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Original research paper

Potential anti-ageing effects of probiotic-derived conditioned media on human skin cells

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ABSTRACT

In this study, the protective functions of bacteria-free conditioned media from *Bifidobacterium* and *Lactobacillus* species against ultraviolet radiation-induced skin ageing and associated cellular damage were investigated. The effects of ultraviolet radiation-induced reactive oxygen species production were suppressed by all conditioned media; particularly, the loss of cell viability and downregulation of collagen gene expression were significantly reversed by the conditioned media from *B. longum* and *B. lactis*. Further examination of potential anti-pigmentation effects revealed that the *B. lactis*-derived conditioned media significantly inhibited tyrosinase activity and alpha-melanocyte-stimulating hormone-induced melanin production in human epidermal melanocytes. Further, the conditioned media suppressed the phosphorylation of extracellular signal-related kinase, which functions as an upstream regulator of melanogenesis. Therefore, *B. lactis*-derived conditioned media can potentially protect against cellular damage involved in skin-ageing processes.

Keywords: anti-ageing, skin cells, probiotics, conditioned media, protection, anti-pigmentation

Ultraviolet radiation (UV) is the major extrinsic cause of skin ageing (1). Melanin pigment is produced within melanocytes in the epidermis, its production is affected by the intercellular interaction between keratinocytes and melanocytes) and functions as a natural sunscreen filter; however, excessive UV radiation causes abnormal synthesis of the pigment, leading to several hyperpigmentation-related dermatological disorders such as melasma, age spots, and freckles (1). Melanin synthesis in the epidermis is regulated by several molecular signalling pathways; for example, alpha-melanocyte-stimulating hormone (α-MSH) and its receptor melanocyte-specific melanocortin-1 receptor (MC1R) participate in a well-known mechanism for UV-induced melanin production in melanocytes (1). Activation of this pathway upregulates its major downstream target, microphthalmia-associated transcription factor (MITF), which is a transcription factor for melanin synthesis

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enzymes such as tyrosinase, tyrosinase-related protein 1 (TYPR1) and tyrosinase-related protein 2 (TYPR2) (1). In UV-induced aged skin, the α-MSH/MC1R pathway is activated, and the enzyme activity of tyrosinase is more upregulated than in normal keratinocytes. Effective treatments for pigmentation disorders are based on an anti-pigmentation strategy that identifies novel compounds that downregulate the pathway and tyrosinase activity (1).

Dermal fibroblasts are the major cells in the dermis, organizing the extracellular matrix (ECM) by producing and secreting collagen proteins such as type 1 (COL1A1) and type 3 collagen (COL3A1) (2). The dynamic structure of ECM is regulated by collagen proteins and collagenases such as matrix metalloproteinase-1 (MMP1) and -3 (MMP3). Therefore, the ECM can be changed during the ageing process by abnormal production of collagens and MMPs (3). Abnormal UV irradiation can lead to ECM degradation through several intracellular events, including reactive oxygen species (ROS) production, downregulation of collagen genes, and upregulation of MMP genes (4). These lead to specific ageing signs such as wrinkles (3). Skin-ageing processes including hyperpigmentation and wrinkles are easily perceived (3); therefore, there is a great demand in the pharmaceutical and cosmetic industries for the identification of agents that can protect against skin ageing.

Probiotics are special microorganisms derived mainly from the human gastrointestinal tract that include *Bifidobacterium* (*Bi*) and *Lactobacillus* (*La*) species. Consumption of adequate quantities of these live microorganisms generates beneficial health effects in the host (4). Several dermatological studies have demonstrated that gut microbiota potentially communicates with the skin and that oral application of probiotics, including *Bi* and *La* species, has beneficial effects on skin dyshomeostasis-related disorders, such as increased level of trans-epidermal water loss (TEWL), psoriasis, atopic dermatitis, and acne (5–7). Recent studies have shown the beneficial effects of probiotics-conditioned media (CM), which is the broth used to grow the probiotic bacteria, subsequently filtered to remove all bacteria (8). Studies show that bioactive components are secreted into CM by certain probiotic bacteria, and these components were shown to exert anti-inflammatory and cytoprotective properties (9, 10). Of note, CM from *Bi* and *La* species showed protective effects against aspirin-induced cellular damage (11) and interleukin-10 deficiency-mediated colon inflammation (12), respectively. Based on these results, we investigated whether CM from *Bi* and *La* species could protect against skin ageing-related cellular dysfunctions.

EXPERIMENTAL

Preparation of probiotics-derived conditioned media

Probiotics used in this study are shown in Table I. *Bifidobacterium lactis* CBT BL3 and *Bifidobacterium longum* CBT BG7 were cultured overnight at 37 °C under anaerobic conditions in a bifidobacterial-optimized media, BL broth (Becton, Dickinson and Company, USA). *Lactobacillus casei* CBT LC5, *Lactobacillus plantarum* CBT LP3, *Lactobacillus reuteri* CBT LU4, and *Lactobacillus rhamnosus* CBT LR5 were cultured overnight at 37 °C under aerobic conditions in a lactobacillus-optimized media, MRS broth (Difco, BD Biosciences, USA). pH-controlled media (pHO) was maintained between pH 6.0 and 6.5. pH-uncontrolled media (pHX) did not have a pH-maintained condition. The supernatant of each culture

medium was obtained by centrifuging at 2700 × *g* for 30 min. Five-fold concentrated powder of the supernatant was made by processing the media through vacuum evaporation and lyophilization.

Cell culture and reagents

Normal human dermal fibroblasts (NHDFs) were purchased from CELLnTEC (Switzerland) and grown in fibroblast growth media (FBM, Lonza, Switzerland) supplemented with an FGM-2 SingleQuot Kit (Lonza) at 37 °C with 5 % CO_2. B16F10 mouse melanoma cells were grown in Roswell Park Memorial Institute 1640 medium (Biowest, France) supplemented with 10 % (*v*/*v*) fetal bovine serum (Biowest), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Gibco, USA) in a humidified 5 % CO_2 incubator at 37 °C.

Water-soluble tetrazolium salt assay

Probiotic CM-induced cytotoxicities were assessed using a water-soluble tetrazolium salt (WST-1) assay (EZ-Cytox cell viability assay kit, Itsbio, Korea). Briefly, NHDFs and B16F10 cells were seeded in a 96-well culture plate and incubated overnight. The cells were then treated with the indicated doses of the probiotic CMs for 24 h. A WST-1 solution was subsequently added to each well, and plates were further incubated for 0.5 h. Cell viability was determined by measuring absorbance at 450 nm with 620 nm (reference filter) using an iMark microplate reader (Bio-Rad, USA).

UVB irradiation and reagent treatment

Protective effects of the probiotic CMs against UV-induced cellular damage (loss of cell viability and ROS production) were assessed with UVB-irradiated and CM-treated NHDFs. Briefly, cells (2×10^5) were seeded in a 60-mm plate and cultured in growth media overnight. Prior to UVB irradiation, the cell medium was replaced twice with phosphate-buffered saline (PBS), then cells were irradiated with 10 mJ cm^{-2} UVB radiation using a UV illuminator (Super Light VI, BoTeck, Korea) without covering the plate dishes. After UVB irradiation, PBS was replaced with fresh growth media containing probiotic CMs for 24 h.

Analysis of intracellular ROS levels

The protective effect of the probiotic CMs against UV-induced intracellular ROS production in NHDFs was assessed using a 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA,

Table I. List of commercial probiotics used in this study

Species	Strains	Origin
Bifidobacterium lactis	CBT BL3	Korean Infant faeces
Bifidobacterium longum	CBT BG7	Korean Infant faeces
Lactobacillus casei	CBT LC5	Korean Human faeces
Lactobacillus plantarum	CBT LP3	Korean fermented vegetable product, kimchi
Lactobacillus reuteri	CBT LU4	Korean Human faeces
Lactobacillus rhamnosus	CBT LR5	Korean Human faeces

Sigma-Aldrich, USA) staining assay. Briefly, UVB-irradiated cells were treated with probiotic CMs for 24 h. The cells were then mixed with 10 μM DCF-DA solution (Sigma-Aldrich) and incubated for 1 h. The fluorescence intensity was determined using a microplate fluorescence reader (Molecular Devices, USA) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) was performed using a Shimadzu HPLC system (Shimadzu, Korea). Fermented supernatant of *B. lactis* CBT-BL3 was prepared under two different conditions. One sample was fermented in a pH-controlled manner by maintaining the pH of the media. The other sample was fermented without pH control of the media. After fermentation, bacterial cells and supernatants were separated by centrifuging the culture media. The ferment supernatant was freeze-dried after separation. Fifty milligrams of freeze-dried samples of each *B. lactis* CBT-BL3 ferment supernatant was dissolved in 6 mmol L^{-1} perchloric acid and filtered through a 0.2-μm filter. The same solvent was pumped into the column (Shodex RSpak KC-811, 6 μm, 8.0 × 300 mm) as the mobile phase. The flow rate of the perchloric acid was 1 mL min^{-1}, and the temperature was 40 °C. Both conditions were maintained until the end of the analysis. The injection amount of the sample was 10 μL. Peaks were detected by a UV detector at 210 nm.

Immunoblot assay

Whole-cell extracts were prepared using a radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, USA) containing a protease inhibitor cocktail (Roche Diagnostics, USA). Extracts were subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membrane. The membrane was then incubated with 5 % skim milk for 1 h, followed by incubation with each indicated primary antibody at 4 °C. For protein detection, horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, USA) were used, followed by enhanced chemiluminescence (ECL, Pierce, Thermo Fisher Scientific) and autoradiography. β-actin was used as an endogenous internal control. The primary antibodies used in these experiments were as follows. Anti-Mitf, anti-tyrosinase, anti-TYRP1, anti-TYRP2, and anti-p38 antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-ERK, anti-p-ERK (Thr202/Tyr204), anti-p-p38 (Thr180/Tyr182), anti-JNK, anti-p-JNK (Thr183/Tyr185) and anti-p-PKA (Thr198) antibodies were purchased from Cell Signaling Technology. Anti-PKA antibody was purchased from Abcam (UK).

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Changes in mRNA levels were measured using RT-qPCR. Total RNAs were prepared using the TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized using an M-MLV reverse transcriptase (Invitrogen, Thermo Fisher Scientific). RT-qPCR was performed using a HOT FIREPol EvaGreen PCR Mix Plus (Solis BioDyne, Estonia) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The primer sequences were as follows: human *COL1A1* forward 5′-AGGGCCAAGACGAAGACATC-3′, reverse 5′-AGATCACGTCATCGCACAACA-3′; human *COL3A1* forward 5′-GTTTTGCCCCGTATTATGGA-3′, reverse 5′-GGAAGTTCAGGATTGCCGTA-3′; human *MMP1* forward 5′-TCTGACGTTGATCCCAGAGAGCAG-3′,

reverse 5′-CAGGGTGACACCAGTGACTGCAC-3′; human *MMP3* forward 5′-AGCAAGGACCTCGTTTTCATT-3′, reverse 5′-GTCAATCCCTGGAAAGTCTTCA-3′. The primer sequences used for *Mitf*, *Tyrosinase*, *Tyrp1*, *Tyrp2* and *β-actin* have been previously described (13). The mRNA expression level of each target gene was normalized to that of β-actin and relative mRNA quantification was calculated using the $2^{-\Delta\Delta Ct}$ method.

Determination of melanin content

Intracellular melanin contents were determined by adding a NaOH lysis buffer and measuring absorbance at 450 nm. Briefly, the cells were treated with different concentrations of probiotic CMs in the presence or absence of 100 nmol L^{-1} of melanocyte-stimulating hormone (α-MSH) for 24 h. The cell pellets were washed with PBS and dissolved in 1 mol L^{-1} NaOH lysis buffer. After 1 h incubation at 60 °C, the melanin content was measured at 450 nm using an iMark microplate reader (Bio-Rad). The protein concentration was measured using the Pierce BCA protein assay kit (USA). The relative melanin content was adjusted to the total protein concentration in the corresponding sample.

Measurement of mushroom tyrosinase activity

To measure *in vitro* tyrosinase activity, 100 U mL^{-1} mushroom tyrosinase and 1 mmol L^{-1} L-DOPA solutions were mixed with various concentrations of probiotic CMs in 0.1 mol L^{-1} sodium phosphate buffer (pH 6.8). After 5 min of incubation at 37 °C, the production of dopachrome was determined by measuring at 450 nm using an iMark microplate reader (Bio-Rad).

Statistical analysis

Statistical analysis was analyzed using non-parametric Dunn's multiple comparison test after Kruskal-Wallis test with GraphPad Prism 9 Software (GraphPad Software, CA, USA). All analyses were performed in triplicates, and p-values < 0.05 were considered to indicate a significant difference.

RESULTS AND DISCUSSION

Intracellular ROS-scavenging effect of CM from Bifidobacterium *and* Lactobacillus *species*

Although ROS is produced by the normal metabolic process of cellular energy generation, abnormal doses of UV radiation trigger increased levels of ROS production, which is one of the major causative factors for photoaging in the skin (14). Therefore, we examined the suppressive effect of CM from Bi and La species on UV-induced ROS production in NHDFs. A WST-1-based cell viability analysis was performed to evaluate non-toxic doses of CM after treatment with different doses (1–100 μg mL^{-1}) of CMs on NHDFs for 24 h. As shown in Fig. 1, the treatment doses of both pHO and pHX CMs from the LP3 strain did not affect cell viability; however, different cytotoxicities were shown for pHO and pHX CMs from the other strains. These results suggest that compositions of CM were potentially changed by the different pH levels in their growth conditions. From these results, non-toxic doses of each CM were chosen for further experiments.

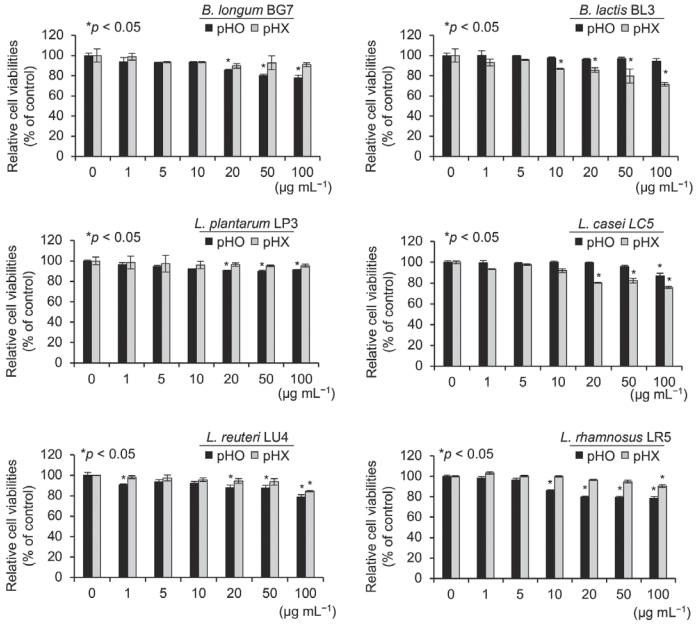


Fig. 1. Cytotoxic effects of conditioned media derived from *Bifidobacterium* and *Lactobacillus* species. The graph indicates the mean ± SD (n = 3). *p < 0.05 compared with non-treated control. pHO, pH-controlled conditioned media; pHX, pH-uncontrolled conditioned media.

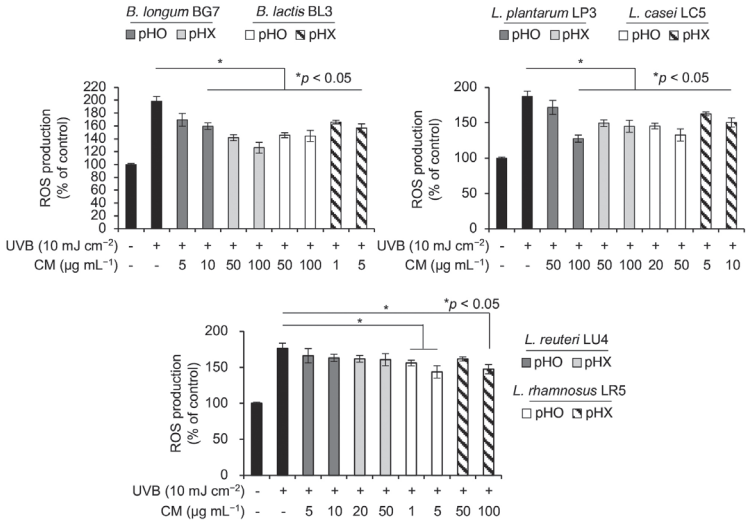


Fig. 2. The inhibitory effect of conditioned media (CM) derived from *Bifidobacterium* and *Lactobacillus* species on UV-induced ROS production in human dermal fibroblasts. The graph indicates the mean ± SD (n = 3). * p < 0.05 compared with non-treated UVB-irradiated cells. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

Next, to examine the intracellular ROS-scavenging effect of CMs, NHDFs were irradiated with 10 mJ cm^{-2} UVB and treated with non-toxic doses of CMs, followed by measurement of intracellular ROS using DCF-DA. As shown in Fig. 2, UV radiation significantly increased the cellular ROS level; however, this value was decreased in all CM-treated groups. Of note, 100 μg mL^{-1} of CM from BG7 pHX, BL3 pHO, and LP3 pHO significantly decreased cellular ROS levels by 72 % (ROS level difference value according to whether or not the CM was treated after UV irradiation), 54 %, and 59 %, respectively, compared to the ROS level (% of control) in UV-irradiated NHDFs. The other CMs decreased the cellular ROS levels by 10–30 %. These results indicate that the CMs had a suppressive effect on UV radiation-induced cellular ROS production in NHDFs.

Protective effect of CM from Bi and La species on UV-irradiated NHDFs

UV-induced ROS production can lead to ECM degradation *via* dysregulation of collagen and MMP expression levels (14). Therefore, we further evaluated whether the above results could be related to the protective function against UV-irradiated cellular damage. NHDFs were irradiated with 10 mJ cm^{-2} UVB and treated with CMs for 24 h. A WST-1 assay was performed to investigate the change in viability. As shown in Fig. 3, UV radiation significantly decreased the viability to 54 ± 0.84 %; however, this loss of viability was reversed by treatment with CMs. Of note, the CMs from BG7 pHO (10 μg mL^{-1}) and pHX (100 μg mL^{-1}) markedly restored the UVB-mediated loss of cell viability, by 13 and 21 %, respectively. Additionally, 100 μg mL^{-1} CMs from BL3 pHO and LP3 pHX restored the loss of cell viability by 18 and 23 %, respectively. The other CMs did not show a restoring effect.

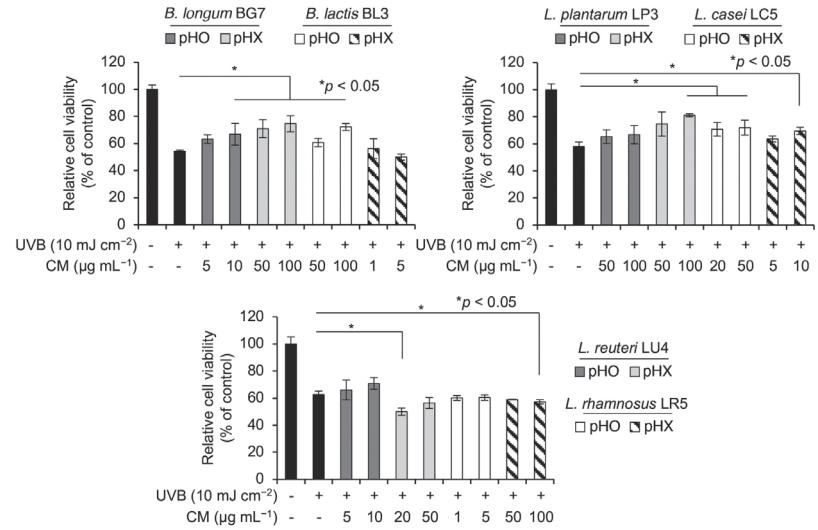


Fig. 3. The protective effect of conditioned media (CM) derived from *Bifidobacterium* and *Lactobacillus* species on UV-induced loss of cell viability in dermal fibroblasts. The graph indicates the mean ± SD (n = 3). * $p < 0.05$ compared with non-treated UVB-irradiated cells. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

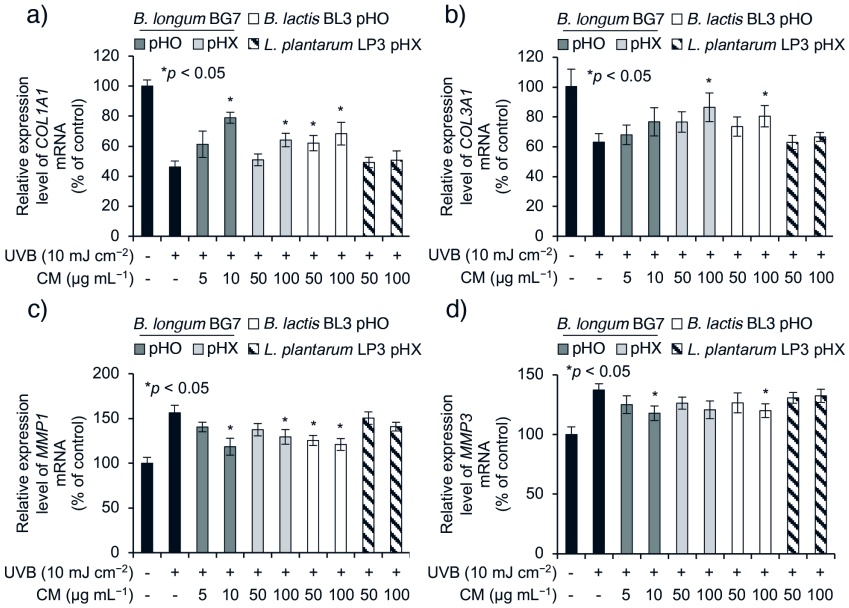


Fig. 4. The regulatory effect of probiotics-conditioned media (CM) on gene expression of collagens in UV-irradiated dermal fibroblasts: a) *COL1A1,* b) *COL3A1*, c) MMPs, *MMP1,* d) *MMP3,* The graph indicates the mean ± SD (n = 3). * $p < 0.05$ compared with non-treated UVB-irradiated cells. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

Next, we further tested whether the UV-protective function with respect to viability is potentially related to protection against UV-induced ECM degradation. After treatment with the CMs from BG7 pHO and pHX, BL3 pHO, and LP3 pHX in UVB-irradiated NHDFs, total RNAs were isolated, and the expression levels of collagen genes (*COL1A1* and *COL3A1*) and MMP genes (*MMP1* and *MMP3*) were analyzed using RT-qPCR experiments. As shown in Fig. 4, UVB irradiation significantly decreased *COL1A1* and *COL3A1* expression levels compared to those in control; however, treatment with CMs from BG7 pHO (10 μg mL^{-1}) and pHX (100 μg mL^{-1}) reversed UVB-induced *COL1A1* and *COL3A1* downregulation. In addition, CMs significantly decreased the extent of UVB-induced MMP1 and MMP3 upregulation. Similar results were observed in the BL3 pHO CM-treated NHDFs, but not in LP3 pHX-treated cells. These results suggest that CMs from BG7 pHO and pHX, and BL3 pHO, exerted a UV-protective effect by regulating cell viability and expression of collagen and MMP genes in NHDFs.

Inhibitory effect of CM from Bi and La species on melanogenesis

Skin hyperpigmentation is an ageing process induced primarily by UV radiation (1). Therefore, we investigated whether CMs have an inhibitory effect on UV-induced melanin production in human epidermal melanocytes. First, we evaluated the inhibitory effect of tyrosinase, a major metabolic enzyme in melanogenesis. Mushroom tyrosinase and its

substrate L-DOPA were incubated with CMs (1–100 μg mL^{-1}), and the inhibition effect of the enzyme was analyzed by measuring the quantities of a metabolite of L-DOPA, L-dopaquinone. As shown in Fig. 5, CMs from BG7 had no inhibitory effect on tyrosinase activity; however, CMs from BL3 showed a significant decrease in enzyme activity in a dose-dependent manner. Specifically, the inhibition rate [(1 − value (sample)/value (L-DOPA-treated control) × 100 %] of the CM from BL3 pHO was increased by approximately 40 %; however, the inhibition rate by the BL3 pHX group showed values up to 30 %, indicating that BL3 pHO CM has a better inhibition rate on tyrosinase activity BL3 pHX CM. In the other group, CM from LP3 pHO, but not from LP3 pHX, showed an inhibitory effect on enzyme activity in a dose-dependent manner. In LU4, LC5, and LR5 groups, inhibition effects were observed, but dose-dependent inhibition did not occur.

We further confirmed the results of the *in vitro* assay by evaluating the melanin quantity in melanocytes after treatment with the CMs, except for BG7 and LR5 CMs, which did not show an inhibitory effect on tyrosinase activity. First, we performed a WST-1 assay to set the treatment doses of the CMs for B16F10 melanoma cells (data not shown). Using non-toxic doses of each CM, intracellular melanin contents were quantified after 24 h of treatment in cells stimulated with α-MSH, a major stimulating factor of melanin synthesis. As shown in Fig. 6, the treatment groups of CMs from BL3 pHO and pHX showed a significant

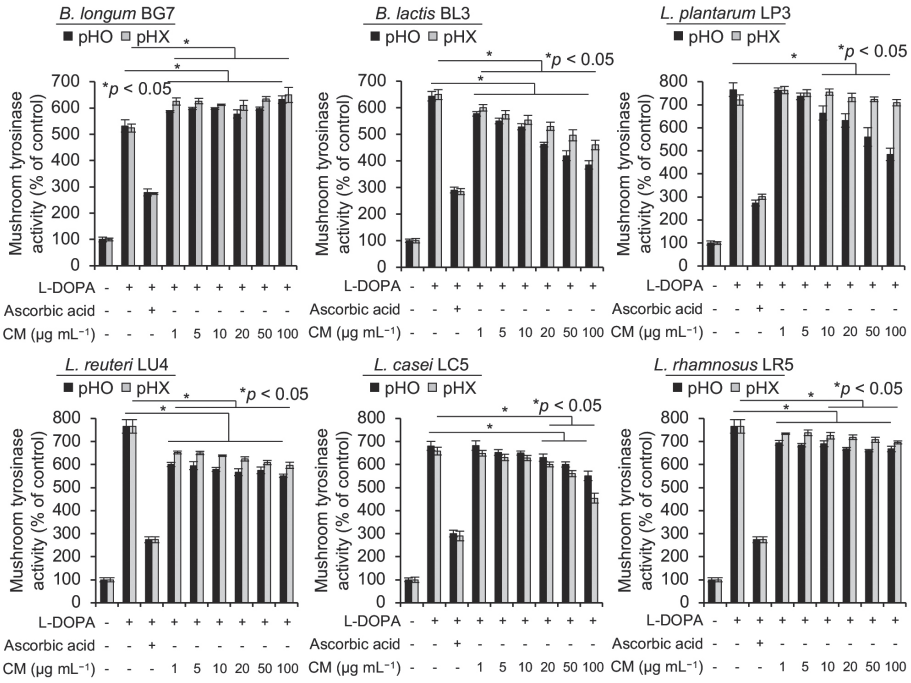


Fig. 5. The inhibitory effect of conditioned media (CM) derived from *Bifidobacterium* and *Lactobacillus* species on mushroom tyrosinase activity *in vitro*. The graph indicates the mean ± SD (n = 3). * $p < 0.05$ compared with cells treated with L-DOPA only. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

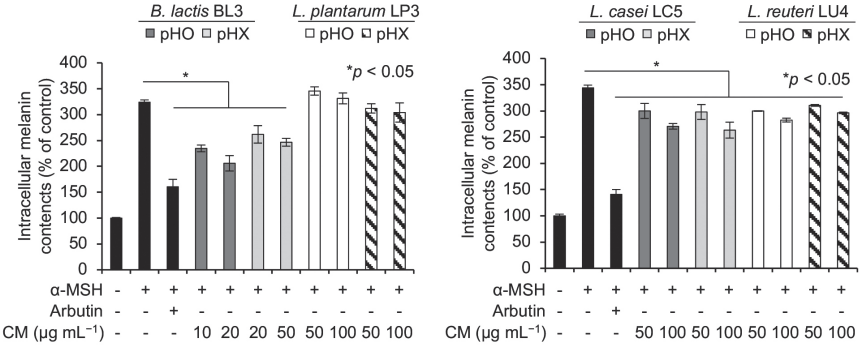


Fig. 6. The inhibitory effect of conditioned media (CM) derived from *Bifidobacterium* and *Lactobacillus* species on intracellular melanin synthesis in B16F10 cells. Melanin contents were determined by measuring the absorbance at 450 nm. Arbutin was used as a positive control. The graph indicates the mean ± SD ($n = 3$). * $p < 0.05$ compared with cells treated with α-MSH only. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

decrease in melanin content; however, the other groups show a low, but not significant, effect on the downregulation of melanin contents. Taken together, these results suggested that the CMs from BL3 had an inhibitory effect on melanin production.

Anti-pigmentation effect of CM from B. lactis via *downregulation of PKA and ERK pathway*

The results in Fig. 5 and 6 showed the inhibition effect of only the CMs from BL3 on *in vitro* tyrosinase activity and melanin contents in melanoma cells. To confirm these results, we further investigated the inhibitory mechanism. We treated the cell stimulated with α-MSH with non-toxic concentrations of the CMs, followed by immunoblotting and RT-qPCR assays. As shown in Fig. 7, α-MSH stimulation led to the upregulation of tyrosinase, TYPR1, and TYRP2 proteins, as well as their mRNA levels; however, the CMs decreased both protein and mRNA levels in a dose-dependent manner. MITF is a major transcription factor for these genes; therefore, we further analyzed its expression level. Results showed that both the protein and mRNA levels of MITF were significantly downregulated by the CMs. These results suggest that the inhibitory effects of the CMs were mediated by the downregulation of *MITF* expression in cells.

It is known that the regulation of *MITF* expression is specifically mediated by the cAMP-mediated PKA/CREB signalling pathway (1). Therefore, we further tested this possibility, and the results from the immunoblot assay showed that treatment with the CMs markedly downregulated phosphorylation levels of PKA and CREB proteins, but not their total protein levels, in a dose-dependent manner (Fig. 7g and 7h). We also evaluated other possible effects of MAPK kinase activity, because phosphorylation of CREB protein is induced by ERK in a PKA-independent manner in melanogenesis (1). As shown in Fig. 7i, α-MSH stimulation led to upregulation of phosphorylation in ERK, but not in p38 or JNK; however, the CMs decreased phosphorylation levels of ERK in a dose-dependent manner. Taken together, these results suggest that the anti-pigmentation effects of the CM from BL3 pHO were mediated by the PKA- and ERK-mediated CREB pathways in melanocytes.

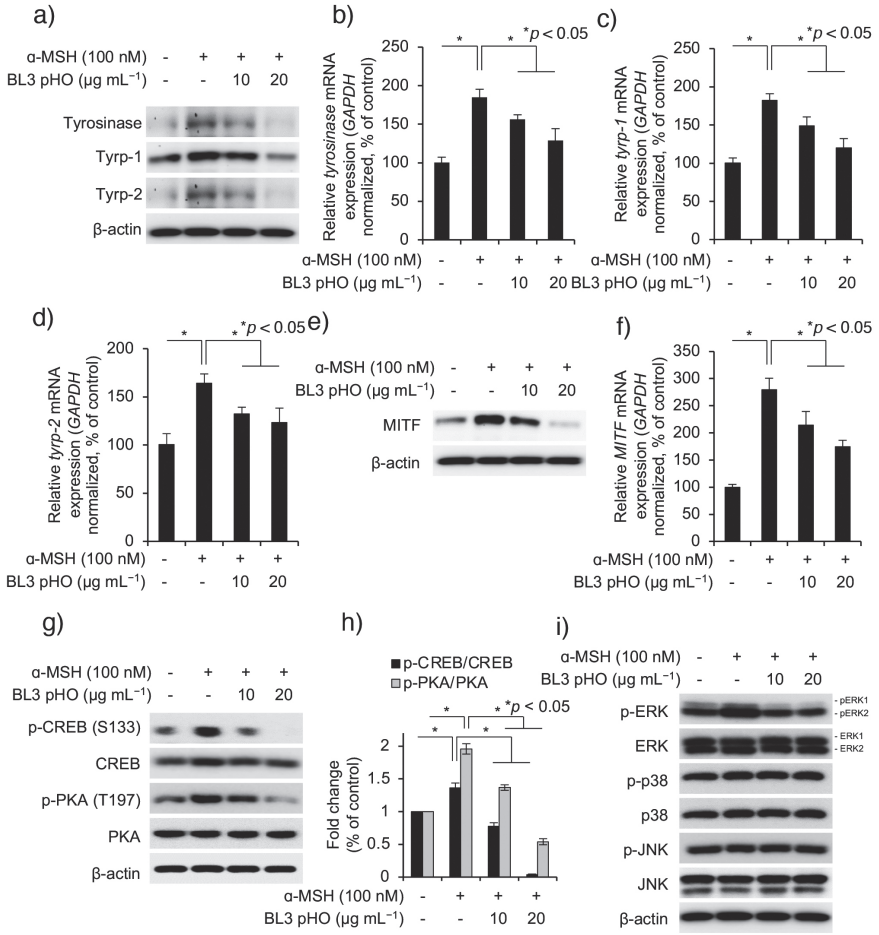


Fig. 7. The inhibitory effect of conditioned media (CM) derived from *B. lactis* BL3 on α-MSH-induced expressions of melanogenesis-related proteins (a) and genes (b to d), including tyrosinase, Tyrp-1, and Tyrp-2. (e and f) CM from *B. lactis* inhibited the upregulation of both protein and mRNA of MITF. (g and h) CM from *B. lactis* inhibited the phosphorylated levels of CREB and PKA in cells. (i) CM from *B. lactis* inhibited the levels of p-ERK protein in cells. The graph indicates the mean ± SD ($n = 3$). *$p < 0.05$ compared with non-treated control or cells treated with α-MSH only. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

Chemical composition of the CM from B. lactis

Although the exact chemical compositions and the biological effect of each component have not been fully elucidated in related studies, one finding regarding the CMs of probiotics is the production of several metabolites such as short-chain fatty acids, secreted proteins, extracellular vesicles, and bacteriocins (15). Therefore, we further analyzed the organic acids distributed in the CM, specifically from the BL3 pHO, because this CM showed

better effects than the other CMs in this study. Prior to performing the analysis, we investigated the impact of filtered MRS and BL broths used in this study on the results of anti-pigmentation and reduced collagen inhibition by UV radiation in skin cells. As shown in Fig. 8a and 8b, treatment with the same concentrations (10 and 20 μg mL^{-1}) of the broth media as the BL3 pHO did not show the inhibitory effect on α-MSH-induced melanin synthesis in B16F10 cells or the restoring effect on UV-induced *COL1A1* mRNA down-regulation in NHDFs. These results indicate that the effects of the CM from BL3 pHO were not affected by the broth media but by components of the CM. To analyze the chemical components in the CM, we compared the compositions of organic acids between the CMs from BL3 pHO and pHX. HPLC analysis showed that both CMs had six different peaks: lactic acid, formic acid, acetic acid, and three unknown peaks (Fig. 8c). Of note, the quantities of unknown first and sixth peaks (peak a and c) did not change between CMs from BL3 pHO and pHX; however, the quantities of unknown second peak (peak b), and the three organic acids were markedly increased in the CM from BL3 pHO compared to those from BL3 pHX CM. Specifically, the quantities of lactic acid, formic acid, and acetic acid showed

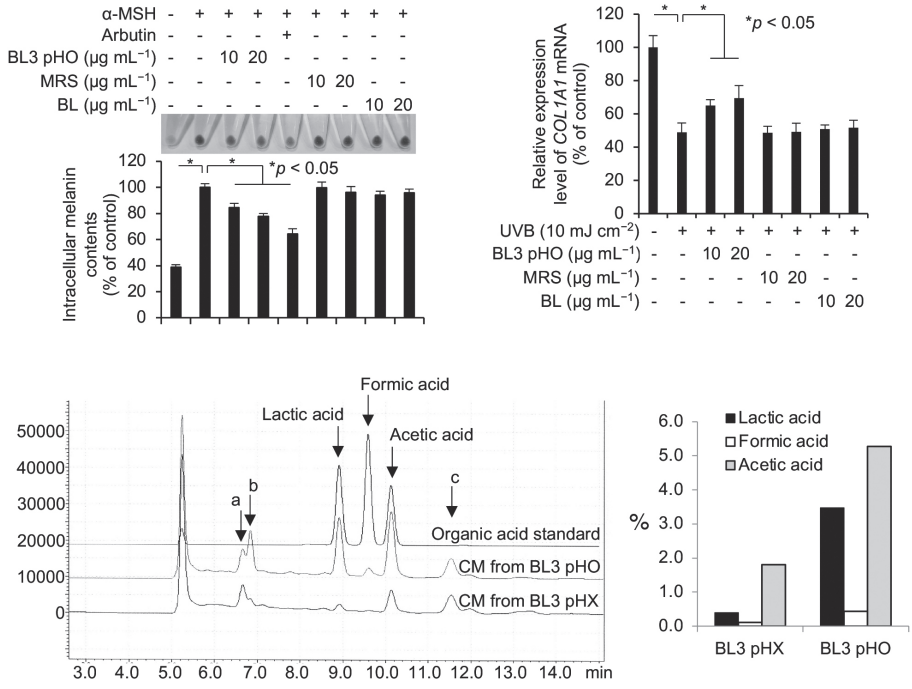


Fig. 8. Chemical composition in conditioned media (CM) from *B. lactis* BL3. Non-regulatory effects of filtered MRS and BL broths on the levels of intracellular melanin content and *COL1A1* expression in B16F10 cells and NHDFs. HPLC chromatogram of CM from *B. lactis*. Composition of individual peaks, including lactic acid, formic acid, acetic acid, and three different unknown peaks. Comparative analysis of the concentration of the acids. Identification and quantification were determined by the individual standard molecules. * $p < 0.05$ compared with non-treated control, or cells treated with α-MSH or irradiated with UVB only. pHO, pH-controlled CMs; pHX, pH-uncontrolled CMs.

approximately 8.9-, 4.3-, and 2.9-fold increases in the pHO sample, compared with values in the pHX sample, respectively (Fig. 8d). These data suggest that these increased components might function as the possible active agents in anti-ageing processes.

In terms of photoaging, the skin of mice administered with *B. breve*-containing milk showed improvement in elasticity and appearance after UV irradiation (16). Similar results were shown in UV-irradiated skin of mice administered *L. plantarum* HY7714 (17). These results indicate the beneficial and anti-ageing effects of probiotics on the skin; however, there are limitations in investigating the effects of probiotics using only oral-administration experimental models: first, safety concerns have been raised in several studies, especially in sick and immunocompromised patients (18–20). Second, some beneficial roles of probiotics are mediated by direct interaction between live probiotic bacteria and epithelial cells, but this is not true for all probiotic benefits (8). With these limitations in mind, studies have tried to find the beneficial effect of probiotics in alternative ways. One of them is to derive and test a CM from probiotics because CM is made from the broth subsequently filtered to remove all bacteria; this feature does not raise the risk of infection and may be relatively safe (8). Recent studies using CMs have demonstrated the various protective effects mentioned in the Introduction section, indicating that CM has the possibility to perform various biological protective functions in human cells. Although no studies have yet reported the possible functions of probiotic CM on skin ageing, we found that CMs from probiotics, especially from *B. lactis*, significantly inhibited UV irradiation-mediated cellular damage, including ROS production, loss of cell viability, and dysregulation of collagen and MMP expression. Moreover, UV-induced melanin synthesis was significantly downregulated by CMs through PKA/ERK signalling pathways. This is the first study to show the biological effect of probiotic CMs and their potential use to suppress UV-induced skin photoaging.

Previous studies have shown the possibility for an anti-oxidative effect of probiotic CM within various experimental models. CM from *L. rhamnosus* GG regulates the production of ROS and nitric oxide in lipopolysaccharide-treated macrophages (21). Additionally, CM from *L. fermentum* shows a protective function against H_2O_2-induced oxidative stress and premature senescence in murine preadipocytes (22). Therefore, we hypothesized that CMs have a restoring effect on UV-induced ROS production in dermal fibroblasts, and we found that the most-tested CMs show anti-oxidative activity in UV-irradiated cells.

As mentioned, ROS production is a causative factor for photoaging in the skin, and it is known that UV-induced ROS production is associated with the results of collagen expression loss (14). This indicates that efficient biological strategies for UV protection could be generated by regulating both ROS production and collagen expression. Indeed, several topical pharmacological and cosmetic agents with anti-ageing properties are based on these two functions (3). In our study, *B. longum* and *B. lactis* CMs show protective effects on UV-induced intracellular ROS production and cellular damage, including cytotoxicity, downregulation of *COL1A1* and *COL3A1* expression, and upregulation of *MMP1* and *MMP3* expression. Although the specific mechanisms have yet to be fully elucidated, our results indicate that *B. longum* and *B. lactis* CMs are potential candidates for biological UV-protection agents that could be used in the pharmaceutical and cosmetic industries.

UV radiation induces not only oxidative stress but also hyperpigmentation in the skin (1). Our results show that *B. lactis* CMs demonstrate the best inhibitory effects on both *in vitro* tyrosinase activity and α-MSH-induced intracellular melanin synthesis. Transcription

levels of tyrosinase genes, *Typr1* and *Tyrp2,* were significantly downregulated by *B. lactis* CMs, and the expression level of *MITF*, a common transcription factor for these genes, was downregulated under the same conditions. These results indicated that *B. lactis* CMs act through biological regulation not only of the catalytic activity of tyrosinase but also *MITF* expression. When melanocytes are exposed to UV radiation, α-MSH secreted by keratinocytes interacts with its receptor, MC1R, located in the plasma membrane of melanocytes, activates (and phosphorylates) PKA and ERK proteins (1). Activated PKA can phosphorylate its target protein, CREB, and p-CREB translocates into the nucleus and binds to the promoter of MITF (23). In addition, ERK activation induces upregulation of MITF transcription (23). Our results show that *B. lactis* CMs inhibited the phosphorylation of both PKA and ERK proteins, indicating that the anti-pigmentation effect of the CMs is mediated by inhibiting the pathways of PKA- and ERK-mediated MITF expression.

From our results, *B. lactis* CM pHO showed the most effective activity in all aspects of the experiments. HPLC analysis revealed that the lactic acid level in *B. lactis* CM pHO was increased by approximately 8.9-fold compared to its level in *B. lactis* CM pHX. Several studies in the literature have shown the anti-ageing effect of lactic acid on the skin (24, 25). Lactate, the anion that results from the dissociation of lactic acid, can be considered a potential antioxidant agent because its ion prevents lipid peroxidation by scavenging oxygen free radicals (26). Furthermore, treatment with lactic acid can stimulate the synthesis of collagen at the cellular level in dermal fibroblasts (27). In addition, in a mouse model, topical application of lactic acid protects UV-induced epidermal thickening and increases the level of antioxidants (28). Furthermore, lactic acid contained in *L. rhamnosus* can reduce melanogenesis *via* inhibition of tyrosinase activity (29), and topical applications of lactic acid have been shown to be effective in treating pigmentary lesions such as melasma (30). These results suggest that the anti-ageing effect of *B. lactis* CM pHO might be induced by lactic acid; however, further validation and identification of the three unknown CM-specific peaks with additional in-depth studies are required to better understand the underlying mechanism.

CONCLUSIONS

We have performed an investigation of the protective effects of probiotic-derived CM against UV-associated cellular damage in human skin cells. Of the twelve CM tested, *B. lactis* CM pHO showed protective effects against UV-associated and skin ageing-related cellular damage, including oxidative stress, hyperpigmentation, and collagen loss in human skin cells. Further experiments revealed that the *B. lactis* CM pHO anti-pigmentation effect was mediated by inhibition of UV-induced MITF and tyrosinase expression *via* dephosphorylation/inactivation of the upstream regulators, PKA, ERK, and CREB in melanocytes. Taken together, our findings indicate that probiotic CMs may be useful for the protection of photoaging and the development of related pharmaceutical and cosmetic products. Therefore, further determination of the active single molecules and clinical validation of the protective effect is crucial.

Conflicts of interest. – The authors declare no conflict of interest.

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Authors contribution. – Conceptualization, Y.K.H., S.A. and S.B.; methodology, Y.H.L., S.A.Y., Y.K.Y. and J.L.; analysis Y.K.H., J.L., G.L. and M.J.C.; investigation, Y.K.H. and S.A., writing, original draft preparation, Y.K.H., S.A. and S.B.; writing, review and editing, S.A. and S.B. All authors have read and agreed to the published version of the manuscript.

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