Effect of Lactobacillus/Phaseolus Coccineus Seed Extract Ferment Filtrate on dry skin.

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Abstract.

Background:

Runner bean (*Phaseolus coccineus* L.) seed was reported that contains various polyphenols such as isoflavones and anthocyanins. In previous study, it was shown that Phaseolus coccineus Seed Extract (PC) developed as a material for cosmetics, promotes collagen production in fibroblasts. To enhance the efficacy, Lactobacillus/Phaseolus Coccineus Seed Extract Ferment Filtrate (PC-FM) was developed by fermenting PC with lactic acid bacteria. Although it was confirmed that PC-FM has anti-aging effects in fibroblasts, the action in keratinocytes has not been clarified yet. In this study, we investigated the effect of PC-FM against epidermal function such as barrier function and moisture retention in keratinocytes.

Methods:

To evaluate the effect of PC-FM, we conducted cell viability assay (WST-8) for keratinocytes treated with PC-FM. In keratinocytes and reconstructed human epidermal keratinization (RHEK) models, we assessed changes in the gene and protein expression involved in skin moisturizing by a RT-qPCR analysis and by Western blotting and immunofluorescence, respectively. To investigate the protective effect against UV, keratinocytes were exposed by UVB and treated PC-FM.

Results:

The cell viability of keratinocytes treated PC-FM was increased. The gene expression of FLG, CASP14, LOR and TGM-1 and the protein expression of FLG and LOR were facilitated, as was RHEK models. Furthermore, PC-FM suppressed the decline of gene and protein expression in NHEKs irradiated UVB.

Conclusion:

These results suggested that PC-FM is expected that approach skin moisturizing functions such as CE maturation and NMF production, and work more effectively as a raw material for dry skin when blended into cosmetics.

Keywords: dry skin; Lactobacillus/Phaseolus Coccineus Seed Extract Ferment Filtrate; Natural Moisturizing Factor; Cornified Envelope; fermentation

Introduction.

The outermost layer of skin, the stratum corneum (SC), is composed of multiple layers of corneocytes stacked like bricks and mortar, and plays the role of “the skin barrier” that prevents water evaporation and matter invasion. Intercellular lipid such as ceramide and cholesterol fill the intercellular spaces, and the SCs are surrounded by cornified envelope (CE), known as insoluble membrane structure. On the other hands, inside the corneocytes, there are keratin and NMF which is natural moisturizing factor, and they retain water into SC.

CE matures with differentiation of the keratinocytes, and intercellular lipids are arranged regularly onto the matured CE as scaffold to make it possible exert a good skin barrier function. CE is mainly composed of involcrin and loricrin (LOR), is of which 65-70% is loricrin[1]. They are produced in keratinocytes. The enzyme transglutaminase-1 (TGM1) crosslink these molecules to insolubilize them and aggregating them under the cell membrane to form strong CE membrane structure. It has been found that CE maturity is reduced in dry skin[2], supporting a close link between CE and the condition of SC.

Filaggrin (FLG) produced in the stratum granulosum of keratinocytes also plays a critical role in the skin barrier. FLG that aggregate keratin filaments, and contributes to strength and flexibility of SC. The aggregated filaments are cross-linked by TGM1 to form an insoluble keratin matrix. This matrix act as scaffold for CE composition attachment[3]. Furthermore, in the process of moving the SC with keratinization, FLG is degraded to amino acids by the proteolytic enzyme CASP14, finally to be NMF. NMF is primarily composed of amino acids, pyrrolidone carboxylic acids (PCA) and lactic acids, and has an important role of retaining water in the SC[4]. Actually, previous study has verified that the amount of amino acids and PCA in the SC showing dry skin is lower than that in healthy subjects[5].

Dry skin is a skin condition which the water contents in SC is reduced. Generally, symptoms such as tautness and bulkiness of the skin are observed, and which caused skin roughness and itchy. Dry skin is induced by not only aging and decrease in humidity, but also various external factors such as ultraviolet rays (UV), inflammatory and reactive oxygen species (ROS). When the skin is damaged by UV, the skin barrier is broken and moisture level in SC degraded to cause dry skin. It has been reported that irradiation of UVB to epidermal cells reduces the expression of FLG and LOR, related barrier function[6], so it is thought that suppressing UVB damage in epidermal cells lead to prevent dry skin.

Runner bean (*Phaseolus coccineus* L.) is perennial plant native to Central America and is called “Hanamame” in Japanese. Currently, it is cultivated all over the world including Japan, and since it is vine and has a plant height of about 3 m, it is cultivated with stanchions. In addition, the ripe seeds are used as edible. The seed is characterized by large black spots on reddish-purple base. *Phaseolus coccineus* L. was said that contains various polyphenols such as isoflavones and anthocyanins, and especially the amount of anthocyanins is high among varieties[7]. Isoflavones and anthocyanins are widely known to have inhibitory effects of inflammatory and oxidant.

Phaseolus coccineus L. has not been researched much in leguminous family, and there are few discovered effects as a cosmetic ingredients.

In previous study, it was reported that Phaseolus coccineus Seed Extract (PC) developed from the seed of *Phaseolus coccineus* L.as a raw materials for cosmetics promotes production of collagen, and inhibits MMP-1 activity and the decrease in production of collagen caused by UV in human fibroblasts[8]. To enhance the efficacy, new materials, Lactobacillus/Phaseolus Coccineus Seed Extract Ferment Filtrate (PC-FM), was developed by fermenting PC with plants-derived lactic acid bacteria. In Pharmaceutical Society of Japan (2022), we reported that PC-FM is superior to PC with regard to synthesis of type I collagen and hyaluronic acid, and the protective effects against skin aging caused by UV and hydrogen peroxide (H_2O_2) exposing in human fibroblasts[9]. However, the effect of PC-FM in human keratinocytes has not been evaluated yet.

In this study, to research the influence of PC-FM in human keratinocytes and human epidermal models, we observed that the mRNA and protein expression related to water retention and skin barrier. Moreover, we verified that the improvement effect of PC-FM against damage caused by UVB irradiation.

Materials and Methods.

Sample Preparation

Phaseolus Coccineus Seed Extract (PC) was obtained by adding purified water to Phaseolus Coccineus Seed, heating and extracting, filtering, concentrating the filtrate and freeze-drying. The PC was added water, and was inoculated *Lactobacillus plantarum* and cultured. Lactobacillus/Phaseolus Coccineus Seed Extract Ferment Filtrate (PC-FM) were obtained by concentrating and freeze-drying the fermented liquid.

Cell and Culture

Normal human epidermal keratinocytes, NHEKs (KURABO Co., Japan) were maintained in Humedia-KB2® (KURABO Co.) supplemented with Insulin (10 µg/mL), hEGF (0.1 ng/mL), hydrocortisone (0.67 µg/mL), gentamicin (50 µg/mL), amphotericin B (50 ng/mL) and 0.4% (v/v) BPE at 37°C, 5% CO_2 atmosphere.

Cell viability

NHEKs (5.0×10^5 cells) were seeded onto 6-well plate and incubated for 24 h. Then, the culture media were changed to fresh media (Humedia-KB2® containing 0.001% BPE) with or without PC-FM. After incubation for 24 h, the medium was removed followed by the addition of 10% water-soluble tetrazolium salt (WST-8) (Wako Pure Chemical Industries, Ltd., Japan) and incubated for 1.5 h. The wavelength absorbance of the media was measured at 450 nm using Cytation5 imaging multimode reader (Biotek Instruments Inc., USA). The cell viability was obtained as a percentage compared to untreated cells.

Real Time RT-qPCR

NHEKs (5.0×10^5 cells/well) were plated on 6-well plate. After incubation for 24 h, the culture

media were changed to fresh media with or without PC-FM and incubated further 24 h. For the UVB irradiation test, NHEKs were seeded in 6-well plates at a density of 4.0×10^5 cells/well, and the cell were irradiated UVB (5 mJ/cm^2) followed by adding PC-FM and incubation for 24 h. Total RNAs were extracted using NucleoSpin® RNA Plus (MACHEREY-NAGEL GmbH & Co. KG, Germany) and transcribed to cDNA using PrimeScript® RT reagent Kit (Takara Bio Inc., Japan). For quantitative PCR, the reaction was performed with SYBR® Premix Ex Taq ™ Ⅱ (Takara Bio Inc.), and the products were analyzed using FX Connect™ Real Time System (Bio-Rad laboratories Inc., USA), normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold change relative to the control was calculate using the 2-⊿⊿CT methods. The primers used for RT-qPCR are provided in Table 1.

	forward	reverse
FLG	5'-GGAATTTCGGCAAATCCTG-3'	5'-TGCTTGAGCCAACTTGAAT-3'
CASP14	5'-GACCTGGATGCTCTGGAACACA-3'	5'-GAATCGATGGCCTGCTGGA-3'
LOR	5'-GGCTGCATCTAGTTCTGCTGTTTA -3'	5'-CAAATTTATTGACTGAGGCACTGG -3'
TGM1	5'-TACAGAGGCCCAAGATCCTCA-3'	5'-GTCTGAGAAGAAGCCCCCATC-3'

Table 1. Primer designs for RT-qPCR.

Western blotting

NHEKs (5.0×10^5 cells/well) were incubated for 24 h on 6-well plate. After irradiation UVB (5 mJ/cm^2), changed to fresh media with or without PC-FM and incubated 48 h (additional 72 h without PC-FM for FLG). Next, NHEKs washed twice with Phosphate Buffer Saline (PBS) (KAC Co., Ltd., Japan) were lysed by radioimmuno-precipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology Inc., USA) containing protease phosphatase inhibitor and protease inhibitor, and crushed with ultrasonic waves. The lysate was centrifuged (4°C, 12,000 ×g, 10 min) and the supernatant was collected. The protein contents of the supernatants were measured by BCA assay kit (Thermo Fisher Scientific Inc., USA). The supernatants mixed with sample loading buffer (62.5 mM Tris-HCl pH 6.8, 2.5% 2-mercaptoethanol, 1% LDS, 10% glycerol and 0.005% bromophenol blue), and heat denaturation at 95°C for 5 min. The sample solution was electrophoresed on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) for separating protein, and then it was transferred to a Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad laboratories Inc.). After blocking with 4% Block Ace (KAC Co., Ltd.) in MilliQ water for 1 h at room temperature, the PVDF membrane was incubated for 3 h at room temperature with primary antibody anti β-actin polyclonal antibody (1:1000; Gene Tex Inc., USA), anti-loricrin polyclonal antibody (1:1000; Proteintech Inc., USA), anti-filaggrin monoclonal antibody (1:200; Santa Cruz Biotechnology Inc.). The membrane washed 3 times by Tris-buffered Saline Tween (TBST) followed by horseradish-peroxidase (HRP) conjugated secondary antibody (1:2000; Cell Signaling Technology Inc., USA) for 1 h at room temperature. Detection was performed using Clarity™ Western Enhanced Chemiliminescence (ECL) Substrate (Bio-Rad laboratories Inc.) and ChemiDoc™ Touch Imaging System (Bio-Rad laboratories Inc.).

Immunofluorescence staining

NHEKs (0.5×10^5 cells/well) were plated on 8-well chamber slide glasses (Matsunami Glass Inc., Ltd., Japan). 24 h after plating (48 h for FLG), the cells were further cultured for 48 h with or without PC-FM. In UVB radiation test, exposed UVB (5 mJ/cm^2) before cultivation with PC-FM. The cells treated were fixed in ice-cold 4% Paraformaldehyde (Wako Pure Chemical Industries, Ltd.) for 15 min, followed by washing PBS. Nonspecific reactivity was blocked with 1% BSA (Wako Pure Chemical Industries, Ltd.) in PBS for 1 h at room temperature. After washing again, the cells were incubated overnight at 4°C with primary antibody anti-loricrin polyclonal antibody (1:100; Proteintech Inc.), anti-filaggrin monoclonal antibody (1:50; Santa Cruz Biotechnology, Inc.), and subsequently with secondary antibody anti-rabbit IgG-Alexa Fluor (H+L) 568 (1:1000; Thermo Fisher Scientific Inc.) and anti-mouse IgG-Alexa Fluor (H+L) 488 (1:1000; Thermo Fisher Scientific Inc.) respectively for 30 min at room temperature. The slide grass were mounted using DAPI Fluoromount-G (Southern Biotechnology Associates Inc., USA), and immunoreactivity was observed using OLYMPUS CKX41 fluorescence microscope (Olympus Co., Japan).

Reconstructed human skin epidermal keratinization (RHEK) models

RHEK model (LabCyte EPI-MODEL 6D, which was cultured under pre-keratinization conditions before the formation of SC layer.) and assay medium were obtained from Japan Tissue Engineering Co., Ltd. (Japan). Each cup of RHEK model were placed into a 24-well plate, and assay medium was added to under the cup, then incubating (37°C, 5% CO_2 atmosphere) for 1 day. The models were treated for 30 min with PC-FM or distilled water (control). The models removed PC-FM or distilled water, incubated 7 days, treated again, additionally incubated for 2 days. The tissue of the RHEK models were dissolved with 350 µL Lysis buffer that is included NucleoSpin® RNA Plus (MACHEREY-NAGEL GmbH & Co. KG), and total RNAs were extracted. The mRNA expression of epidermal function related factors were measured by Real Time RT-qPCR. For immunofluorescence staining, the models were applied with 0.01% PC-FM or distilled water (control) every 2-3days for 30 min and cultured until 5 days later. we embedded the tissue in Tissue-Tek® O.C.T. compound (Sakura Finetek Inc., USA), frozen at -20°C, and cut with cryostat (Thermo Fisher Scientific Inc.) to obtain tissue section. The section were incubated 1 h at room temperature with primary antibody anti-loricrin polyclonal antibody (1:100; Proteintech Inc.), anti-filaggrin monoclonal antibody (1:100; Santa Cruz Biotechnology, Inc.), followed by with secondary antibody anti-rabbit IgG-Alexa Fluor (H+L) 568 (1:1000; Thermo Fisher Scientific Inc.) and anti-mouse IgG-Alexa Fluor (H+L) 488 (1:1000; Thermo Fisher Scientific Inc.) for 1 h at room temperature.

Statistical Analysis

All results are expressed as means ± standard deviations (SD). The significances of differences were examined by Student's t-test. Significant differences are indicated by asterisks as follows. *P<0.05, **P<0.01, ***P<0.001.

Results.

Effect of PC-FM on cell proliferation in NHEKs

We carried out WST-8 assay to assess cell viability after treatment with PC-FM in NHEKs. NHEKs were treated two difference concentration (0.001% and 0.005%). The cell viability of NHEKs was 109.5% at 0.001% and 116.7% at 0.005%, which increased in a concentration dependent as compared with the untreated cells (Control) (Fig. 1).

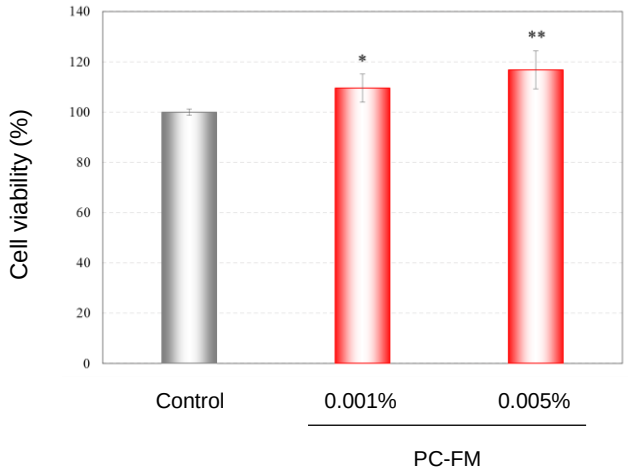


Fig. 1 Cell activation effect of PC-FM in NHEKs.

NHEKs were treated with PC-FM for 24 h. The cell viability was measured by absorbance at 450 nm. Each value represents the means ± standard deviations (SD) %. *P<0.05, **P<0.01 compared with control.

PC-FM upregulated the expression of factors related to epidermal function in NHEKs

To examine change of the gene expression associated with skin moisturizing in keratinocytes, we performed RT-qPCR. We focused on FLG, CASP14, LOR and TGM1 which are factors involved in water retention and CE maturation. NHEKs treated with PC-FM for 24h were compared with untreated cells (Control). As results, PC-FM promoted the gene expression of FLG, CASP14, LOR and TGM-1. In NHEKs treated with 0.005% PC-FM, the expression level of compared to control increased by 110.2% in FLG, 79.7% in CASP14, 137.1% in LOR, and 55.8% in TGM1 (Fig. 2A-D).

The effect on protein expression was observed using western blot and immunofluorescence. Regarding protein expression, we elucidated changes of only FLG and LOR, which mainly active in barrier function. As shown in Fig. 3A and B, both FLG and LOR expression were increased compared with that in untreated NHEKs. Fluorescent staining showed that FLG and LOR proteins increased when adding 0.005% PC-FM to NHEKs (Fig. 3C and D).

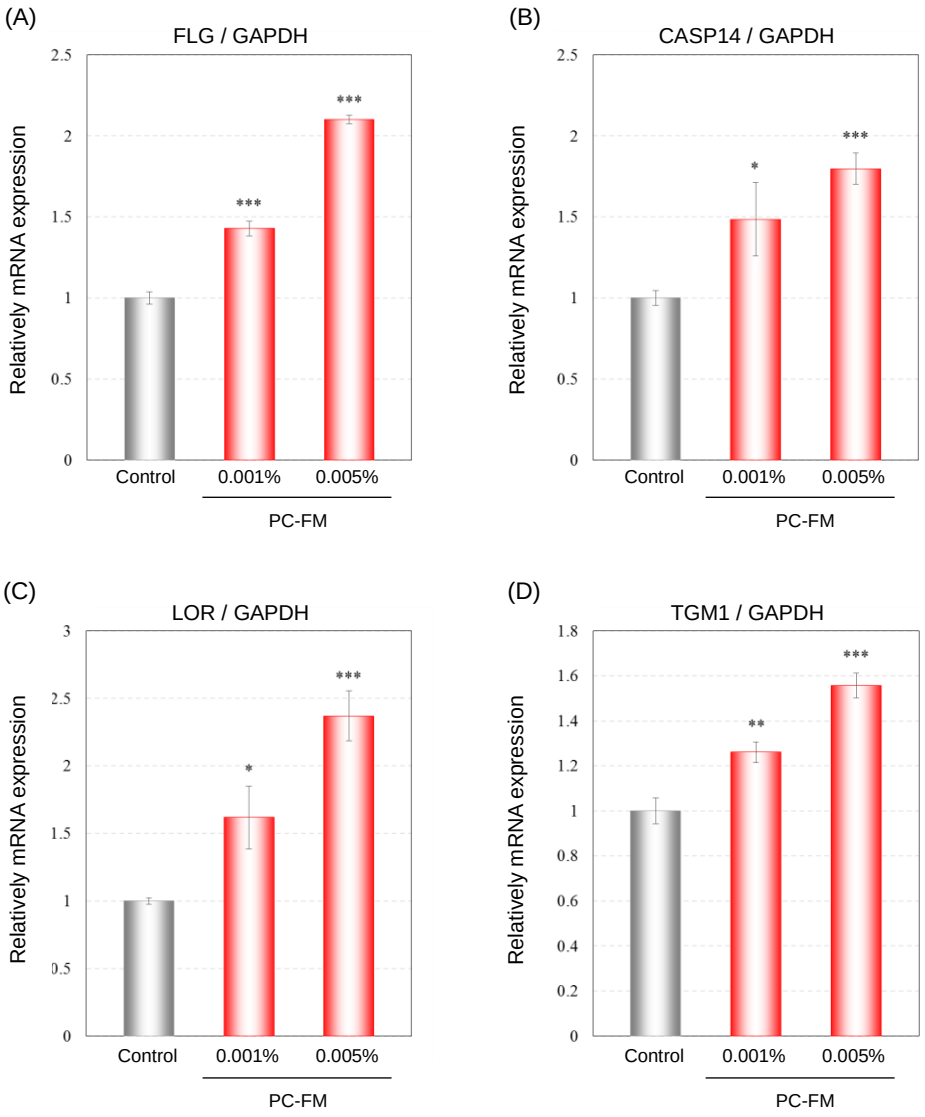
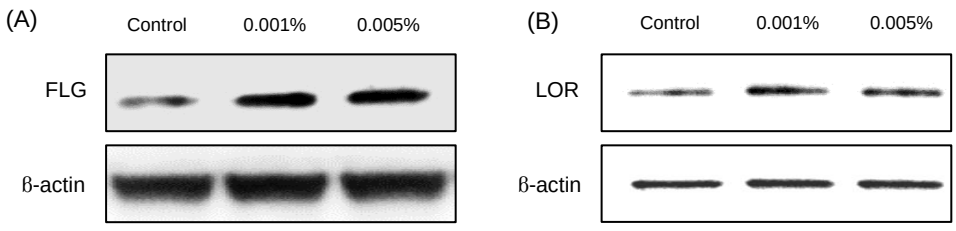


Fig. 2 The change of skin moisturizing-related gene expression in NHEKs.

NHEKs were treated with PC-FM for 24 h. After treatment with PC-FM, we extracted mRNA and carried out RT-qPCR. (A) Evaluation of FLG gene expression. (B) Evaluation of CASP14 gene expression. (C) Evaluation of LOR gene expression. (D) Evaluation of TGM1 gene expression. *P<0.05, **P<0.01, ***P<0.001 compared with control.



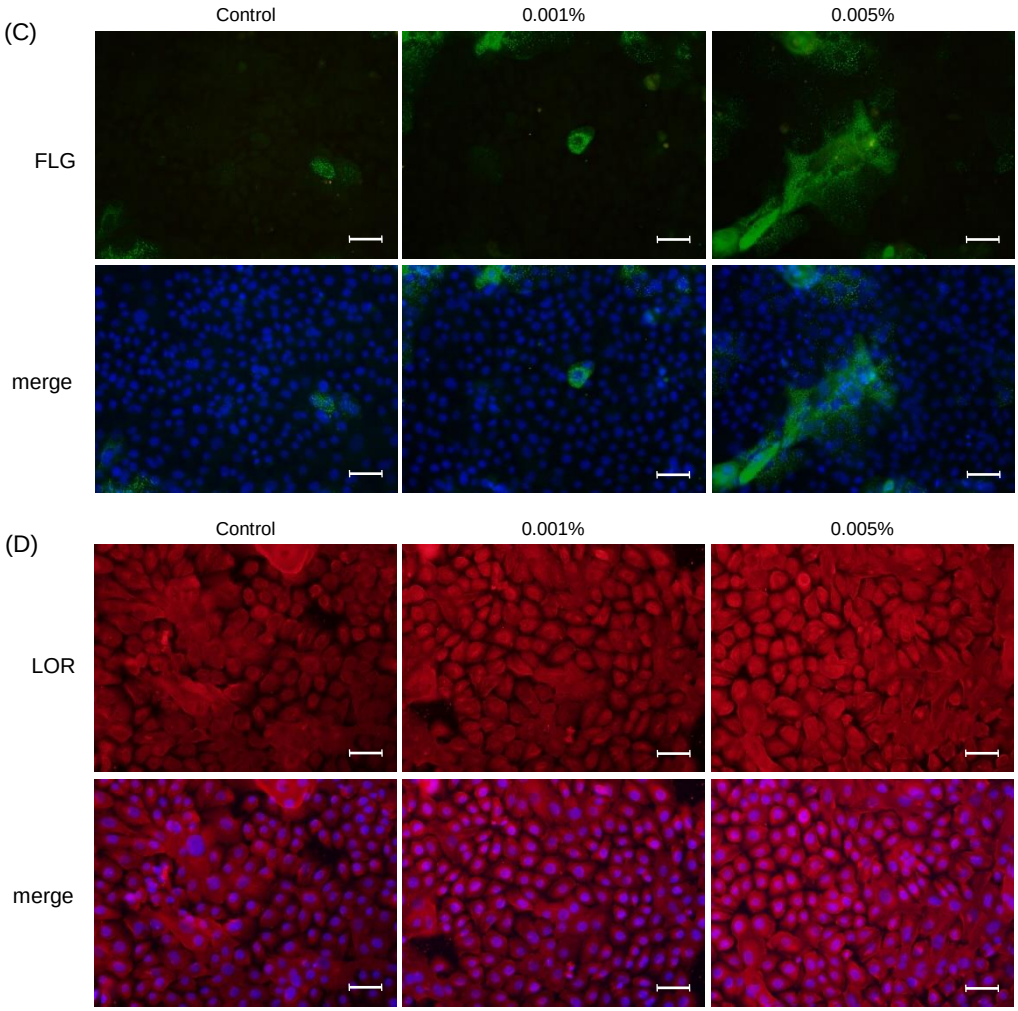


Fig. 3 The change of protein expression that mainly works in epidermal function in NHEKs.

NHEKs were treated with PC-FM for 48 h. After treatment with PC-FM, we extracted protein for western blotting, and fixed the cells for immunofluorescent staining. (A)(C) Evaluation of FLG protein expression. (B)(D) Evaluation of LOR protein expression. For immunofluorescence, merge is composite image with nuclear staining (Blue). Scale bars = 50 μm.

PC-FM suppressed damage caused by UVB in NHEKs.

To verify the protective effect of PC-FM against UVB that causes dry skin, NHEKs were exposed by UVB (5 mJ/cm^2) and cultured 24 h, after that the expression of gene and protein were confirmed. The expression of gene under UVB decreased significantly, which of FLG by 61.8%, CASP14 by 65.1%, LOR by 41.8% and TGM1 by 39.8%. Treatment with PC-FM to UVB-irradiated NHEKs tended to reduce the damage in gene expression in a concentration-dependent manner. Compared with non-treated cells under UVB, treatment with 0.005% PC-FM suppressed the decline of FLG was by 16.1%, CASP14 was by 25.2%, LOR was by 63.6% and TGM1 was by 33.2% (Fig. 4A-D).

The protein expression of FLG and LOR under UVB didn't decrease significantly, but it was

observed that FLG and LOR production level increased by treatment with PC-FM compared to non-treatment under UVB (Fig. 5A-C).

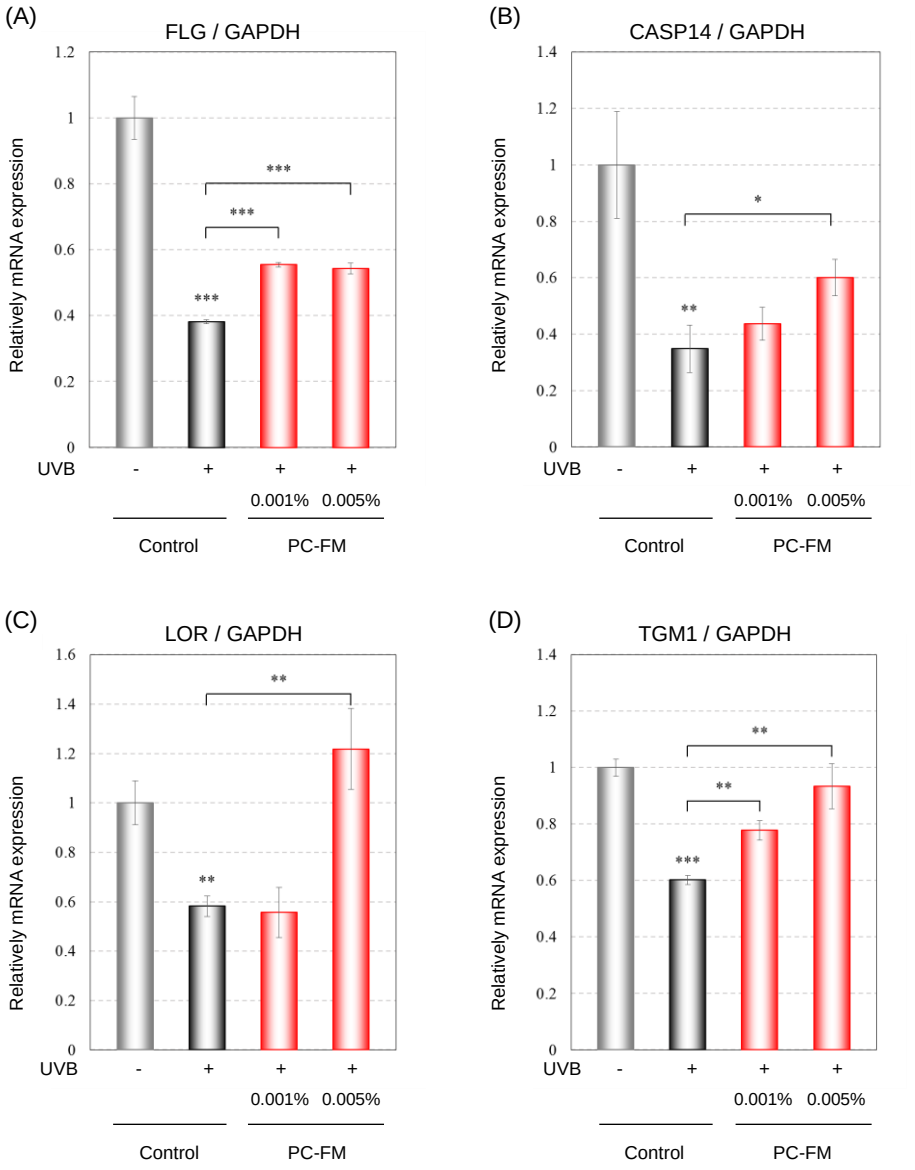


Fig. 4 The inhibitory effect of decline gene expression with PC-FM in NHEKs.

NHEKs were treated with PC-FM for 24 h. After treatment with PC-FM, we extracted mRNAs and conducted RT-qPCR. The values of UVB (+) control were significantly different from non-irradiated control. (A) Evaluation of FLG gene expression. (B) Evaluation of CASP14 gene expression. (C) Evaluation of LOR gene expression. (D) Evaluation of TGM1 gene expression. *P<0.05, **P<0.01, ***P<0.001 compared with control.

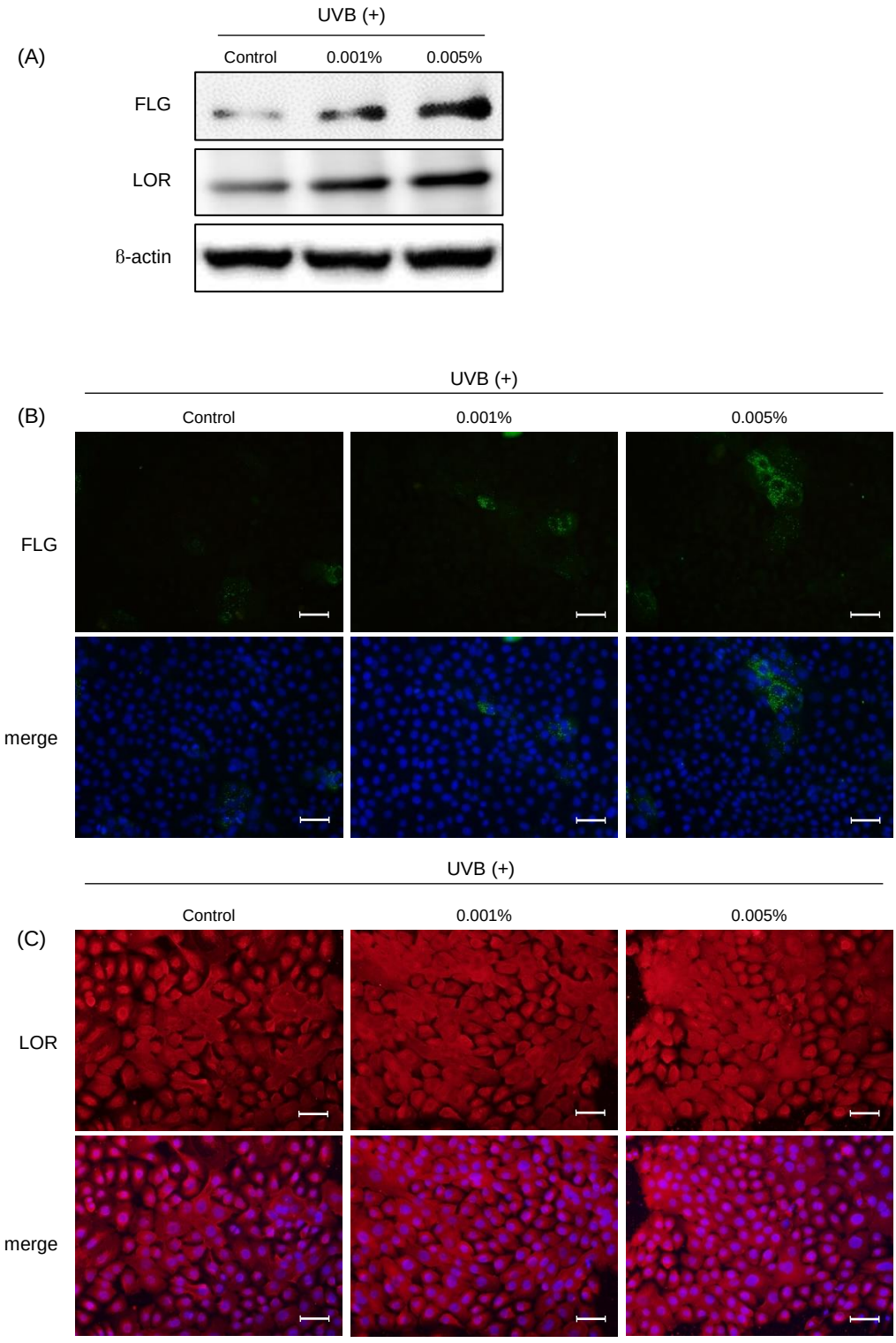


Fig. 5 The change of FLG and LOR protein expressions in NHEKs.

NHEKs were treated with PC-FM for 48 h after. After treatment with PC-FM, we extracted protein for western blotting, and fixed the cells for immunofluorescent staining. (A)(C) Evaluation of FLG protein expression. (B)(D) Evaluation of LOR protein expression. For immunofluorescence, merge is composite image with nuclear staining (Blue). Scale bars = 50 μm.

Effects of PC-FM on gene and protein expression in RHEK models

PC-FM (0.01%) was added to surface side of LabCyte EPI-MODEL 6D, culturing for 7 days after removal. After exposing to PC-FM again and cultivation for 2 days, we assessed the change of gene expression and protein expression. As results, FLG, CASP14 and LOR gene expression were facilitated by treatment with 0.01% PC-FM. The expression level compared to untreated model (Control) increased by 40.1% in FLG, 71.5% in CASP14 and 119.6% in LOR (Fig. 6A-C). Observation of FLG and LOR protein expression using immunofluorescence staining denoted that both of them were higher than control (Fig. 7).

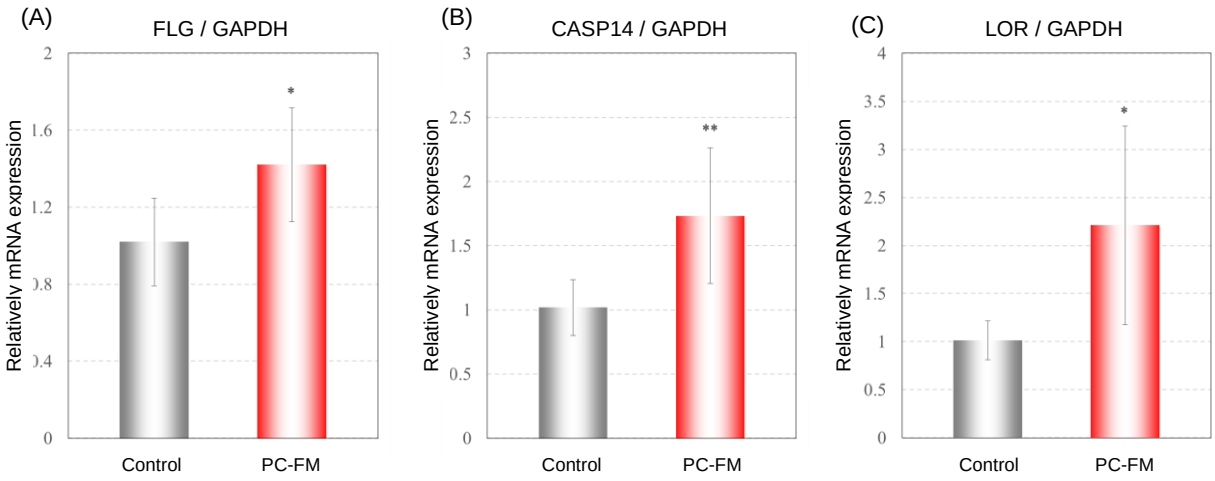


Fig. 6 The effect of PC-FM on gene expression in RHEK models.

PC-FM were added to RHEK models (LabCyte EPI-MODEL 6D) for 30min. After incubation, we extracted mRNA and carried out RT-qPCR. (A) Evaluation of FLG gene expression. (B) Evaluation of CASP14 gene expression. (C) Evaluation of LOR gene expression. *P<0.05, **P<0.01 compared with control.

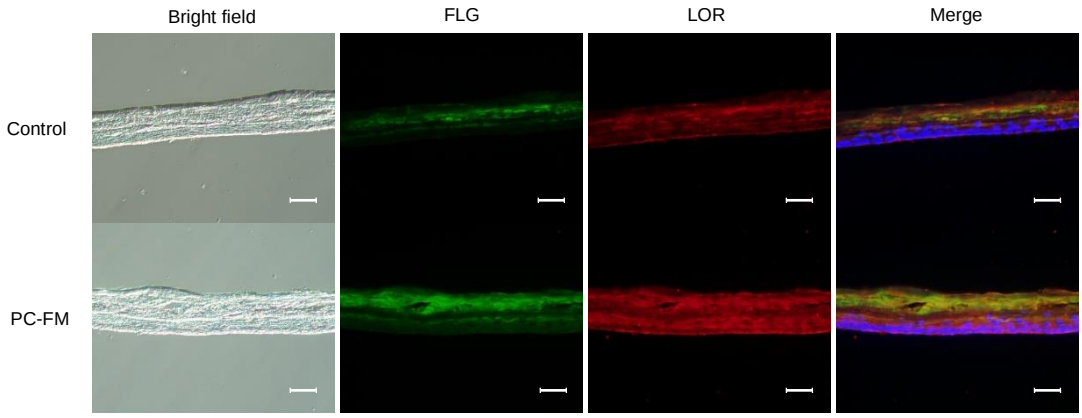


Fig. 7 The effect of PC-FM on protein expression in RHEK models.

The tissue of RHEK models were embedded in compound and frozen at -20°C. Merge is composite image FLG (Green) and LOR (Red) with nuclear staining (Blue). Scale bars = 50 µm.

Discussion.

The present study developed PC-FM by fermenting PC with lactic acid bacteria. Fermentation refers to the process by which microbiota such as lactic acid bacteria and yeast produce metabolites, for example they causes decomposition and low molecular weight of protein, resulting in an increase in amino acids and free amino acids. The technique has been applied not only in the fields of foods and pharmaceuticals, but also in the fields of cosmetics to generate many beauty ingredients that have a positive effect on the skin[10]. Among them, in lactic acid fermentation, it is known that lactic acid bacterium assimilated glucose as an energy and finally converts it into lactic acid, and it has been suggested that the fermentation products promote the differentiation of NHEKs and improve epidermal function[11]. Because of its safety and high recognition of its effect, the utilization of cosmetic ingredients using lactic acid fermentation technology has increased over the last decade[12]. In this time, we confirmed that lactic acid fermentation increased multiple amino acids such as arginine, lysine, glycine, proline and glutamic acid ware increased in PC-FM. They are commonly components of NMF and have been cleared to be effective on the epidermis.

In *Phaseolus coccineus L.,* the amount of polyphenols such as isoflavones and anthocyanins is higher than that of varieties[7]. Both isoflavones and anthocyanins have been demonstrated that have anti-inflammatory and anti-oxidant properties, thus exhibit photo-protective effects[13][14][15]. Isoflavones also have an active effect in NHEKs. Isoflavones are usually exist as inactive form in the state of glycosides to which sugars such as glucose are bound, but when the sugars are separated by β-glucosidase, it is converted to active form called aglycone type. The aglycone type is small molecule and liposoluble, and has higher absorption into the organism than the glycosides[16]. Due to its nature, the effect of aglycone type on the permeability into the SC has been confirmed[17]. It is thought that since lactic acid bacillus has β-glucosidase activity, lactic acid fermentation converts the isoflavones to aglycone type[18] and raise the action.

PC-FM showed a cell proliferation promoting effect in NHEKs (Fig. 1), and upregulation the gene expression of FLG, CASP14, LOR and TGM1 (Fig. 2) and the protein expression of FLG and LOR mainly acting in epidermal function (Fig. 3). In PC-FM, it is expected that are increased amino acids and lactic acids, and converted isoflavone to aglycon type that have high active by lactic acid fermentation. Amino acids and lactic acids are constituents of the NMF, accounting for about 40% and 12% of NMF, respectively. Also, lactic acid has been shown to promote the expression of skin moisturizing-related gene such as FLG, CASP14 and keratin 10[19]. Therefore, it is considered that NHEKs were activated by isoflavones, and increased the production of factors involved in skin moisturization by uptaking amino acids and lactic acids contained in PC-FM. In Reconstructed human skin epidermal keratinization (RHEK) model, it is indicated that PC-FM upregulate the gene expression of FLG, CASP14 and LOR (Fig. 6), and the protein expression of FLG and LOR (Fig. 7), which is the basis for possibility that PC-FM has a good effect on the actual human skin. FLG, LOR and TGM1 is greatly involved in CE mature to form good skin barrier function, while FLG and CASP14 is important factors in NMF production required for water retention in SC. As dry skin is caused by decreasing water contents and barrier function in SC, PC-FM may lead to improve the dry skin.

PC-FM suppressed UVB damage in gene and protein expression (Fig. 4 and 5). UVB causes inflammatory cytokines and ROS production in NHEKs, and is a factor that ultimately induces dry

skin. It is suggested that the action of polyphenols contained in PC-FM, isoflavones and anthocyanins, that have anti-inflammatory and anti-oxidant effects, may suppress the downregulation of skin-moisturizing factors by reducing cell damage caused by UVB radiation.

Conclusion.

In this research, we revealed that PC-FM may have an effect on CE maturation and NMF production. In NHEKs and RHEK models, PC-FM raised the expression of epidermal function-related factors. Besides, PC-FM has inhibitory effect against UVB damage in NHEKs. In summary, PC-FM not only raises the water retention capacity in SC by increasing the amount of NMF, but also strengthens the barrier function by promoting the CE maturation. These two approach of PC-FM hold water in SC and improve skin condition.

In recently, the number of people who are conscious of skin care is increasing, so “skin moisturizing” is a good appeal point to consumers. This study indicated that PC-FM is expected to have high potential as a more useful and effective cosmetic ingredient for dry skin. We will continue to search for and verify further effectiveness from now on.

Conflict of Interest Statement.

NONE.

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