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Improvements in Human Keratinocytes and Antimicrobial Effect Mediated by Cell-Free Supernatants Derived from Probiotics

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Abstract: The skin acts as a physical and physiological barrier, thereby protecting the body from various environmental components and stimuli. Cell-free supernatants (CFS) derived from probiotics can improve skin functions and retain moisture. In this study, to assess the efficacy of CFS derived from *Ligilactobacillus salivarius* and *Limosilactobacillus fermentum*, we investigated the barrier strengthening and moisturizing effects of CFS in keratinocytes along with their antibacterial effects. We also determined the adhesive effects of probiotics on colorectal cells. To confirm improvements in moisturization and barrier function mediated by CFS in keratinocytes, hyaluronic acid (HA) production, and mRNA expression of HA synthases (*HAS*)*2*, *HAS3*, and *FLG* were measured. The results showed that CFS from *L. salivarius* MG242 and *L. fermentum* MG901 increased the expression of these genes along with the production of HA (2.40- and 1.95-fold of control). Additionally, CFS derived from *L. salivarius* MG242 and *L. fermentum* MG901 inhibited the growth of *S. aureus* and *E. coli*, thereby demonstrating inhibitory effects against harmful pathogens observed on the skin. These results indicate that the use of CFS derived from *L. salivarius* MG242 and *L. fermentum* MG901 may increase moisturization in the skin and improve barrier function of keratinocytes along with elimination of potential pathogens.

Keywords: postbiotics; skin; antimicrobial; *Ligilactobacillus salivarius*; *Limosilactobacillus fermentum*

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1. Introduction

The skin is organized into three layers: epidermis, dermis, and hypodermis [1]. The epidermis, comprising the outermost layer of the skin, plays an important role in physiological immune reactions and as a physical barrier in the human body [2]. The epidermis prevents the absorption of chemicals released in various environments and protects against microorganisms and water loss [3]. The main constituents of the epidermis are keratinocytes (90–95%) [1,4]. Keratinocytes aid the immune system in the skin via the expression of toll-like receptors and nod-like receptors and maintain skin moisture by synthesizing hyaluronic acid (HA) [3,5]. Skin diseases, including atopic dermatitis, psoriasis, and skin inflammation, occur when the immune system, including immune reactions mediated by keratinocytes, is disrupted by pathogen infiltration [4].

According to the World Health Organization, probiotics are living microorganisms that provide benefits to the host when administered at a suitable dose [6]. The most common genera of probiotics are *Lactobacillus* and *Bifidobacterium*. Among members of the Lactobacillaceae family, *Ligilactobacillus salivarius* (*Lactobacillus salivarius*, *L. salivarius*) and *Limosilactobacillus fermentum* (*Lactobacillus fermentum*, *L. fermentum*) have the basic properties of Lactobacilli, such as inhibition of pathogen growth in the intestine, immunomodulatory activity, and intestinal barrier regulation, and are also known to have superior antibacterial effects against *S. aureus* compared to other species [7]. Additionally, they have demonstrated beneficial effects, such as protection from periodontitis, atopic dermatitis, intestinal

inflammation, and obesity [8–11]. Probiotics produce bioactive metabolites and products, such as short-chain fatty acids (SCFA), organic acids and antimicrobial peptides [12]. In particular, cell-free supernatants from *L. fermentum* and *L. salivarius* contain SCFA, such as D-, and L-lactic acid, acetic acid, propionic acid and butyric acid [13,14]. These metabolites are characterized as postbiotics; cell-free supernatants (CFS) containing bioactive soluble factors produced during the fermentation of microorganisms demonstrate anti-inflammatory, anti-tumor, and anti-oxidant effects and aid in the treatment of diarrhea [15]. Probiotics produce metabolites, especially SCFA and organic acids, at a low pH [16]. The acidic pH (pH 4–6) of the skin modulates the skin barrier function by protecting against the invasion of various pathogens [17]. Therefore, we hypothesized that CFS with SCFA would improve and protect the skin.

Previously, we found that *L. salivarius* MG242 and *L. fermentum* MG901 effectively reduced bacterial vaginosis in mice models [13]. In this study, we investigated the skin improvement potential, including HA production in keratinocytes and anti-microbial effects, of CFS derived from the growth of two probiotics, *L. salivarius* and *L. fermentum*.

2. Materials and Methods

2.1. Preparation of CFS

All lactic acid bacteria (LAB), including *L. salivarius* MG242 (NCBI accession number, MN055708.1), MG4265 (NCBI accession number, MN060992.1), MG4227 (NCBI accession number, MF597747.1) and *L. fermetum* MG901 (NCBI accession number, MN055709.1), MG4231 (NCBI accession number, MW947163.1), MG4244 (NCBI accession number, MW947154.1) and CFS of LAB used in this study were supplied by MEDIOGEN Co., Ltd. (Jecheon, Korea). The CFS was collected by centrifugation (4000× *g*, 10 min at 4 °C) [18]. The supernatants were filtered and sterilized using a 0.22 µm polytetrafluoroethylene membrane filter (ADVANTEC, Tokyo, Japan). Filtered supernatants were kept at -80 °C after freeze-drying in vacuo following the study.

2.2. Cell Culture and Viability

HaCaT human keratinocytes were purchased from the Korean Cell Line Bank (Seoul, Korea). HaCaT keratinocytes were incubated in Dulbecco's modified Eagle medium (DMEM; Gibco, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37 °C in an incubator containing 5% CO_2. Keratinocytes were sub-cultured every two to three days.

Cell viability was measured using an MTT assay. HaCaT keratinocytes were seeded at 8×10^4 cells/well in 96-well plates for 24 h. After removing the supernatants, keratinocytes were treated with CFS (200 and 400 µg/mL) in serum-free media for 24 h. The MTT stock (5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to obtain a concentration of 0.1 mg/mL, followed by culturing of the cells for 2 h. Formazan crystals were dissolved in DMSO (Sigma-Aldrich). A microplate reader (EPOCH2, Biotek, Winooski, VT, USA) was used to evaluate the absorbance at 550 nm.

2.3. Quantification of HA Production

HA production was measured using a commercial ELISA-like assay kit (BioVision Inc., Milpitas, CA, USA). Briefly, the cells (2×10^5 cells/mL) were incubated in the presence or absence of CFS in a 12-well plate for 24 h. After incubation, the absorbance of the supernatants was measured at 450 nm using a microplate reader according to the manufacturer's instructions.

2.4. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA from HaCaT keratinocytes was isolated using the NucleoZOL reagent (MACHEREY-NAGEL, Dueren, Germany) according to the manufacturer's instructions. RNA was reverse-transcribed using the cDNA reverse transcriptase premix (iNtron, Gyeonggi-do, Korea). qRT-PCR (Bio-Rad, Hercules, CA, USA) was performed using the amfiSure qGreen Q-PCR Master Mix (GenDepot, Katy, TX, USA). The primers used for analyzing skin hydration

are listed in Table 1. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a control for the normalization of gene expression. The mRNA expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences of human origin used for qRT-PCR.

Gene [1]	Primer	Sequence (5′→3′)
HAS2	Forward	ATTACCCAGTCCTGGCTTCG
	Reverse	CCTGTGGAAGACTCAGCAGAA
HAS3	Forward	TGTCCAGATCCTCAACAAGTACGA
	Reverse	CTGGAGGAGGCTGTTGC
FLG	Forward	GGCTAAGTGAAAGACTTGAAGAGA
	Reverse	AATAGACTATCAGTGGTGTCATAGG
GAPDH	Forward	GTCTTCACCACCATGGAGAA
	Reverse	AGGAGGCATTGCTGATGAT

[1] *HAS2*, hyaluronan synthase 2; *HAS3*, hyaluronan synthase 3; *FLG*, filaggrin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

2.5. Antimicrobial Effect against Escherichia coli and Staphylococcus aureus

To evaluate the inhibition of the growth of *E. coli* DH5α and *S. aureus* KCTC 3881, 1.0×10^7 CFU/mL of pathogenic bacteria were inoculated in the LB and MRS broth, respectively and CFS (400 and 1000 μg/mL in peptone water, each) was added to the broths [19]. After incubation for 12 h at 37 °C, the viable cells were counted on LB and MRS agar, respectively, using the plate count method.

2.6. Adherence to HT-29 Colorectal Cells

Adherence of probiotics to HT-29 colorectal cells was analyzed according to the protocol reported in a previous study [20]. Briefly, 1 mL of 1×10^6 HT-29 colorectal cells were grown in 12-well plates at 37 °C in an atmosphere containing 5% CO_2 until a cell monolayer was formed. LAB cultured in MRS broth (Difco, MI, USA) were resuspended in DMEM at an optical density 600 of 1.0 in DMEM media and treated in 12-well plates inoculated with HT-29 colorectal cells. After 2 h, the non-adherent LAB was discarded, washed twice with PBS, and LAB adherents to colorectal cells were scraped. The LAB attached to the cells were counted using the plate count method on MRS agar.

2.7. Statistical Analysis

The results are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical differences were evaluated using one-way ANOVA, and post hoc analysis was conducted using Duncan's test at a value of $p < 0.05$. Statistical analyses were performed using the Statistical Package for the Social Sciences Statistics 21 (IBM Co., Armonk, NY, USA).

3. Results and Discussion

3.1. Effect of CFS Derived from L. salivarius and L. fermentum on Cell Viability and Hyaluronic Acid Production in HaCaT Keratinocytes

Various metabolites produced by probiotics have been retrieved from CFS [21]. An MTT assay was used to confirm the viability of HaCaT keratinocytes after treatment with CFS. CFS derived from *L. salivarius* and *L. fermentum* (200 and 400 μg/mL, respectively) showed no cytotoxicity against HaCaT keratinocytes (Figure 1A). Thus, further experiments were performed using 400 μg/mL of CFS.

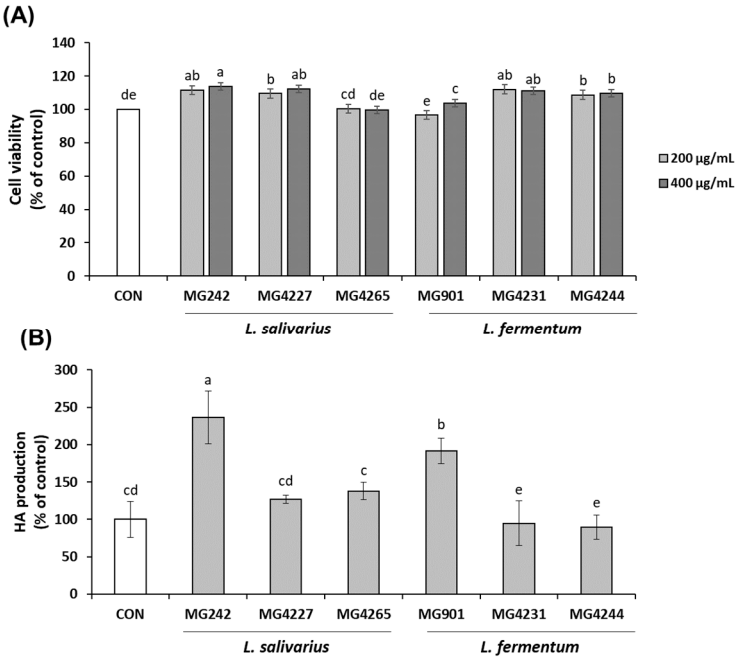

Figure 1. Effect of CFS derived from probiotic bacterial strains on (**A**) cell viability, and (**B**) hyaluronic acid (HA) production in HaCaT keratinocytes. Data are represented as mean ± SD (n = 3). Statistical significance is indicated by different letters over the column ($p < 0.05$).

Hyaluronic acid (HA), which is found in the extracellular matrix of epidermal keratinocytes, is the key component essential for skin moisturization. HA is a ubiquitous glycosaminoglycan with the unique ability to bind and retain water molecules [22]. In Figure 1B, HA production by HaCaT keratinocytes treated with CFS derived from *L. salivarius* and *L. fermentum* is shown. CFS derived from *L. salivarius* MG242 and *L. fermentum* MG901 significantly increased HA production by 2.40- and 1.95-fold, respectively, compared to that observed in the control.

3.2. CFS Derived from L. salivarius and L. fermentum Affects mRNA Expression Associated with Skin Moisturization and Barrier Maintenance in HaCaT Keratinocytes

The polydispersity of HA determines its biological properties, and this polymer is formed by hyaluronic acid synthases (HAS) [23]. HAS comprise three isoenzymes, HAS1, HAS2, and HAS3. Each isozyme shows different properties. They produce HA of different sizes; HAS1 and HAS2 can synthesize HA polymers up to 2×10^6 Da in length, and HAS3 synthesizes polymers with shorter chains than HAS1 and HAS2 [24]. The mRNA expression of *HAS2* and *HAS3* in MG242 (1.40- and 1.58-fold of control), and MG901 (1.45- and 1.71-fold of control) were higher than those of other CFSs (Figure 2A,B). Most HA was produced by CFSs of *L. salivarius* MG242 and *L. fermentum* MG901 (Figure 1). HA is primarily produced by *HAS2* and *HAS3*. Intrinsically, HAS2 and HAS3 are more actively catalyzed than HAS1 [24]. Therefore, the increase in HA production observed upon treatment with CFSs of *L. salivarius* MG242 and *L. fermentum* MG901 can be attributed to increased expression of *HAS2* and *HAS3*. The results also demonstrated that the influence of HAS3 on HA production was higher than that of HAS2.

A weakened skin barrier can lead to xerotic cutis. Filaggrin is a natural moisturizing agent that preserves moisture when combined with hydrophilic components in the skin [25]. *FLG* showed similar mRNA expression (1.40- and 1.58-fold of control) after treatment with all CFS, except with that derived from MG4244 (Figure 2C). Low-molecular-weight hyaluronan polymerized by HAS3 is involved in the production of FLG in the epidermis [26]. Thus, the effect of CFS derived from probiotics on *FLG* was mediated via modulation of the expression of HAS. In summary, the results confirmed that CFS derived from *L. salivarius* MG242 and *L. fermentum* MG901 improved skin hydration and barrier capacity.

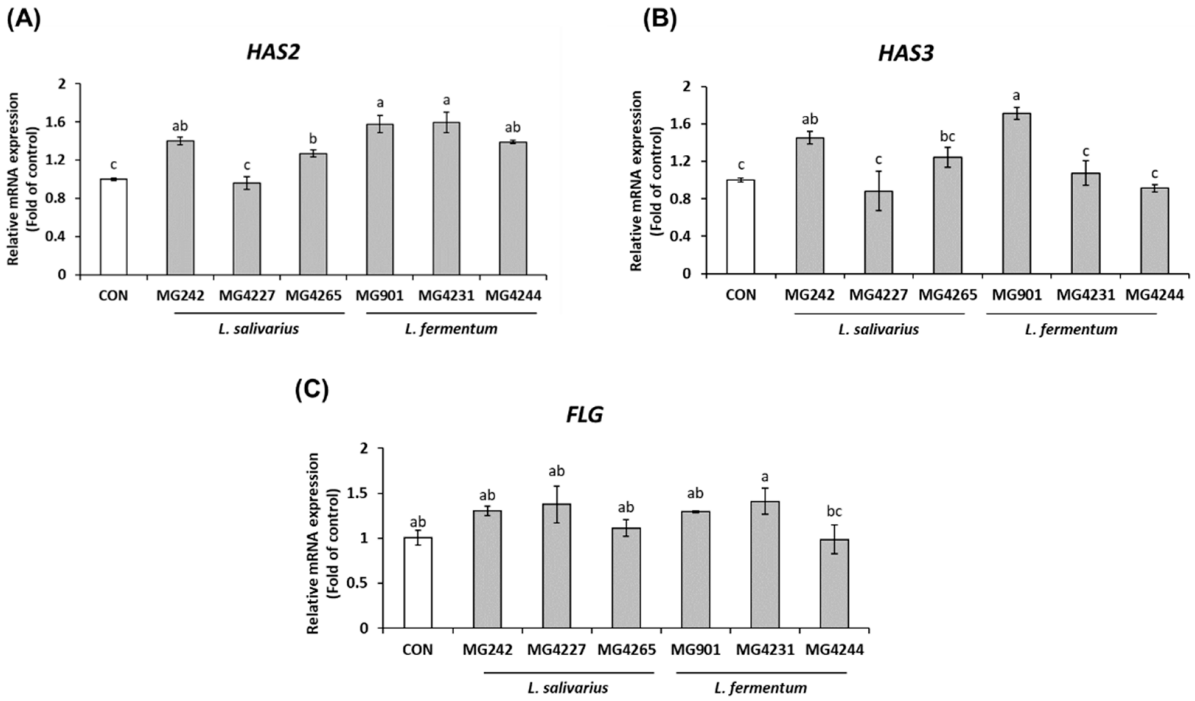

Figure 2. Effect of CFS derived from probiotics on (**A**) *HAS2*, (**B**) *HAS3*, and (**C**) *FLG* mRNA expression in HaCaT keratinocytes. Data are shown as mean ± SD (n = 3). Statistical significance is indicated by different letters on the column ($p < 0.05$).

3.3. Antibacterial Effect of CFS Derived from *L. salivarius* MG242 and *L. fermentum* MG901

S. aureus, a gram-positive bacterium, is present in the skin, and skin infection by *S. aureus* can lead to severe conditions, such as abscesses and sepsis [27]. Common skin and soft tissue infections are mainly caused by gram-negative bacteria, such as *E. coli* [28]. The antibacterial effects of CFSs derived from *L. salivarius* MG242 and *L. fermentum* MG901 on *S. aureus* and *E. coli* are shown in Figure 3. The amount of *S. aureus* was reduced by up to 50% upon treatment with CFSs of *L. salivarius* MG242 and *L. fermentum* MG901. The CFS of *L. salivarius* MG242 and *L. fermentum* MG901 also decreased the amount of *E. coli* up to 99%. This antibacterial effect of CFS suggests that they can help improve skin health by reducing bacterial infections in the skin.

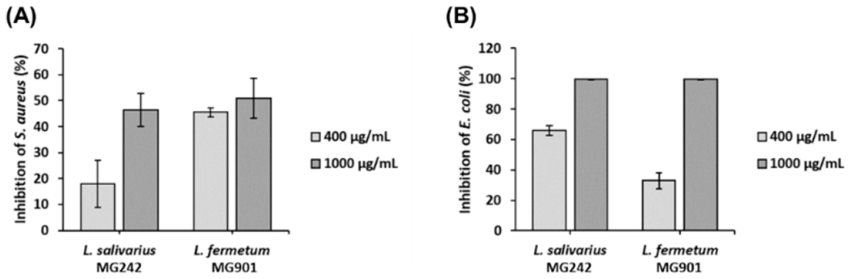

Figure 3. Effect of CFS derived from *L. salivarius* MG242 and *L. fermentum* MG901 on the growth of (**A**) *S. aureus* and (**B**) *E. coli* in the media. Data are shown as mean ± SD (n = 3).

3.4. Adherence of Probiotics to HT-29 Colorectal Cells

Recently, studies examining the gut microbiome and gut-skin axis have increased. Findings on the gut-skin axis suggest that skin health may be affected by the gut immune system [29]. Some studies have reported that probiotics improve skin health [30]. Adherence of *L. salivarius* MG242 and *L. fermentum* MG901 to HT-29 colorectal cells was 85.79 and 82.69%, respectively (Table 2). Other probiotics have been analyzed, with *L. fermentum* and *L. salivarious* showing approximately 55 to 80% adherence. Pathogens present in colorectal

cells produce inflammatory cytokines, which affect various organs, such as the oral cavity, skin, and respiratory tract. However, probiotics attach to colon cells and form biofilms to inhibit the invasion of pathogens, indirectly affecting skin health [31]. Metabolites produced by probiotics can be delivered directly to the skin through the bloodstream or can affect the skin microbiome [29]. Therefore, MG242 and MG901, which show remarkable adhesion to the intestine, produce metabolites that maintain skin health and protect against the invasion of pathogens by strengthening the skin barrier.

Table 2. Adhesion of *L. salivarius* MG242 and *L. fermentum* MG901 to HT-29 colorectal cells.

Strains	Initial Counts (CFU/mL)	Adhesion Counts (CFU/mL)	Adherence (%)
L. salivarius MG242	8.54 ± 0.05	7.41 ± 0.07	85.79 ± 1.55%
L. fermentum MG901	8.62 ± 0.01	7.15 ± 0.02	82.69 ± 0.41%

Data are represented as mean ± SD (n = 3).

4. Conclusions

In this study, we confirmed the effects on skin of CFS, which contain various metabolites produced from probiotics. The CFS of *L. salivarius* MG242 and *L. fermentum* MG901 improved skin moisture and barrier function by modulating mRNA expression of hyaluronic acid synthase and filaggrin in keratinocytes and showed antimicrobial effects against *S. aureus* and *E. coli*. In addition, *L. salivarius* MG242 and *L. fermentum* MG901, which produced various metabolites with excellent intestinal wall adhesion, were demonstrated to have great potential for improving skin health. However, further research is needed to determine the components of these strains that are beneficial for skin health.

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