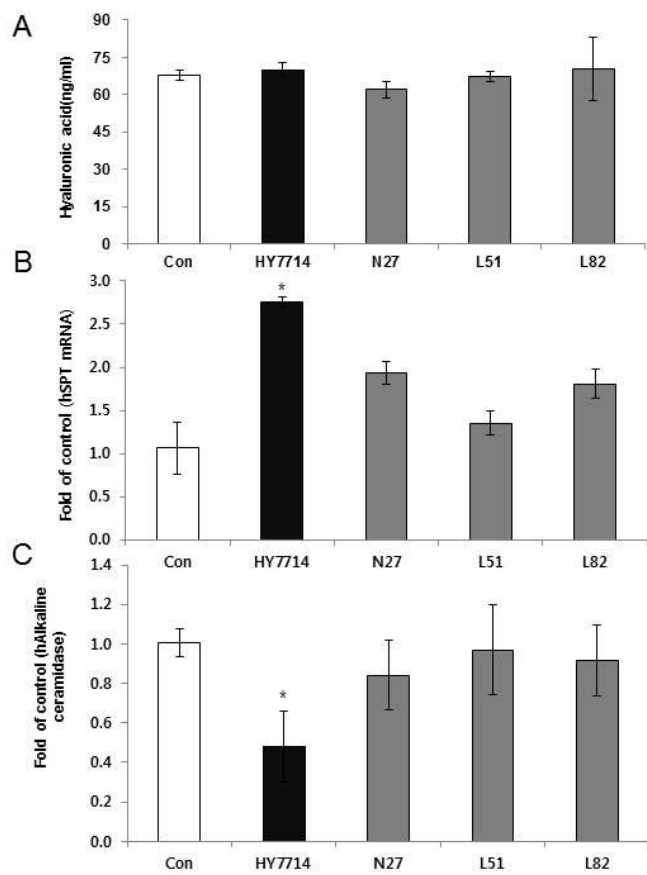
(A) SPT and (B) ceramidase. Mouse SPT and ceramidase mRNA levels were determined by real-time PCR. Total RNA was prepared from the epidermis of hairless mice. Data are presented as mean $\pm$ SEM (n = 8). Con, the control group; UVB, the group treated with UVB alone; HY7714,

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442 *plantarum* HY7714.

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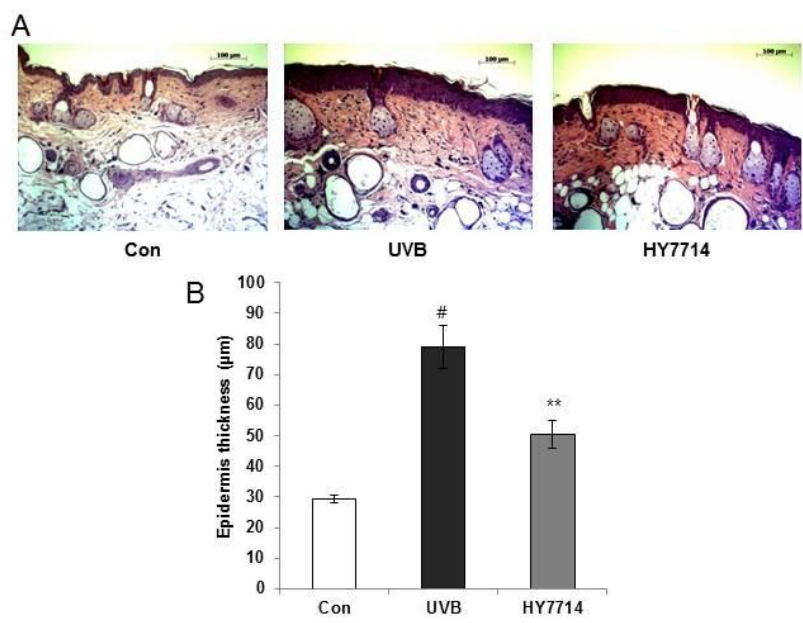
Figure 1



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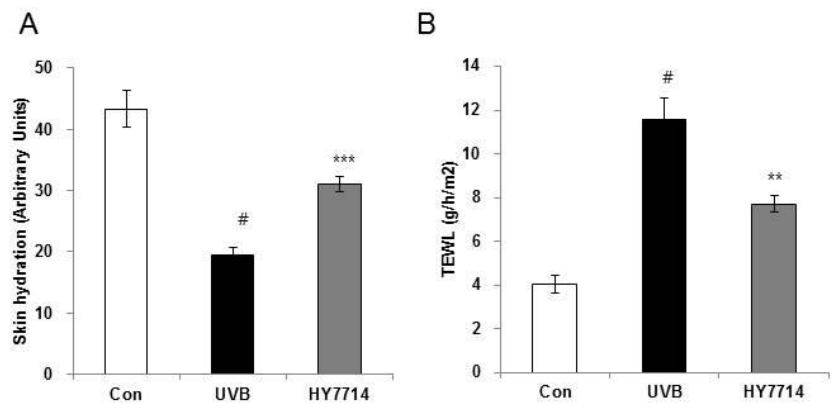
Figure 2



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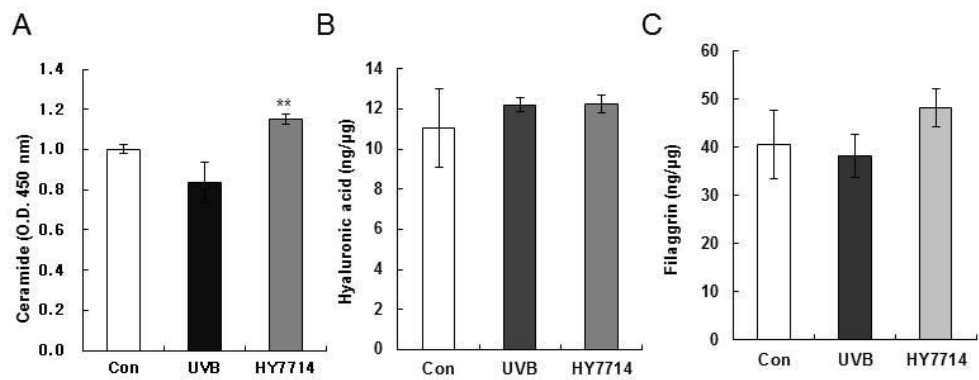
Figure 3



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Figure 4



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Figure 5

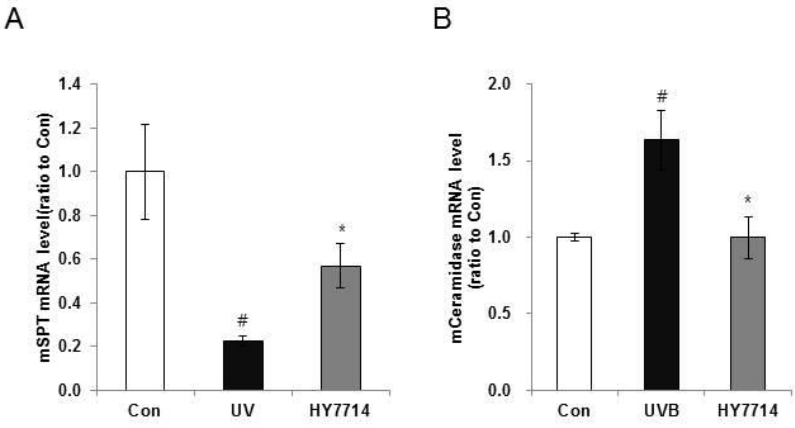


Figure 1

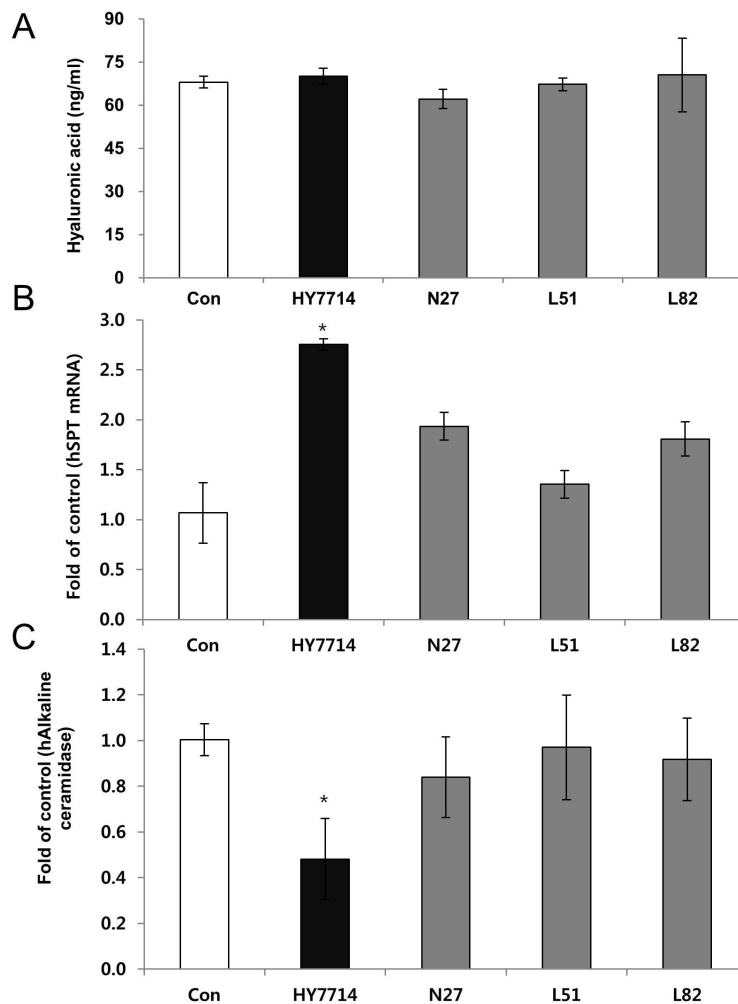


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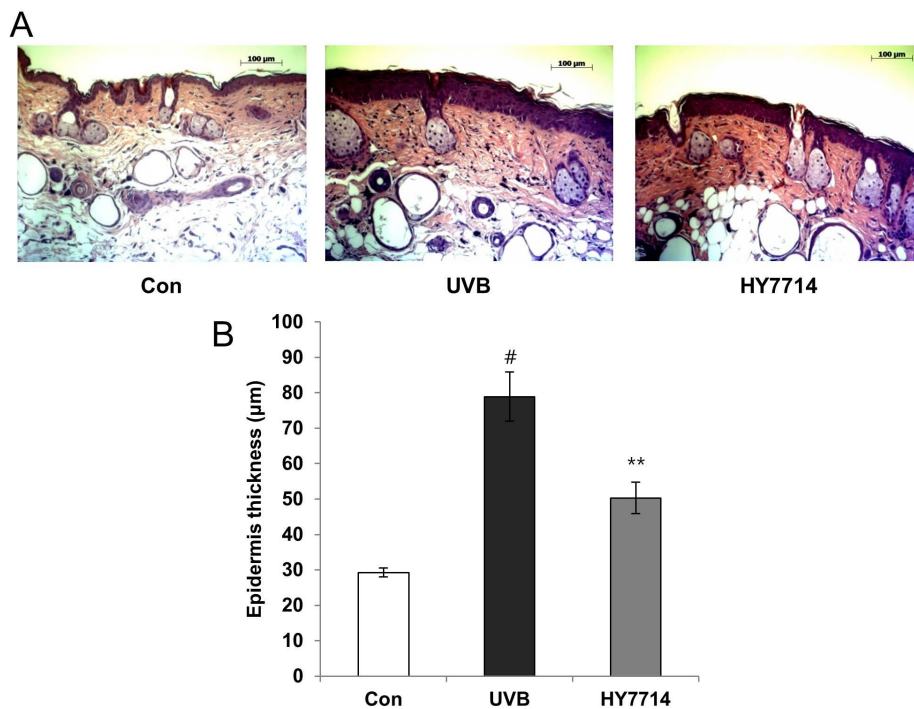


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Figure 3

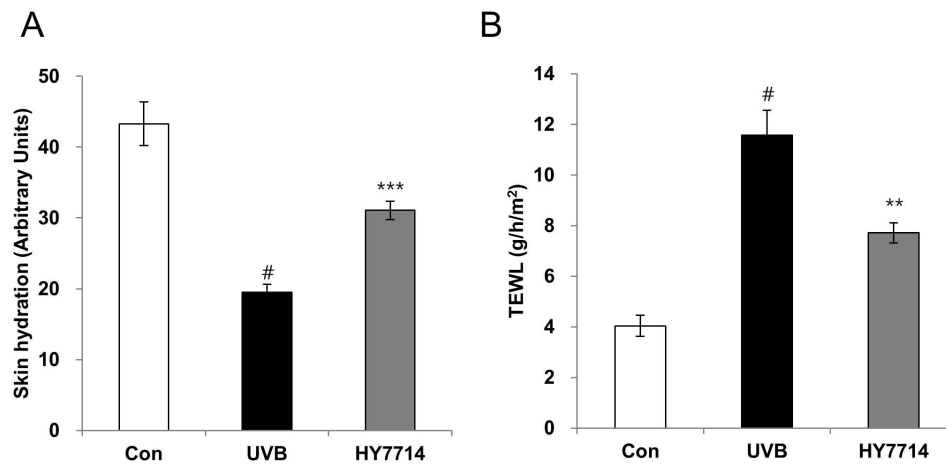


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Figure 4

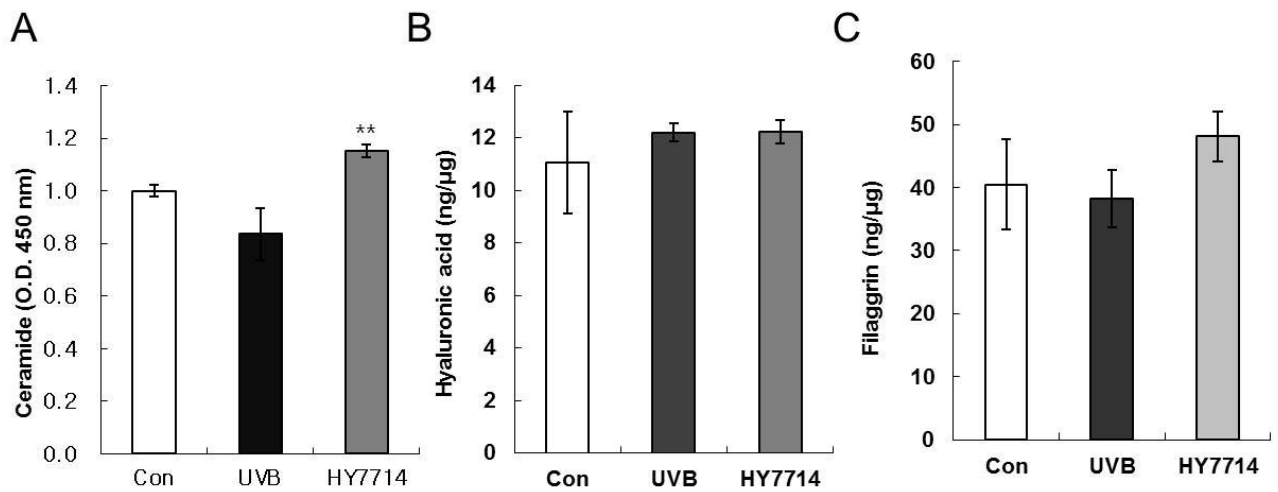


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Figure 5

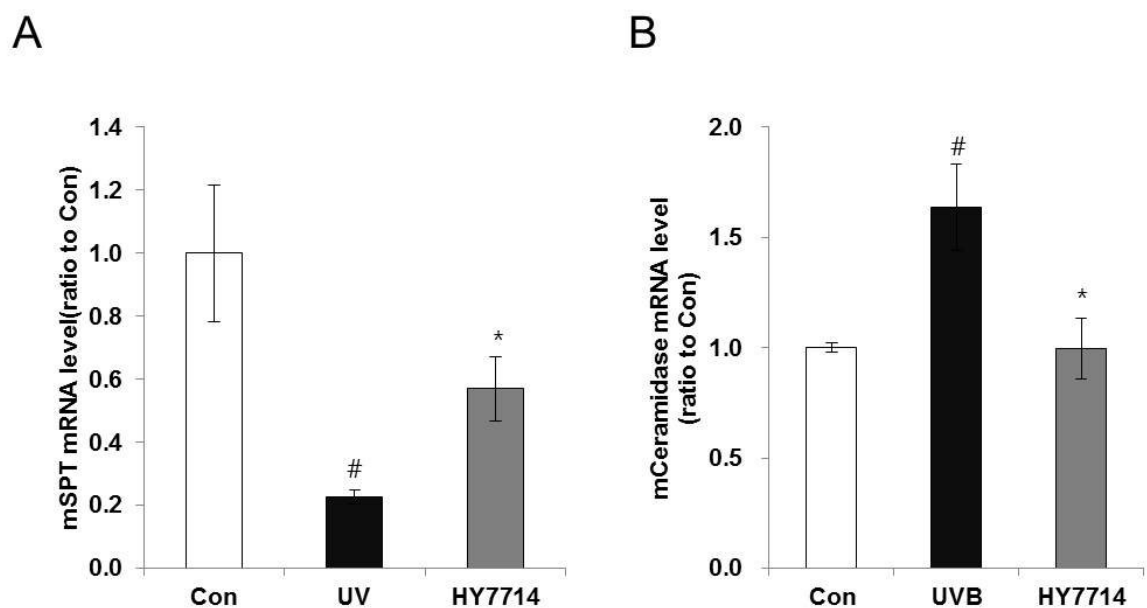


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