Effect of Oral Administration of \textit{Lactobacillus plantarum} HY7714 on Epidermal Hydration in Ultraviolet B-Irradiated Hairless Mice

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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: (i) Acute exposure is dangerous to the skin, causing DNA damage and connective tissue degradation; and (ii) accumulated damage by chronic exposure causes premature skin aging (photoaging) [10, 12]. 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, 33]. 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 [22, 31, 35]. 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 [37].

Probiotics are defined as “living microorganisms that, when administered in adequate amounts, confer health benefits on the host” [9]. Most studies on probiotics focus on \textit{Lactobacillus} spp. Lactobacilli, lactic acid bacteria associated with fermented foods, contribute mainly to raw food preservation via acidification, along with contributing to product characteristics such as flavor and texture [20].

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

Keywords: Skin hydration, \textit{Lactobacillus plantarum}, photoaging, probiotic, ultraviolet B

Received: August 11, 2014
Revised: August 27, 2014
Accepted: August 31, 2014

First published online
September 1, 2014

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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 [6, 26]. Specifically, probiotics consumption has been considered as a new strategy in systemic photoprotection. 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, 36].

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 [32]. However, only a 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 (BD, 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 × 10^9 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

HaCaT human dermal fibroblasts were purchased from the American Type Culture Collection (Manassas, 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. HaCaT 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 × 10^9 CFU/ml for 24 h. The cell culture medium was collected, and hyaluronic acid was quantified using an enzyme immunoassay kit. For mRNA assay, HaCaT cells were cultured in a 6-well plate (2 × 10^4 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 µl of PBS. The mice in the UVB plus L. plantarum HY7714 treatment group were orally administered 100 µl 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, 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% (v/v)), embedded in paraffin under vacuum, and sectioned at 4 µm 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, USA) with a magnification of 200×.

Measurement of Skin Hydration and Skin Transepidermal Water Loss (TEWL)

Skin hydration and TEWL were measured after irradiation. Skin hydration was measured using a CM 820 corneometer (Courage & Khazaka Electronic GmbH, Germany) and was automatically calculated and expressed in arbitrary units (AU),
following the method described by Blichmann [7]. TEWL was measured quantitatively using a Tewameter (TM300; Courage & Khazaka) and was automatically calculated and expressed in g/h/m² [5]. 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, 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, 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'-TACGACAGCCTCTTGTGCTGT-3' (forward), 5'-GGAGAATTGGCCTTTGGAAG-3' (reverse), gene accession number NM175731; mouse ceramidase, 5'-TCCGTGATGGCTAAGGACCAC-3' (forward), 5'-ACAGAAAGTCGCCGAGGA-A-3' (reverse), gene accession number NM011479; mouse ceramidase, 5'-TCCGTGATGGCTAAGGACCAC-3' (forward), 5'-ACAGAAAGTCGCCGAGGA-A-3' (reverse), gene accession number NM011479; mouse ceramidase, 5'-TCCGTGATGGCTAAGGACCAC-3' (forward), 5'-ACAGAAAGTCGCCGAGGA-A-3' (reverse), gene accession number NM011479; mouse ceramidase.

Results

Lactic Acid Bacteria Increase the SPT mRNA Level and Decrease the 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 with 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. In this study were purchased from Applied Biosystems Corp.: 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 sec, 60°C for 30 sec, and 72°C for 30 sec. 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
The epidermis samples 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. 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 with a hyaluronic acid ELISA kit (Echelon Bioscience Inc, USA) and filaggrin ELISA kit (D. L. Develop, Wuxi, China), according to the manufacturer’s instructions by using epidermis homogenates.

Statistical Analysis
Where appropriate, data are expressed as the mean ± 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.

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 × 10⁹ 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 the UVB group (78.9 ± 6.9 µm) compared with that in the Con group (29.3 ± 1.3 µm) (p < 0.001). However, the epidermal thickness induced by UVB was decreased (50.3 ± 4.5 µm) in the \textit{L. plantarum} HY7714 group (p < 0.01, Fig. 2).

\textbf{L. plantarum HY7714 Rescues UVB-Induced Reduced Skin Hydration and Suppresses UVB-Induced TEWL}

Since oral \textit{L. plantarum} HY7714 administration reduced UVB-induced epidermal thickness, we further evaluated the effects of oral \textit{L. plantarum} HY7714 administration on skin hydration and TEWL in hairless mice. UVB irradiation induced a decrease in skin hydration (19.5 ± 3.5 AU) and an increase in TEWL (11.6 ± 1.0 g/h/m²), compared with that in the Con group (43.3 ± 9.2 AU and 4.04 ± 0.42 g/h/m², respectively). The \textit{L. plantarum} HY7714 group improved reduced skin hydration by UVB (31.1 ± 1.3 AU, p < 0.001, Fig. 3A) and suppressed UVB-induced TEWL (7.72 ± 0.40 g/h/m², p < 0.01, Fig. 3B).

\textbf{L. plantarum HY7714 Induced the 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 OD) compared with that in the Con group (1.00 ± 0.02 OD, Fig. 4A), but did not have any effect on the levels of hyaluronic acid and filaggrin. The \textit{L. plantarum} HY7714 group showed an increased level of ceramide (1.15 ± 0.03 OD) compared with that in the UVB group, but showed no difference in other indices (Figs. 4B and 4C).
We confirmed the effects of oral _L. plantarum_ HY7714 administration on the SPT and ceramidase mRNA levels in hairless mice. UVB irradiation decreased the SPT mRNA level by 0.23 ± 0.02 times and increased the ceramidase mRNA level by 1.64 ± 0.19 times, compared with that in the Con group (p < 0.05 and p < 0.05, respectively). Interestingly, oral administration of _L. plantarum_ HY7714 rescued the SPT mRNA level by 0.57 ± 0.10 times (Fig. 5A) and suppressed the ceramidase mRNA level by 0.99 ± 0.14 times, compared with 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 [13, 21, 38]. In animal studies, oral administration of probiotics can modulate inflammatory immune responses to alleviate allergic and inflammatory diseases [15]. 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 strain HY7714 may provide benefits to skin by having a positive influence on 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 the TEWL and prevented the onset of sodium dodecyl sulfate-induced dermatitis [4]. It is also reported that oral intake of L. rhamnosus decreased the TEWL and skin inflammation [18]. 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 with that in the UVB group. Ceramides play a key role in maintaining the structural integrity of the epidermal barrier and epidermal hydration [8, 14, 34]. Epidermal ceramides are essential not only for protection against desiccation, but also for protection against microbial infections [30]. The higher susceptibility to pathogenic infections and various skin disorders like atopic dermatitis or harlequin ichthyosis can be explained by reduced ceramide levels [2, 39]. In chronic UV-irradiated skin of hairless rats, decreased ceramide levels were observed and attributed to abnormal sphingomyelinase [25]. It is also reported that high ceramidase expression causes a decrease in ceramide levels in the epidermis [17]. 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 [11, 23]. We investigated the effects of L. plantarum HY7714 on SPT and ceramidase mRNA expression in the epidermis. The mRNA expression level does not represent the production amount of specific proteins from mRNA. However, reported studies showed that the mRNA expression level of SPT and ceramidase correlated consistently with their protein level [19, 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 the 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 the HY7714 effects on skin protection are needed.
References


