Skin Barrier-enhancing, Antiwrinkle, and Antimelanogenic Effects of Probiotic Lysates Composed of Nucleotides

Kyung-Min Kim¹, Ha-Yeon Kim¹, So-Yoon Cha¹, Ye-Hyang Kim¹, Ji-Won Song¹, and Seunghun Lee²,*

¹Researcher, R&D Center, HYUNDAI BIOLAND Co., Ltd.
²Research Director, R&D Center, HYUNDAI BIOLAND Co., Ltd.

Several previous studies have investigated the skin aging prevention effects of ceramide, hyaluronic acid, and natural or fermented plant materials. Recently, oral administration and dermal application of probiotics or probiotic lysates have shown antiaging effects. The purpose of this study is to optimize the preparation of probiotic lysates with a high concentration of nucleotides and to confirm the effects of probiotic lysates on the skin. Probiotic lysates were prepared by heating at 121°C for various periods with adding of sodium hyaluronic acid. Probiotic lysates of *Bifidobacterium longum* HDB7072, *Lactobacillus paracasei* HDB1196, and *Lactobacillus acidophilus* HDB1014 were applied to normal human epidermal keratinocytes (NHEKs), fibroblast cells, and B16F1 cells, respectively. Cell viability, antioxidant effects, and mRNA expression were evaluated by using MTT assays, DPPH assays, and qRT-PCR. Probiotic lysates prepared by heating the culture medium at 121°C for 2 h with 0.5% sodium hyaluronic acid showed the highest nucleotide concentration. In the three tested skin cells, the cell viability of filtered lysates was similar or higher to that of unfiltered lysates. HDB7072 lysates increased filaggrin expression in NHEKs. HDB1196 lysates showed DPPH radical-scavenging and antiwrinkle effects through the downregulation of matrix metalloproteinase-1 and upregulation of collagen type 1 in fibroblasts. HDB1014 lysates had antioxidant and antimelanogenic effects in B16F1 cells. Cell wall-removed probiotic lysates could be used as novel ingredients to improve skin aging and skin barrier issues.

Keywords: Antimelanogenic, Antiwrinkle, Probiotics, Skin aging, Skin barrier

I. Introduction

The skin is the largest sensory organ in the human body and consists of three layers; the epidermis, dermis, and subcutaneous fat layer. Skin protects the human body from the external environment, synthesizes vitamin D, prevents water loss, and maintains a constant body temperature (Jung et al., 2019). As the skin ages due to age, diet, and environmental factors such as ultraviolet (UV) radiation, chemical substances, and fine particles, skin structures begin to function abnormally (Krutmann et al., 2017). The most common symptoms of skin aging are skin barrier weakness and the formation of wrinkles, melasma, and freckles (Puisina-Ivuc, 2008).

Previous research on protection from skin aging has focused largely on the effects of natural plant and animal materials or their fermented products. Recently, however, microorganisms have also been investigated for their ability to suppress and slow down skin aging. Lactic acid bacteria (LAB) are representative probiotic strains that can provide health-promoting effects such as antiobesity, antiinflammation, antioxidant, and cholesterol-lowering effects (Kim et al.,...
In addition to intestinal benefits, recent studies have reported that LAB and their extracts can improve skin conditions and prevent skin problems. Applying LAB and their lysates directly to the skin can improve skin conditions. For instance, Lactobacillus rhamnosus lysate crushed with a microfluidizer improved the skin barrier function in reconstructed human epidermis (Jung et al., 2019). Additionally, Bifidobacterium longum and Lactobacillus spp. lysed by a bead beater induced the upregulation of tight junction barrier genes in human keratinocytes (Sultana et al., 2013). The extract of Lactobacillus paracasei and L. rhamnosus showed antiwrinkle activities by decreasing production of matrix-metalloproteinase-1 (Kim et al., 2015). Furthermore, heat-killed Lactobacillus acidophilus KCCM12625P was able to regulate wrinkle-related and melanogenesis-related genes regulation in UVB-irradiated cells (Lim et al., 2020). Finally, Lactobacillus reuteri DSM 17938 lysed by a mechanical high-pressure homogenizer caused antiinflammatory and hydration effects in reconstructed human epidermis (Khmaladze et al., 2019).

Although several probiotic lysates prepared by various means have been evaluated, their indicators and functional substances have yet to be clarified. In addition, for most probiotic lysates, the whole component, including culture media and cell wall, has typically been applied after cells were crushed. Some researchers have reported that lipopolysaccharides (LPS) from the cell wall and lactic acid in culture media could irritate the skin (Aramaki et al., 2002; Cruz et al., 2001). Therefore, the objective of this study is to identify the functional substances of probiotic lysate and optimize a lysate preparation method to include a high concentration of nucleotides and remove cells walls and culture media. Probiotic lysates were prepared using an optimized method with species previously reported to be effective, namely, L. acidophilus, L. paracasei, and B. longum, and the skin effects of these probiotic lysates were compared.

II. Materials and Methods

1. Bacterial strains and cell culture

L. acidophilus HDB1014, L. paracasei HDB1196, and B. longum HDB7072 were provided by Cell Bank (HYUNDAI BIOLAND Co., Ltd., Korea). Lactobacillus strains were grown in de Man, Rogosa, and Sharpe (MRS; BD BBL, Franklin Lakes, NJ, USA) broth, and the Bifidobacterium strain was grown in BL (MB-B1602, KisanBio Co., Ltd., Korea) broth at 37°C for 24 h.

Human dermal fibroblasts were obtained from American Type Culture Collection (ATCC; USA) and cultured using Iscove’s modified Dulbecco’s medium (WELGENE, Korea). B16F1 cells (mouse melanoma cells) were purchased from Korean Cell Line Bank (KCLB; Korea) and cultured using Dulbecco’s Modified Eagle Medium (WELGENE). These cells were cultured with 10% fetal bovine serum (Gibco, USA) and 1% (v/v) streptomycin/penicillin solution in an incubator with 5% CO₂ at 37°C. Normal human epidermal keratinocytes (NHEKs) were obtained from Cascade Biologics, Inc. (USA), and cultured in Epilife medium (Cascade Biologics) supplemented with human keratinocyte growth supplement. NHEKs were grown in culture dishes in an incubator with 5% CO₂ at 37°C, and the culture medium was replaced every 2 days.

2. Optimization of probiotic lysates preparation

All probiotic strains were grown in MRS or BL broth at 37°C for 24 h. Each overnight culture was adjusted to approximately 10⁸ CFU/mL, centrifuged at 5,782 × g for 10 min, and then washed three times with phosphate-buffered saline. The harvested cells were inoculated into distilled water or sodium hyaluronate solutions [0.25%, 0.50%, and 1% (w/v) sodium hyaluronate in distilled water] and incubated at 37°C for 24 h. To produce probiotic lysates, the overnight cultures were heated at 121°C for 15, 30, 60, 120, and 150 min. After filtration with a 0.45 µm filter to remove cell walls, the probiotic lysates were completed. The nucleotide concentration, i.e., the active components of probiotic lysates, were measured using a NANODROP 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

To determine the necessity of filtration, the cell cytotoxicity of filtered and unfiltered probiotic lysates was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Promega, USA) assay (Kim et al., 2020). The probiotic lysates were applied to each well of 24-well plates containing the three tested skin cells and incubated at 37°C for 24 h. After the supernatant was removed, MTT solution (0.25 mg/mL) was applied to each well at 37°C for 4 h before DMSO was added to the wells. The absorbance of the
mixtures was then measured at 570 nm.

3. Radical-scavenging activity assay

The radical-scavenging activity of probiotic lysates was measured using a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Kim et al., 2015). Distilled water and 0.1 mg/mL ascorbic acid (Vit C; Sigma Aldrich, USA) were used as control groups. Samples (150 µL) were mixed with the same amount of DPPH solution (0.1 mM in methanol). The mixtures were then incubated at room temperature for 30 min in the dark, and then the absorbance of the mixtures was measured at 517 nm.

4. Skin barrier enhancement assay

To evaluate filaggrin (FLG) expression, NHEKs were inoculated and incubated for 24 h. After the culture media were removed, ceramide NP (Evonik Degussa, Germany, 100 µg/mL), as a positive control for keratinocyte differentiation, or *B. longum* HDB7072 lysate (HDB7072) were treated to NHEKs and then incubated for 24 h.

5. Antiwrinkle assay

To evaluate the inhibition of matrix metalloproteinase-1 (MMP-1) gene expression, fibroblast cells were inoculated and incubated for 24 h. Cells were irradiated with 6 J/cm² UVA and incubated for 24 h with 2.5 µg/mL epigallocatechin gallate (EGCG; Sigma Aldrich) and *L. paracasei* HDB1196 lysate (HDB1196). To evaluate collagen type 1 (COL-1) gene expression, fibroblast cells were inoculated and incubated for 24 h. Subsequently, 75 µg/mL Vit C or HDB1196 was inoculated into each well and incubated for 24 h. EGCG and Vit C were used as positive controls.

6. Antimelanogenic assay

B16F1 cells were inoculated and incubated at 37°C for 24 h. The culture media were changed to media with 100 nM α-melanocyte stimulating hormone (α-MSH; Sigma Aldrich) to stimulate melanogenesis. *L. acidophilus* HDB1014 lysate (HDB1014) was then applied, and cells were incubated for 72 h. To evaluate the melanin secretion rate, the absorbance of the culture medium was measured at 450 nm. To evaluate melanin content, cells were dissolved with 250 µL of 1 N NaOH at 50°C for 15 min. The absorbance of the mixtures was measured at 450 nm. Arbutin was used as a positive control.

7. Quantitative real-time polymerase chain reaction (qRT-PCR)

After cells were cultured with the samples for each experiment, total mRNA was extracted using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Subsequently, mRNA concentrations were measured using a Qubit RNA BR Assay Kit (Invitrogen, USA), and cDNA was synthesized using a qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, UK). To quantify the expression of the barrier gene (FLG) and wrinkle gene (MMP-1 and COL-1), the primers shown in Table 1 were used. A real-time PCR kit (qPCRBO SyGreen Blue Mix Lo-ROX, PCR Biosystems, BioD Co., Ltd., Korea) was used to perform aRT-PCR on a 7300 Real-Time PCR system (Applied Biosystems, USA).

8. Statistics

All results are shown as the mean ± standard deviation (SD) of at least three experiments. The control and experi-

<table>
<thead>
<tr>
<th>Target</th>
<th>Orientation</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filaggrin (FLG)</td>
<td>Forward</td>
<td>CACGTGGGACGTCTGACAGGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTTTTCCTGCTGCTGCC</td>
</tr>
<tr>
<td>Matrix metalloproteinase-1 (MMP-1)</td>
<td>Forward</td>
<td>GTTCAGGGACGAGAATGCTCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTAGGGAACCCCAAGGGCT</td>
</tr>
<tr>
<td>Collagen type 1 (COL-1)</td>
<td>Forward</td>
<td>AGCAAGAACCCCAAGGACAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGAATGCGTCACTTACGGTC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>GCCACCCAGCACAATGAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCGATCCACACCGGACTTGT</td>
</tr>
</tbody>
</table>
mental groups were compared using Mann-Whitney U test in SPSS Static 19.0 (SPSS, Inc., Chicago, IL, USA).

III. Results and Discussion

1. Optimization of probiotic lysate manufacturing

Studies on the skin health effects of probiotics and their cultures are actively being conducted, and lipoteichoic acid (LTA), phospholipids in cell membranes, oligosaccharides, short chain fatty acids, and peptides are known to be functional substances (Kim & Lee, 2015). However, using the entire bacteria and culture may have disadvantages as it may contain harmful substances. Thus, it is important to purify functional substances that can easily be analyzed and quantified.

Nucleotides are the units of DNA and RNA, which are natural polymers and play a pivotal role in metabolism in living organisms. Many researchers have studied the functionality of nucleotides or their components such as adenosine. Salmon milt DNA has been used for medical therapy and is reported to have wound healing, antiinflammatory, and UV protection effects on the skin (Nakamichi et al., 2019; Sasaki et al., 2010; Sato et al., 2017; Shen et al., 2008). Besides salmon milt DNA, bacterial genomic DNA of heat-killed \textit{L. plantarum} has been shown to have antiinflammatory effects through the secretion of elafin on Caco-2 cells mediated by toll-like receptor 9 (TLR9) (Hiramatsu et al., 2019). In addition, adenosine is known to have wound healing, antiinflammatory, and dermal collagen production effects (Cronstein, 2011). Collectively, these results of research indicate that the nucleotides of LAB could be substances that promote skin health.

To increase the nucleotide concentration of probiotic lysates, the heating time and addition of sodium hyaluronate were varied in the present study. Until 2 h of heating and 0.5% of sodium hyaluronate, the concentration of nucleotides increased as heating time and sodium hyaluronate concentration increased (Fig. 1A). Specifically, probiotic lysate heated at 121°C for 2 h with 0.5% (w/v) sodium hyaluronate had the highest nucleotide content of 268.51 ng/µL. It is presumed that nucleotide content increased with heating time due to the continued disruption of the cell wall of probiotics. At the second fermentation step, sodium hyaluronate likely increased nucleotide content because, when used as a nutrient, it promotes LAB growth. In previous studies, the growth of LAB such as \textit{L. acidophilus}, \textit{L. rhamnosus}, and \textit{Streptococcus thermophilus} was enhanced in MRS broth with 0.03%-0.10%

![Fig. 1. The nucleotide concentration and cell viability of probiotic lysates.](image)

(A) The nucleotide concentration of \textit{L. paracasei} HDB1196 lysates. Cell viability of unfiltered (NF) and filtered (F) probiotic lysates in normal human epidermal keratinocytes (B: treated with \textit{B. longum} HDB7072 lysate), fibroblasts (C: treated with \textit{L. paracasei} HDB1196 lysate), and B16F1 cells (D: treated with \textit{L. acidophilus} HDB1014 lysate). Data are means ± SDs of three independent experiments. Different letters on bars indicate significant differences (at \(p < 0.05\)) among values: * \(p < 0.05\), ** \(p < 0.01\), and *** \(p < 0.001\) vs. control.
sodium hyaluronate in a dose-dependent manner (Cong Nguyen, 2010; Di Cerbo et al., 2013).

The MTT assay showed that filtration was a necessary process for reducing skin irritation on skin cells. Although the cytotoxicity of unfiltered and filtered HDB7072 treatments was not significantly different to that of untreated NHEKs (control) at concentration up to 9% (v/v) (Fig. 1B). However, filtered HDB1196 and HDB1014 treatments were less cytotoxic than unfiltered lysates in B16F1 and fibroblast cells. Filtered HDB1196 did not affect viability at concentrations up to 9% (v/v), whereas unfiltered lysates affected viability at 9% (v/v) (Fig. 1C). In addition, filtered HDB1014 had no effect viability at concentrations up to 3% (v/v), whereas unfiltered lysates affected cell viability at 3% (v/v) (Fig. 1D).

Considering these data, the probiotic lysates used in the subsequent experiments were prepared by first heating probiotics cells at 121°C for 2 h with 0.5% (w/v) sodium hyaluronate and then by filtering (Fig. 2). In the subsequent functional evaluation, samples were treated at a maximum of 3% according to the cell safety concentration of the HDB1014 strain.

2. Skin barrier-enhancing effects of B. longum HDB7072 lysates on NHEKs

The stratum corneum is composed of a “brick and mortar” structure wherein proteins form the brick (e.g., keratin microfibrils and filaggrin) and lipids form the mortar (e.g., ceramides, free fatty acids, and cholesterol) (Jung et al., 2019). The skin barrier on which this theory is based is important not only for protection from the outside but also for protection of water loss from the skin. Skin barrier dysfunction, which causes conditions such as atopic dermatitis, psoriasis, and ichthyosis, is related to mutant filaggrin gene (flg), tight junction disorder, and reductions in ceramides, serine protease inhibitors, and antibacterial peptides (Bläsler et al., 2016).

The mRNA expression of FLG was increased in the HDB7072-treated cells in a dose-dependent manner (Fig. 3). The FLG expression of ceramide-treated and 3% (v/v) HDB7072-treated cells increased by 123.1% and 144.5% compared with 100% of the control (distilled water), respectively. Thus, HDB7072 was shown to have a skin barrier-improving effect.

In previous research, the skin barrier-improving effects of other probiotic lysates have been reported. For instance, the microfluidized lysate of L. rhamnosus was shown to improve the expression of tight junction proteins [claudin (CLDN)-1, occludin (OCC)] and skin barrier proteins (FLG and loricrin) in a reconstructed human epidermis (Jung et al., 2019). In addition, L. rhamnosus and B. longum lysates, lysated with a bead beater, increased transepithelial electrical resistance and expression of tight junction proteins such as CLDN-1, CLDN-4, OCC, and ZO-1 (Sultana et al., 2013).

3. Antiwrinkle effects of L. paracasei HDB1196 lysates on fibroblast cells
Wrinkles are the most common symptom of aging skin. Collagen and elastin are the main components of the extracellular matrix in the dermis, and these give the skin elasticity. Therefore, inhibiting the loss of collagen and elastin is an important factor in preventing skin aging. In particular, collagen production and MMP expression and activity are the most important factors in wrinkle formation (Park et al., 2019). Reactive oxygen species (ROS) accelerate the skin aging by promoting the degradation of collagen and the production of MMP-1, which is an interstitial collagenase that breaks down collagen fibers (Hong et al., 2015).

HDB1196 showed DPPH radical-scavenging activity in a dose-dependent manner (Fig. 4A). The mRNA expression of MMP-1 was markedly increased in the UVA-irradiated cells (Fig. 4B). Compared with the expression in the UV-irradiated cells, HDB1196 reduced MMP-1 expression levels by about 60%, whereas EGCG reduced these levels by 24%. By contrast, 3% (v/v) HDB1196-treated and Vit C-treated cells showed increased COL-1 mRNA expression (124.9% and 118.9%, respectively) compared with 100% of untreated cells (Fig. 5C). Furthermore, the antioxidant effect of HDB1196 seemed to suppress wrinkle formation by counteracting ROS and decreasing MMP-1 levels.

In previous studies, the antiwrinkle effects of other probiotic lysates have been reported. In one study, lysates of L. paracasei and L. rhamnosus manufactured using an ultrasonic shredder showed DPPH radical-scavenging activity, upregulation of collagen, and downregulation of MMP-1 (Kim & Lee, 2015). In another study, heat-killed L. acidophilus KCCM12625P showed antioxidant effects, increased levels of COL-1, and inhibition of elastase and MMP expression, which occurred through the inhibition of the phosphorylation of extracellular signal-regulated kinases (ERKs) and c-Fos in the activator protein-1 (AP-1) pathway (Lim et al., 2020).
4. Inhibition of melanogenesis by \textit{L. acidophilus} HDB1014 lysates in B16F1 cells

Melanogenesis is the process by which melanin is produced by melanocytes in melanosomes. Melanin, which is related to the color of the skin, hair, and eyes, protects the skin from UV radiation (Jang et al., 2020). However, oversynthesis and accumulation of melanin lead to the formation of melasma and freckles on the skin (Park et al., 2019). Tyrosinase is involved in the melanogenesis pathway, and it degrades tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), which is, in turn, converted to dopachrome and melanin via an oxidation reaction with ROS (Kim & Lee, 2015). Thus, antioxidant effects are related to antimelanogenic effects.

HDB1014 showed DPPH radical-scavenging activity in a dose-dependent manner (Fig. 5A). Melanin content and secretion were noticeably increased in α-MSH-induced cells compared with levels in untreated cells. HDB1014 treatment inhibited melanin content in the cells and secretion from the cells. Melanin content and secretion in arbutin-treated cells were reduced by 57.8% and 15.7%, respectively, whereas HDB1014 at 3% (v/v) inhibited melanin production by 27.6% and secretion by 40% compared with the levels in α-MSH-induced cells (Fig. 5B, 5C). Moreover, the antioxidant effect of HDB1014 seemed to suppress the oxidation reaction of melanogenesis by removing ROS and thereby reduced melanin content in B16F1 cells.

The antimelanogenetic effect of other probiotic lysates has previously been reported. For example, tyndallized \textit{L. acidophilus} KCCM12625P showed ABTS radical-scavenging activity and antimelanogenic effects through the regulation of the cyclic adenosine monophosphate (cAMP) signaling-mediated pathway elements such as protein kinase A (PKA), cAMP response element binding (CREB), microphthalmia-associated transcription factor (MITF), and tyrosinase family members (Lim et al., 2020). In addition, \textit{L. paracasei} and \textit{L. rhamnosus} lysates showed DPPH radical-scavenging activity and the ability to reduce melanin content (Kim & Lee, 2015).

Researchers have reported that LTA, a major component of the cell wall of gram-positive bacteria, has antioxidant, anti-wrinkle, and antimelanogenic effects. LTA of \textit{L. plantarum} and \textit{L. sakei} has been shown to produce antioxidant effects and inhibit MMP-1 expression in UV-induced cells by inhibiting AP-1 and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-kB) signaling pathways (Hong et al., 2015; You et al., 2013). In addition, LTA isolated from \textit{L. plantarum} is reported to produce antimelanogenic effects through the activation of ERK and the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, e.g., via elements such as ERK, PI3K p85α, PI3K p110β, AKT, MITF, and tyrosinase family members (Kim et al., 2015). In this study, probiotic samples were filtered, so the cell wall, including LTA, was largely removed. However, these probiotic lysates exerted the same skin functionality effects as lysates with intact cells. Therefore, in addition to the cell wall, nucleotides can be considered to act as functional substances. In conclusion, the probiotic lysates studied here could be potential ingredients for treatments of specific skin aging.

IV. Conclusion

Skin aging is one of the important concerns of modern people, which is accompanied by symptoms such as collapse of the skin barrier, wrinkles, and freckles depending on internal and external factors. In this study, probiotic lysate from which cell wall components have been removed, each effective in skin barrier enhancing, antiwrinkle, and whitening, were developed as skin antiaging cosmetic materials. Three types of probiotic lysates (\textit{B. longum} HDB7072, \textit{L. paracasei} HDB1196, and \textit{L. acidophilus} HDB1014) were prepared by extracting the nucleotide content, expected as an active material, through post-fermentation process with 0.5% sodium hyaluronate. HDB7072 significantly increased the gene expression of FLG, a skin barrier protein, in the NHEKs. HDB1196 significantly reduced collagen reduction and MMP-1 enzyme expression by UV treatment in the fibroblast. HDB1014 decreased the amount of melanin synthesis and secretion, which is an indicator of MSH-induced melanogenesis in the B16F1 cells. Through these results, it was confirmed that three types of cell wall-removed probiotic lysates (HDB7072, HDB1196, and HDB1014) showed efficacy for skin barrier enhancing, antiwrinkle, and whitening, respectively, as a function for the development of antiaging cosmetic materials.

References
